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Molecular Basis for Pathogenesis of Steatohepatitis: Contemporary Understanding and New Insights

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is characterized by a broad spectrum of clinical and histological presentations, ranging anywhere from simple steatosis to steatohepatitis. Of the patients with NAFLD, only a small fraction goes on to develop inflammation and fibrosis (i.e. NASH). Hence, understanding the underlying molecular mechanisms, which play part in progression of NAFLD and determine the disease severity, is extremely important. Almost two decades ago, Day and colleagues first described the “two-hit hypothesis” to explain progression of NAFLD. However, since then, the advances in field of molecular research have identified that NAFLD development and progression involves complex interplay of numerous determinants, including gut-derived signals, endoplasmic reticulum stress, adipose-derived adipokines, nutritional factors, hormonal imbalances and components of innate immunity which act in concert on genetically pre-disposed individuals to induce liver inflammation. This chapter reviews the different players of this “multiple-hit model”.

Keywords: NAFLD, NASH, molecular basis, “multiple-hit model”, steatohepatitis

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) encompasses a broad spectrum of clinical and histo-pathological presentations, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), the latter being characterized by inflammation, macrovesicular steatosis and apoptosis, with or without fibrosis. NAFLD can further progress to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [1]. The prevalence of NAFLD in Western countries ranges from 30–46% [2], whereas in Asian populations, it is about 15% [3]. About 30% of patients with NAFLD may have NASH [4].

Regarding progression of NASH to cirrhosis, estimated 10% of patients with NASH can progress to decompensated liver disease over 10 years and about 25% of patients develop cirrhosis over a span of 9 years [5]. It is very important to understand and uncover the underlying molecular mechanisms which explain the variable incidence and severity of steatohepatitis in only few patients with NAFLD, while most patients with steatosis never progress to steatohepatitis [6]. After Day and Colleagues first described “two-hit” model for pathogenesis of steatohepatitis in 1998, wherein steatosis (“first hit”) progresses to steatohepatitis due to rampant lipid peroxidation in liver (“second hit”) [7], recent advances in field of molecular research have identified numerous other culprits, collectively summed up in “multiple-hit” hypothesis [8]. The “multiple-hit” hypothesis examines multiple insults which act collectively and in-parallel on genetically predisposed individuals to induce development of NAFLD and expedite progression to further adverse pathologies. This chapter provides a review of literature for multiple culprits identified in development of NAFLD and NASH.

2. Development of hepatic steatosis

Several mechanisms are involved in development of hepatic steatosis [9–11], including increased fatty acid supply due to increased lipogenesis from both visceral and subcutaneous adipose tissue, increased dietary intake of fats, increased *de novo* hepatic lipogenesis, decreased free fatty acid oxidation, and decreased secretion of VLDL from liver. Increased free fatty acid delivery to liver and elevated *de novo* lipogenesis are major contributors to fatty acid accumulation in NAFLD.

Elevated hepatic *de novo* lipogenesis may be due to activation by transcription factors such as *SREBP-1* (activated by Insulin, regulated via *Insulin Receptor Substrate* (IRS) and maintains cellular cholesterol homeostasis), *ChREBP* (activated by glucose and increased hepatic *de novo* lipogenesis) and *PPAR-γ*. Studies have demonstrated that *de novo* lipogenesis in liver is elevated in insulin-resistant state and NAFLD [12, 13].

2.1. Lipoapoptosis: free fatty acids and cholesterol

Free fatty acid and Cholesterol are considered main players in lipotoxicity. Increased concentration of serum free fatty acids (16 Carbons and more; saturated or unsaturated) are seen in patients with NAFLD [14]. Apoptosis of hepatocytes, which is morphologic and pathologic feature of human NASH, is partly due to free fatty acids, as explained below.

Hepatocytes can undergo apoptosis via extrinsic pathway (activated by FAS ligand and tumor necrosis factor related apoptosis-inducing ligands) or intrinsic pathway (activated by intracellular stress of membrane-bound organelle, such as mitochondria, endoplasmic reticulum and mitochondria) [15]. Free fatty acids can induce apoptosis via following mechanisms, as demonstrated in **Figure 1**:

- *Mitochondrial pathway* [16] (palmitic acid and stearic acid activate intrinsic apoptotic pathway via C-jun N-terminal kinase and Bim, leading to Bax activation, mitochondrial permeabilization, release of cytochrome c, and activation of Caspase 3 and 7),

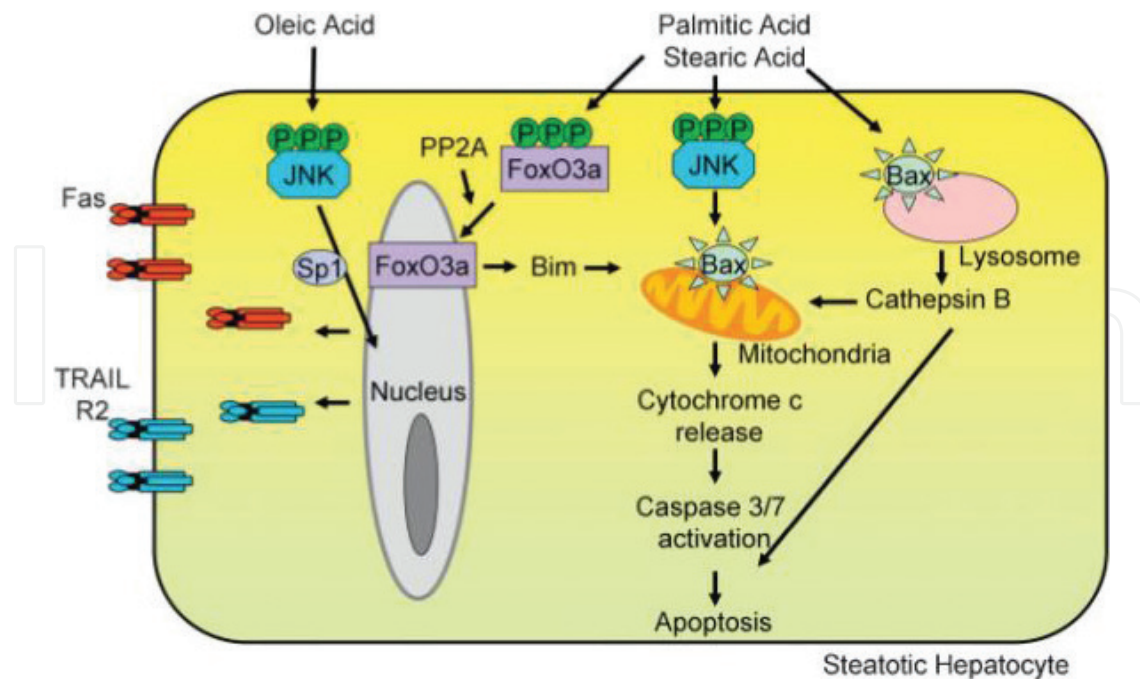


Figure 1. The figure demonstrates different mechanisms by which fatty acids impart lipotoxicity to hepatocytes (copyright © 2008 Georg Thieme Verlag KG) [15] (permission requested from Thieme publications).

- *Induction of Bim expression* [17] (palmitic acid and stearic acid can activate transcription factor FoxO3a, which further induces expression of pro-apoptotic protein Bim),
- *Lysosomal pathway* [18, 19]: Oleic acid and palmitic acid activate Bax which trans-locates to lysosomes, increases the permeability of lysosomes and causes release of cathepsin B, which further increase permeability of mitochondria and activates Caspases. Furthermore, Lysosomal permeabilization is also associated with activation of NF- κ B, which results in generation of tumor necrosis factor- α (TNF- α) in hepatocytes,
- *Endoplasmic Reticulum* [20]: Palmitic acid and stearic acid lead to activation of Endoplasmic reticulum (ER) stress pathway, which can lead to apoptosis, as explained later,
- *C-Jun N-Terminal Kinase*: JNK belongs to family of intracellular mitogen-activated protein (MAP). The murine dietary models of obesity were associated with increased activation of JNK in liver [21]. JNK leads to activation of pro-apoptotic protein Bax [22] while it inactivates anti-apoptotic protein, Bcl-2 [23]
- *Death Receptors*: Tumor Necrosis Factor Receptors, TNFR-1 and TNFR-2, and Fas are implicated in pathogenesis of steatohepatitis [24]. Obesity, being a chronic inflammatory state [25], is characterized by infiltration of adipose tissue by macrophages which release of inflammatory mediators, including TNF- α [26], with increased levels of TNF- α being observed in obesity. Upon activation by TNF- α , TNFR-1 activates NF- κ B which leads to activation of pro-inflammatory genes and further apoptosis, if NF- κ B mediated survival signals are inhibited. TNF- α can also lead to JNK activation, which can also lead to apoptosis if its activation is sustained [15].

Fas is expressed on hepatocytes and upon binding by Fas ligand, it signals apoptotic cell death [15]. In dietary murine models, such as methionine and choline-deficient diet and

high sucrose diet, steatosis is accompanied by increased expression of hepatic Fas [27], and increase in Fas expression confers increased Fas ligand-mediated apoptosis. Also, Mono-unsaturated fatty acid, oleic acid, under the transcriptional control of JNK, increases the expression of Fas and TRAIL-R2 on hepatocytes [15]. This is another mechanism by which fatty acids impart sensitivity to death receptor mediated extrinsic pathway of apoptosis,

- *Ceramide*: Ceramides are composed of sphingosine and fatty acid, and availability of long chain fatty acids is a rate limiting step in synthesis of ceramide in ER [28]. In nutritional obesity with associated elevation of palmitic acid and stearic acid, excess synthesis of ceramide is possible. Palmitic acid and stearic acid-induced *de novo* ceramide synthesis in hematopoietic precursor cell line is associated with apoptosis. However, more studies are needed to highlight the exact contribution of ceramide to wide spectrum of NAFLD pathologies [29]
- *Toll-Like Receptors*: toll-like receptors (TLR) are family of pattern recognition receptors that respond to microbial pathogens by activating innate arm of immune system [15]. Palmitic acid activates TLR4, leading to activation of NK- κ B. This leads to upregulation of its target genes- i.e. TNF- α and Interleukin-6 (IL-6)- in macrophages and adipocytes [30]. When a high-fat diet is fed to mice lacking TLR4, there is a lack of inflammatory gene expression induction by high-fat diet, pointing towards the role of TLR4 in hepatic inflammation seen in NASH [31].
- *Oxidative Stress*: Enhanced mitochondrial and microsomal fatty acid β oxidation and cytochrome P450 (CYP2E1) induction can lead to oxidative stress via generation of Reactive Oxygen species (ROS), as observed in human models of steatohepatitis [15, 32]. 4-hydroxy-2-noneal (HNE) and Thiobarbituric acid reacting substrate (TBARS), both markers of lipid peroxidation, are increased in patients with NAFLD and NASH [33, 34]. Thus, oxidative stress may contribute towards development of steatosis and steatohepatitis.
- *Long chain poly unsaturated fatty acids (LCPUFA)*: Oxidative stress leads to depletion of n-3 LCPUFA (e.g Eicosapentaenoic acid, EPA, and docosahexaenoic Acid, DHA) due to increased peroxidation or defective desaturation processes. Depletion of n-3 LCPUFA leads to upregulation of lipogenic and glycogenic effects from SREBP-1c and down-regulation of fatty acid oxidation effects from peroxisome proliferator activated receptor- α (PPAR- α), ultimately promoting hepatic steatosis [35]. In addition, depletion of LCPUFA can also lead to insulin resistance due to disturbance in membrane mediated processes such as insulin signaling [36]. From dietary prospective, a study aimed at assessing the influence of high-fat diet on $\Delta 5$ and $\Delta 6$ desaturase enzymes involved in LCPUFA formation, found that HFD lead to enhanced oxidative stress and macrovesicular steatosis, with diminution in desaturase activity and hence, depletion of LCPUFA [37].

The role of cholesterol in lipoapoptosis requires special mention. In an analysis conducted on human liver samples, subjects with NAFLD and NASH exhibited almost a 2-fold increase in free cholesterol, as compared to controls [38]. Furthermore, in a study to evaluate the effect of dietary free cholesterol loading in rodents, rats fed high cholesterol diet developed microvesicular steatosis and were sensitized to apoptotic effect of TNF- α , which may explain the lipoapoptosis due to cholesterol [39].

2.2. Triglycerides

Triglycerides are the main lipids stored in liver of patients with hepatic steatosis and recent studies suggest that triglycerides may in fact have protective functions. Diacylglycerol acyltransferase 1 and 2 (DGAT) catalyze final step in triglyceride synthesis. In a model of diet-induced obesity, mice with over-expression of DGAT1 in adipocytes and macrophages were protected from macrophage activation and accumulation in white adipose tissue, and from systemic inflammation and insulin resistance [9, 40]. In another study, DGAT2 antisense oligonucleotides lead to inhibition of triglycerides synthesis which improved liver steatosis but worsened liver damage, further strengthening the notion that liver triglycerides are protective in nature [41]. Thus, in summary, accumulation of triglycerides in liver might not actually be a pathology but in fact, an adaptive, beneficial response in situations where hepatocytes are exposed to toxic triglyceride metabolites and fatty acid excess due to increased caloric consumption [9, 42].

2.3. Inflammation leads to Steatosis or vice versa: chicken or the egg?

- Treatment with anti-TNF antibody and metformin (an anti-diabetic drug that inhibits hepatic TNF α expression) in ob/ob mice (the laboratory model of nonalcoholic fatty liver disease) showed marked improvement in hepatic steatosis [43, 44].
- In patients with alcoholic steatosis, treatment with anti-TNF antibody can improve hepatic steatosis [45].
- Similarly, loss of Kupffer cells lead to decreased production of anti-inflammatory cytokine (IL-10), which lead to hepatic steatosis [46].

The above examples support the notion that Inflammation activates stress response in hepatocytes, which leads to lipid accumulation. In fact, hepatic steatosis may be considered a “bystander phenomenon” following inflammatory attacks. It may be a possibility that inflammation proceeds steatosis in NASH and, benign and non-progressive simple steatosis and NASH are different disease entities altogether [9].

2.4. Insulin resistance in NAFLD

NAFLD is strongly associated with hepatic and adipose tissue insulin resistance, as well as reduced whole body insulin sensitivity [47]. Different underlying molecular mechanisms have been identified which account for insulin resistance [8]:

1. Serine phosphorylation of insulin receptor substrate (IRS) by inflammatory signals, such as *c-jun N-terminal protein kinase 1* (JNK) or *inhibitor of nuclear factor- κ B kinase-b* (IKKb) [48]
2. Activation of nuclear factor kappa B (NF- κ B) and Suppressors of cytokine signaling (SOCS) [49]

Insulin Resistance would mean that ability of Insulin to suppress lipolysis has been suppressed, leading to increased delivery of free fatty acids to liver [50]. The free fatty acid can further

exacerbate hepatic insulin resistance by causing translocation of PKC- γ isoform from cytosol to the membrane where it impairs hepatic *insulin receptor substrate* (IRS)-associated phosphatidylinositol activity [51].

2.4.1. Oxidative stress and insulin resistance

Prolonged excess oxidative load in steatosis, due to carbohydrates and lipids, leads to redox disequilibrium characterized by lower than normal hepatic anti-oxidative potential, for example, decreased hepatic glutathione (GSH) and reduced superoxide dismutase (SOD) activity, which further triggers insulin resistance (IR) [52]. This is validated by a data from study where increased reactive oxygen species (ROS) in 3T3-L1 cultured pre-adipocytes preceded the onset of Insulin resistance [53], by molecular mechanisms listed above. The insulin resistance due to exacerbated hepatic oxidative stress can in turn lead to upregulation of pro-oxidative CYP2E1 expression, the response which is normally attenuated by repressive effects of Insulin on CYP2E1 expression [54]. Thus, there is increasing evidence for positive reinforcement and interdependency between oxidative stress and insulin resistance in patients with Hepatic steatosis [52].

Thus, due to impairment in IRS activity and further down-regulation of IRS due to insulin resistance, SREBP is unregulated and over-expressed, leading to increased hepatic *de novo* lipogenesis [55]. Insulin Resistance, due to its ability to induce lipotoxicity, oxidative stress and inflammatory cascade, may be one of the “multiple hit” in pathogenesis of NAFLD and progression towards NASH [8, 56].

3. The gut-derived factors

The gut microbiota is implicated in the pathogenesis and progression of NAFLD, through so-called gut-liver axis [8]. A study aimed at analyzing human gut microbiome recognized different “enterotypes” [57] and “obese microbiome”, which has an ability to harvest increased amount of energy from diet, has been demonstrated in obese mice [58]. In fact, colonization of germ-free mice with “obese microbiome” leads to greater increase in total body fat as compared to colonization with “lean microbiome” [58]. The **Figure 2** summarizes the role of gut-liver axis and adipose tissue in pathogenesis of NAFLD.

The liver receives more than 50% of its blood supply from splanchnic circulation [8], and hence, it is always exposed to gut-derived toxins. The ability of gut-derived factors like lipopolysaccharide (LPS) to flow in portal vein requires intestinal permeability, which in NAFLD is due to disrupted intercellular tight junctions in the intestine [59]. In Murine models of NAFLD, intestinal mucosa has bacterial overgrowth with increased intestinal permeability and concurrent reduction in expression of tight junction proteins [60]. Consequentially, plasma endotoxin levels are significantly high in patients with NAFLD and NASH [61], and high-fat diet is associated with 2–3 fold increase in plasma LPS levels [62]. LPS may act as a ligand for TLR with consequent activation of inflammatory cascade, including stress- and mitogen-activated protein kinases-JNK (explained later), p38, Interferon regulatory factors 3 and nuclear factor- κ B – each having

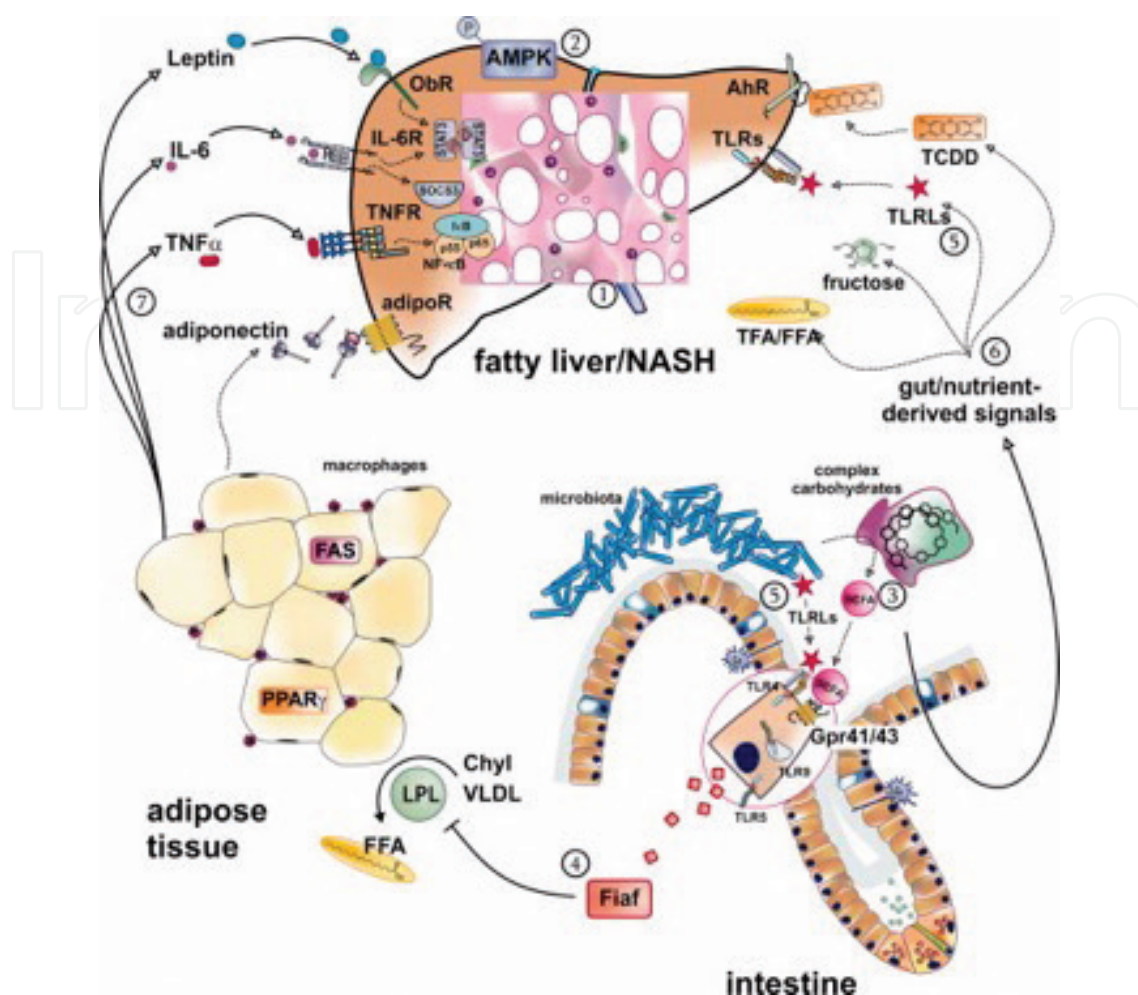


Figure 2. “The multiple parallel hits model. Lipotoxicity: (1) a liver loaded with lipids consisting primarily of triglycerides might reflect a benign process because triglycerides might exert mostly protective effects. Furthermore, hyperleptinemia leads to oxidation of hepatic lipids, thereby also protecting this organ from lipotoxicity. When the capacity of peripheral and central organs of detoxifying “aggressive lipids” fails, lipotoxic attack of the liver might begin. Inflammation may precede steatosis in NASH. Gut-derived signals: Many signals beyond endotoxin might affect hepatic steatosis and inflammation. Several pathways have been identified how the gut microbiota might influence host energy metabolism: (2) Absence of the microbiota in germ-free mice correlates with increased activity of phosphorylated AMPK in the liver and the muscle (not shown). (3) Some of the breakdown products of polysaccharides are metabolized to SCFAs. SCFAs such as propionate and acetate are ligands for the G protein-coupled receptors Gpr41 and Gpr43. Shortage of SCFAs might allow the evolution of systemic inflammatory events. Such mechanisms elegantly combine diet, microbiota, and the epithelial cell as “nutrient sensor.” (4) The microbiota decreases epithelial expression of fasting-induced adipocyte factor (Fiaf), which functions as a circulating lipoprotein lipase (LPL) inhibitor and therefore is an important regulator of peripheral fat storage. (5) Several TLRs, such as TLR5 or TLR9, are not only able to affect microbiota but also to regulate metabolism, systemic inflammation, and insulin resistance, thus highlighting the role of the innate immune system in metabolic inflammation as observed in NASH. (6) Various nutrients such as trans fatty acids (TFAs), fructose or aryl hydrocarbon receptor (AhR) ligands such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD) may directly lead to steatosis/liver inflammation. Adipose tissue-derived signals: Signals derived from the adipose tissue beyond toxic lipids might play a central role in NAFLD/NASH. (7) here, adipocytokines such as adiponectin and leptin, certain pro-inflammatory cytokines such as TNF α or IL-6, and others (the death receptor Fas, PPAR γ) are of key relevance. The cytokine/adipocytokine milieu might be critical because ob/ob-adiponectin tg mice, although becoming severely obese, are not insulin-resistant. This suggests that in the hierarchy of processes soluble mediators play the central role. Adipose-derived mediators might indeed affect target organs such as the liver, because JNK1 adipose-deficient mice are protected from diet-induced obesity, and experiments have demonstrated that this effect is mediated mainly by IL-6 (a cytokine), which is of key importance in human obesity.” [9] (figure and associated caption used after permission from “John Wiley and Sons” [9]).

significant contribution towards insulin resistance, hepatic fat accumulation, obesity and NASH development and progression [63, 64]. Evidently, a continuous infusion of LPS for 4 weeks in mice mimicked high-fat diet phenotype, with noticeable increase in insulin resistance, increased liver triglyceride content and adipose tissue inflammation [62]. Similarly, the use of antibiotics reduced the intensity of inflammation in high-fat diet and ob/ob mice [65].

Another mechanism for gut microbiota to influence the host immune system is via their capacity to digest dietary fibers, such as resistant starch and nonstarch polysaccharide, to short chain fatty acids (SCFAs, mainly propionate, butyrate and acetate), which are absorbed by intestinal epithelium [66]. SCFAs, via their interactions with G protein-coupled receptor 43 (Gpr43), have anti-inflammatory function in various models of human ulcerative colitis [67, 68].

Enteric bacteria also suppress the synthesis of fasting-induced adipocyte factor (Fiaf), resulting in increased lipoprotein lipase activity and increased triglyceride accumulation in the liver [8, 9]. Gut microbiota also produce enzymes which cause the conversion of dietary choline to toxic compounds, particularly methylamines which, in the liver, are transformed to trimethylamine-N-oxide and induce inflammation and liver fibrosis [69]. Intestinal microbiome is a major source for production of hepatotoxic compounds such as alcohol, phenols and ammonia which are delivered to the liver by portal circulation. These compounds activate kupffer cells and stimulate the production of nitric oxide and other inflammatory cytokines [70]. Patients with NASH show abundance of alcohol-producing bacteria as compared to healthy children and children with simple steatosis [71]. This endogenous production of alcohol has a well-established role in generation of ROS and liver inflammation [72].

Furthermore, NLRP6 and NLRP3 inflammasomes, through their production of IL-18, play an important role in modulation of gut microbiota. In different mouse models, inflammasomes deficiency is associated with modifications in configuration of gut microbiota, and exacerbation of hepatic steatosis and inflammation. This is due to increased influx of TLR4 and TLR9 ligands into the portal circulation, leading to enhanced tumor necrosis factor- α (TNF- α) production in liver which leads to NASH progression [73].

3.1. Bile acids

The primary bile acids cholic acid and chenodeoxycholic acid are conjugated to glycine and taken up in distal ileum for transport to the liver [74]. By binding to cellular receptor farnesoid X receptor (FXR) in various organs of the body, bile acids act as signaling molecules to control overall metabolism of the host [75]. Upon activation of FXR by primary bile acids, downstream signals are generated which lead to inhibition of hepatic *de novo* lipogenesis, increased insulin sensitivity and protection of hepatocytes from bile acid-induced cytotoxicity [76]. However, the gut microbiota in distal ileum can deconjugate the bile acids and can further metabolize them to secondary bile acids, and thus, contribute towards obesity by altering lipid metabolism, through changes in bile acid pools and modulation of FXR signaling [74].

3.2. Dietary factors

The recent decades saw a dramatic increase in consumption of trans-fatty acids, and as evident from studies on mice, trans-fatty acids consumption leads to larger liver with NASH-like lesions and Insulin resistance [77]. Similarly, Fructose is a lipogenic, pro-inflammatory dietary factor associated with oxidative stress and upregulation of TNF- α [78], and daily fructose consumption is associated with liver inflammation and fibrosis [79]. Fructose diet can induce oxidative stress and hepatocellular damage by different mechanisms, including induction of protein fructosylation which activates SREBP and generates reactive oxygen species (ROS) [80]. Also fructose phosphorylation leads to depletion of ATP, which stimulates increased uric acid synthesis which in turn stimulates production of ROS [81]. Lastly fructose can induce mitochondrial disturbance which lead to disequilibrium between *De novo Lipogenesis* and VLDL, which promotes alteration of respiratory chain and uncoupling of oxidative phosphorylation with excess ROS production [82, 83].

Another receptor, aryl hydrocarbon receptor (AhR), is a ligand activated transcription factor which is activated by many constituents of our diet such as indolo-(3,2-b)-carbazole and 3,3'-diindolylmethane (metabolized from indole 2-carbinol), or flavonoids, and this pathway plays an important role in inflammation [84]. This is evident in transgenic mice with constitutively activated AhR as they develop spontaneous hepatic steatosis and increased hepatic oxidative stress [85].

Studies show that Low-Calorie, Low-carbohydrate soy-containing diet and Mediterranean diet rich in antioxidants and polyunsaturated fatty acids of n-3 series are known to be protective in reducing hepatic steatosis [86].

4. Adipose tissue-derived signal

Adipose tissue, with its ability to generate cytokines and adipocytokines, can be classified as a complex endocrine and immune organ which mediates different metabolic, immunological and inflammatory responses.

4.1. Adiponectin

Adiponectin is an anti-inflammatory cytokine with anti-lipogenic effects which protect non-adipocyte tissue, such as liver, from lipid accumulation [87]. Reduced levels of adiponectin are seen in conditions associated with development of NAFLD, namely obesity [88] and insulin resistance [89]. Hence, adiponectin levels are inversely related to visceral obesity and insulin resistance, and weight loss is an inducer of adiponectin synthesis [90]. The levels of adiponectin are significantly reduced in patients with NASH as compared to simple steatosis [91]. Thus adiponectin protects the liver against steatosis.

By activating cyclic-AMP dependent protein kinase (AMPK), adiponectin opposes fatty acid synthesis and promotes mitochondrial β -oxidation [92]. The anti-inflammatory effects of

adiponectin are due to its ability to block activation of NF- κ B which inhibits the release of pro-inflammatory cytokines such as TNF α and IL-6 [93]. The anti-inflammatory and hepatic lipid modulating effects of adiponectin may also be due to activation of peroxisome proliferator activated receptor- α (PPAR- α), as pharmacological treatment with PPAR- α agonist reverses experimental steatohepatitis [94]. Furthermore, PPAR- γ agonist, such as thiazolidinedione, stimulate adiponectin synthesis, and latter activates PPAR- α [95].

4.2. Leptin

Leptin is a gene product of *ob* gene and is produced by visceral adipocytes [96]. The levels of leptin directly correlate with body fat mass and adipocyte size [97]. Leptin has a potential dual action on NAFLD experimental models, exerting anti-steatotic, and pro-inflammatory/pro-fibrogenic actions [98]. In non-adipose tissue, such as liver, it prevents lipid accumulation by decreasing the expression of SREBP-1. Leptin exerts pro-fibrogenic effects by activating stellate cell in liver through hedgehock [99], mTOR [100] or kupffer cell-mediated TGF- β 1 secretion which then activates stellate cell [101]. In mice models of NASH, gut-derived endotoxins can induce hyper-responsiveness to leptin, with subsequent upregulation of CD14 and accelerated fibrosis [102]. Experimental studies have demonstrated that leptin deficiency in mice may lead to hepatic steatosis which can be reversed by leptin replacement. On the other hand, excess leptin contributes towards hepatic inflammation and fibrosis [98]. It may be a possibility that anti-steatosis effects of leptin may predominate in initial stages of NAFLD while pro-inflammatory and pro-fibrotic effects might take over during disease progression phase [103]. However, the exact magnitude of contribution by Leptin towards NAFLD remains to be elucidated.

4.3. IL-6 and TNF α

In severe obesity, adipocytes are major source of IL-6 production, as evident by a study results where IL-6 expression was 100-fold elevated in adipose tissue as compared to liver in obese patients [9]. Similarly, elevated TNF- α production has been observed in cultures of peripheral blood cells collected from obese patients with NASH [104]. These two important pro-inflammatory cytokines are found to be elevated in obese patients and weight loss is associated with dramatic decrease in serum levels of these cytokines [105, 106].

The liver is the target organ for adipose tissue-derived IL-6 and TNF α . It is known that high-fat diet, also called “inflammatory diet”, stimulates JNK1 (mitogen-activated protein kinase, associated with apoptosis) signaling in adipocytes, which leads to IL-6 secretion by adipocytes. IL-6 further acts on hepatic cell, leading to hepatic steatosis and hepatic insulin resistance [48]. Continuous exposure to elevated levels of IL-6/TNF- α leads to many of the histological features of NASH such as hepatocyte necrosis and apoptosis, neutrophil chemotaxis and activation of hepatic stellate cells [107]. Also, they caused insulin resistance by upregulating hepatic suppressor of cytokine signaling 3 (SOCS3) [108].

Transcription factor *nuclear factor- κ B kinase β* (NF- κ B) and its IKK2 subunits is also important mediators of chronic inflammatory states. Persistent activation of NF- κ B has been shown in animal models of NAFLD [109] and NASH [110].

4.4. Inflammasomes

Inflammasomes are large caspase-1 –activating multiprotein complexes that sense both endogenous and exogenous danger signals via intracellular NOD-like receptors (NLRs) [111]. Among the three prototypes of inflammasomes, NALP3 is associated with NAFLD and responds to danger signals by activating Caspase 1. Active Caspase-1 promotes cleavage and maturation of pro-inflammatory cytokines, such as IL-1 β , IL-18, IL-33, which further promote inflammation [111]. Gut-derived endotoxin and free fatty acid may act as danger associated molecular pattern (DAMP) which may lead to activation of inflammasomes [112].

To validate the role of inflammasomes in NASH, it was seen that there was increased gene expression of inflammasomes in livers of patients with NASH as compared to liver of healthy controls [113]. Furthermore, LPS and saturated fatty acids amplify the expression and activation of inflammasomes, and free fatty acids sensitize the hepatocytes to LPS-induced IL-1 β secretion [112]. It was seen observed that saturated fatty acids also directly induce hepatocyte apoptosis and activation of Caspase 8, which triggers the release of danger molecules from hepatocytes [112].

IL-1 β induces the suppression of peroxisome proliferator activated receptor- α (PPAR- α), activates the stellate cells to promote fibrosis and promotes TNF- α -induced cell death [114].

5. Toll-like receptors and innate immunity

Toll-like receptors are sensors of microbial and endogenous danger signals which are expressed in innate immune cells and liver parenchyma and contribute towards progression of NASH [90]. Upon activation by gut microbiota-released pathogen- or damage- associated molecular pattern (PAMP and DAMP), downstream signals are activated which lead to progression of NASH. TLR2, TLR4 and TLR9 are most commonly associated with NASH [90].

The gut-derived bacterial endotoxin is brought to liver via portal circulation where they activate the kupffer cell by way of TLR4 receptor complex. This interaction leads to activation of nuclear transcription factors, leading to release of pro-inflammatory mediators such as TNF α which can induce liver injury and fibrosis [115]. The role of TLR4 in pathogenesis of NASH is further supported by study where TLR4-deficient mice, which were fed high fructose diet, were protected from formation of reactive oxygen species, induction of TNF α expression in liver and insulin resistance [116].

TLR9 is located on endoplasmic reticulum and is activated by unmethylated CpG DNA particles that are released from bacteria [90]. It is known that TLR9 is involved in steatohepatitis

as TLR9-deficient mice are protected from liver inflammation [114]. In *CDA*A diet-(Choline-deficient amino acid defined diet) induced NASH, translocated bacterial DNA from gut binds to TLR9 receptor on kupffer cell to produce IL-1 β which activate hepatic stellate cells to induce liver fibrosis, and also stimulate hepatocytes for lipid accumulation and cell death [114]. The induction of hepatic steatosis is independent of TLR2, however, functional TLR2 receptors are found on kupffer cells which mediate liver inflammation and fibrosis in *CDA*A diet-induced NASH [117].

Recently, another data that implicated TLR5 in pathogenesis of metabolic syndrome was presented. It was reported that mice deficient in TLR5 developed all features of metabolic syndrome including, hyperphagia, obesity, insulin resistance, pancreatic inflammation and hepatic steatosis. It was proposed that TLR5 altered the gut microbiota and the finding were reproducible when microbiota from TLR5 $-/-$ mice was transferred to healthy mice [118]. However, another study did not find any such results in TLR5-deficient mice [119]. Indeed, more studies are needed to elicit role of TLR5 in steatohepatitis.

6. Endoplasmic reticulum (ER) stress

Endoplasmic reticulum (ER) is an important intracellular organelle involved in production, folding, post-translational modification and trafficking of secretory and membrane proteins. Also present in the ER is endoplasmic reticulum-associated degradation (ERAD) machinery that ensures that misfolded proteins are re-translocated back to cytoplasm for degradation by proteasomes [120]. Thus, ER serves as a quality control checkpoint, allowing only properly folded proteins to be transported to Golgi apparatus [121, 122] (**Figure 3**).

Any event that disturbs ER protein folding capacity- be it due to excessive protein synthesis, accumulation of misfolded proteins, depletion of calcium in ER, disturbance in redox regulation, glucose depletion, viral infection or high-fat diet (saturated fatty acid such as palmitic acid and stearic acid) [123] - leads to induction of evolutionarily conserved ER stress response, known as Unfolded protein response (UPR). The role of sensing ER stress and activating UPR is performed by three ER transmembrane proteins, mentioned as following:

1. RNA-dependent Protein kinase-like ER eukaryotic initiation factor-2 α Kinase (PERK)
2. Inositol-requiring ER-to-nucleus signaling protein1 (IRE1) and,
3. Transcription factor 6 (ATF6)

Each of these transmembrane proteins has an ER luminal domain to sense unfolded protein, a transfolded domain for targeting to the ER membrane, and a cytosolic domain to transmit signals to the transcriptional and/or translational apparatus [124]. In an unstressed cell, these ER proteins are maintained in an inactive state via their association with the ER chaperon protein, glucose-regulated protein 78 (GRP78)/Bip [125] and upon ER stress, unfolded/ misfolded proteins accumulation enhances the release of GRP78 from these stress-sensing proteins, leading to respective activation of PERK, ATF6 and IRE1 [126].

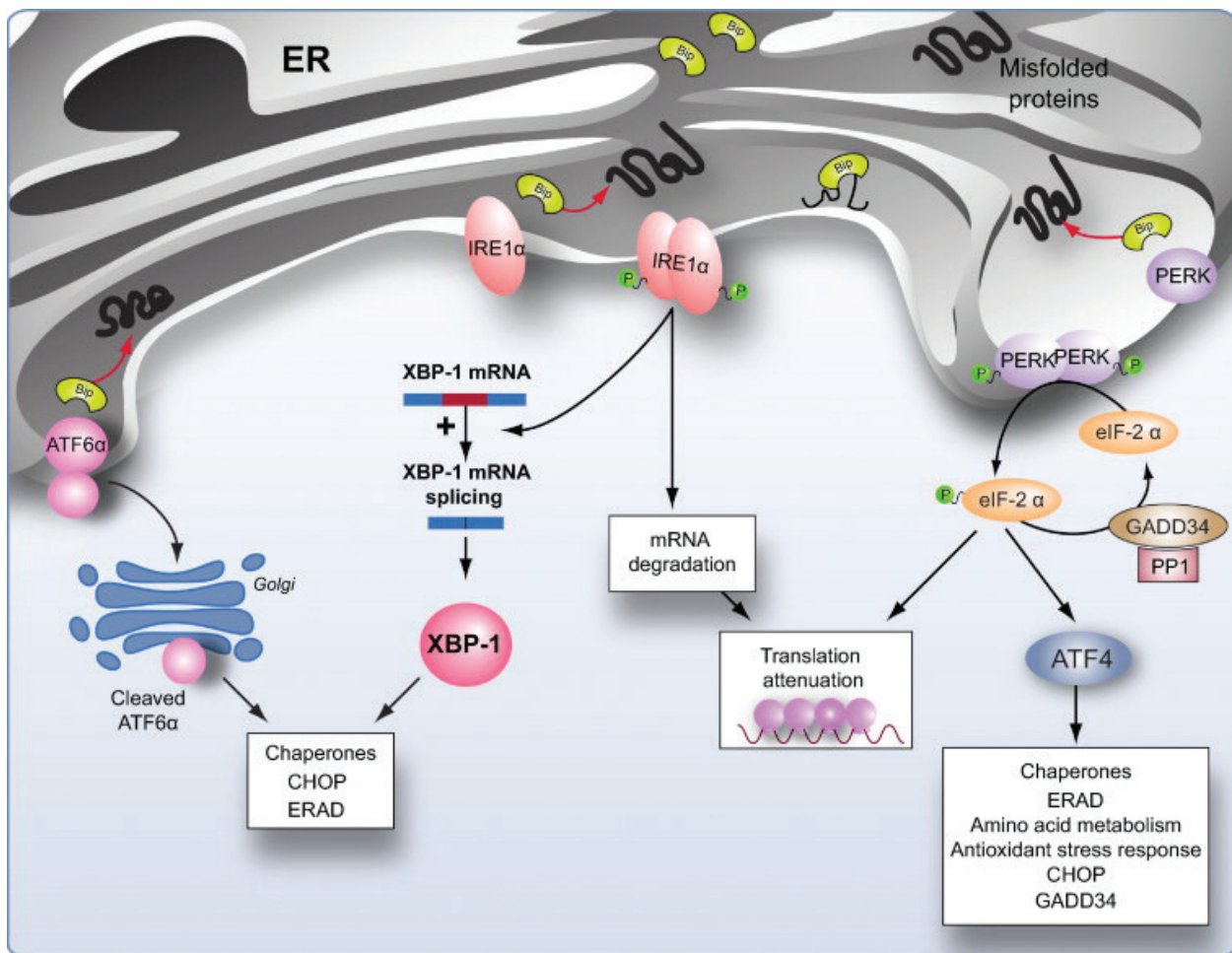


Figure 3. The figure demonstrating the three different pathways of unfolded protein response [135] (<https://doi.org/10.1016/j.jhep.2010.11.005>) (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Upon activation, PERK, IRE1 α and ATF6 induce signal transduction events that attenuate the accumulation of misfolded proteins in the ER by increasing expression of ER chaperons, inhibiting protein load on ER by decreasing mRNA translation, and stimulating retrograde transport of misfolded proteins from ER into cytosol for ubiquination and destruction by a process named ERAD [127]. However, under conditions where ER stress is chronically prolonged and the cell fails to restore homeostasis in ER, the UPR will initiate cell apoptosis [126].

6.1. Protein kinase RNA-like endoplasmic reticulum kinase (PERK)

Activation of PERK leads to phosphorylation of α -subunit of eukaryotic Initiation Factor 2 α (eIF-2 α), leading to its inactivation and hence, attenuation of mRNA translation and decreased protein load on the ER [128]. The phosphorylated eIF-2 α also causes preferential translation of UPR-dependent genes, such as activation transcription factor 4 (ATF 4). Further, ATF4 induces expression of several genes, including amino acid transporter, chaperons and CHOP [129] ("*C/EBP homologous protein*", also known as '*growth arrest and DNA damage (GADD 15)*').

CHOP is an ER-derived transcription factor which is an important mediator of ER stress-induced apoptosis, as evident from studies where deletion of CHOP lead to attenuation of hepatocellular apoptosis in alcohol- and cholestasis-induced liver disease [130]. Apoptosis-relevant targets of the CHOP transcription factor include:

1. *GADD34* [131]: promotes dephosphorylation of eIF2 α , thus reversing translation inhibition. This leads to accumulation of unfolded proteins in ER compartment, and, simultaneously, permits translation of mRNA encoding pro-apoptotic genes,
2. *DR5*: a caspase activating cell-surface death receptor, and
3. *Ero 1 α* (Endoplasmic Reticulum Oxidoreductase-1): hyperoxidises the ER and also activates the inositol triphosphate receptor (IP₃R), causing excessive transport of Calcium from ER to the mitochondria and thus causing cell death) [132].

CHOP also induces apoptosis via direct inhibition of Bcl-2 transcription [133] and induction of Bim expression [134]. Bcl-2 proteins are localized within the ER membrane and are protective (*anti-apoptotic*) against ER stress. This cytoprotective function is mainly due to the ability of Bcl-2 to lower steady-state levels of ER Ca²⁺ via IP₃Rs. The protective role of Bcl-2 in regulating ER Ca²⁺ can be inhibited by JNK-mediated phosphorylation of Bcl-2. The Phosphorylated Bcl-2 loses its anti-apoptotic function by being unable to bind *pro-apoptotic* “BH₃-only” members of Bcl-2 family (i.e. Bim), leading to increasing calcium release from ER, which is associated with mitochondrial calcium uptake. This leads to increased mitochondrial permeabilization, release of cytochrome C, and hence apoptosis [127]. Calcium release from ER can also activate Caplains, which may further proteolytically activate Caspase-12 to induce apoptosis [135].

6.2. Inositol-requiring ER-to-nucleus signaling protein1 (IRE1)

Accumulation of unfolded proteins in ER leads to activation of IRE1, which further processes an intron from X-box binding protein-1 (XBP-1) mRNA and permits synthesis of XBP-1 protein. XBP-1 binds to promoters of genes involved in UPR (encoding ER chaperons) and ERAD to restore homeostasis and prevent cellular toxicity [127]. Apart from cytoprotective effects, IRE1 can also recruits inflammatory factors (JNK and NF- κ B) which induce inflammatory response signaling [136], and apoptotic signal kinase-1 (ASK1) which causes downstream activation of stress kinases Jun-N-terminal Kinase (JNK) and p38 MAPK, that promotes apoptosis [137].

Activated JNK translocate to mitochondria and causes activation of Bim and Inhibition of Bcl-2. Activated JNK also induces the expression of pro-inflammatory genes by phosphorylating transcription factor activating protein-1 (AP-1) [138]. Activated p38 MAPK phosphorylates and activates CHOP [127] to causes apoptosis.

6.3. Transcription factor 6 (ATF6)

ATF6 belongs to CREB family of transcription factors. Activation of ATF6 leads to its release from ER membrane, processing in the Golgi and entry into the nucleus. It trans-activates ER stress related genes such as ER chaperones, XBP-1, foldases and CHOP [124].

6.4. Endoplasmic reticulum stress and Steatosis

Hepatocytes, being rich in both smooth and rough EPR, perform diverse metabolic functions, including lipoprotein and very-low-density lipoprotein (VLDL) assembly and secretion, cholesterol biosynthesis and xenobiotic metabolism [121]. The Sterol regulatory element-binding protein (SREBPs) are key regulators of lipid homeostasis and play crucial role in *de novo* lipogenesis [139], where SREBP-1 regulates fatty acid and triglycerides (TG) metabolism and SREBP-2 controls cholesterol metabolism and low density lipoprotein (LDL) receptor expression [140]. SREBP are transcription factors bound to ER membrane in inactive form and their activity is controlled within ER by interaction of *SREBP-Cleavage Activating Protein* (SCAP) and *Insulin regulated proteins* (Insigs). Insigs cause SREBP-SCAP complex to be retained within the ER and prevents SREBP-1 activation [141]. Under conditions of low sterols, Insigs are dissociated with SCAP, leading to migration of SREBP-SCAP complex to Golgi apparatus, where SREBP is processed to its active form by S1P and S2P [142]. Activated SREBP translocate to nucleus and regulates the various genes involved in lipid metabolism.

However, under ER Stress, rapid activation of precursor form of SREBP-1c and SREBP-1c target genes takes place, even in absence of Insulin [143]. Furthermore, ER stress induces proteolytic activation of SREBPs by increasing turnover of Insigs [142]. Hence, the recent data suggests that ER stress leads to hepatic steatosis by increasing *de novo* lipogenesis and upregulating the transcription of genes encoding for key lipogenic trans-activators and enzymes [121, 144].

Due to its high capacity for protein synthesis, ER stress plays an important part in mediating pathological changes in various liver diseases [135]. The signaling pathway activated by ER stress are implicated in lipotoxicity, Insulin Resistance, Inflammation and apoptotic cell death which are common to both NAFLD and NASH [123]. The presence of ER stress and activation of UPR in chronic disease (such as NAFLD) suggests that ability to resolve ER stress has been compromised. Inducing ER stress in individuals with genetically ablated eIF2 α , IRE1 α or ATF6 α leads to hepatic steatosis [145], suggesting that steatosis results from impairment in the capacity to oxidize fatty acids and augmented by impaired lipoprotein secretion. Thus, initially UPR aims to prevent steatosis and re-establish *ER homeostasis* after ER stress but selective impairment to the UPR that reduce the ability of UPR to resolve ER stress leads to development and exacerbation of hepatic steatosis. However, further work is needed to investigate this Homeostatic Model hypothesis.

It is now well-established that various arms of UPR and its downstream signaling molecules play role in regulation of lipid metabolism and induction of various hepatic pathologies.

6.4.1. PERK-eIF2 α -ATF4 pathway

The PERK-eIF2 α -ATF4 pathway is reported to regulate lipogenesis and hepatic steatosis. PERK-dependent signaling has been crucial to sustained expression of lipogenic enzymes such as fatty acid synthase (FAS), ATP-citrate Lyase, and stearoyl-CoA Desaturase-1 (SCD1) [146]. Phosphorylated eIF2 α (activated form) is associated with enhanced expression of adipogenic nuclear receptor peroxisome proliferator activated receptor γ (PPAR γ) and its upstream

regulators, and dephosphorylation of eIF2 α using GADD34 leads to diminished hepatosteatosis in animals fed high-fat diet [147]. Furthermore, activated ATF4 increases expression of lipogenic genes, such as PPAR γ , sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase (ACC), and FAS, in liver and white adipose tissue. Similarly, ATF4 knockout mice are protected from diet-induced hepatic steatosis [148, 149]. Thus, the current evidence suggests that PERK-eIF2 α -ATF4 pathway plays an important role in promoting lipogenesis.

6.4.2. IRE1 α -XBP1 pathway

The IRE1 α -XBP1 pathway plays an important part in maintenance of hepatic lipid homeostasis under ER stress and regulation of hepatic VLDL assembly and secretion [150]. IRE1 α is also required for efficient synthesis of ApoB-containing lipoproteins [151]. Mice with hepatocyte specific deletion of IRE1 α show increased hepatic steatosis and reduced plasma lipids under ER stress condition due to altered expression of key metabolic players (such as PPAR γ and C/EBP β) and of enzymes involved in Triglycerides biosynthesis [151]. Thus, these results indicate IRE1 α represses lipid accumulation in liver, especially under ER stress condition. However, the deletion of IRE1 α leads to loss of this protective role of IRE1 α , resulting in unresolved ER stress and hence, hepatic steatosis.

The role of XBP1 in lipogenesis is emphasized in a study where conditional disruption of XBP-1 in the liver of mice lead to reduced plasma level of triglycerides, cholesterol and free fatty acids, possibly due to decreased de novo lipogenesis [152]. XBP1 regulates lipogenesis in hepatocytes by directly binding to promoters of lipogenic genes such as SCD1 (Stearoyl-CoA Desaturase 1), DGAT2 (Diacylglycerol Transferase 2) and ACC2 (Acetyl-CoA carboxylase), thereby activating their transcription [152]. Thus under appropriate conditions, XBP1 promotes lipogenesis and contributes to hepatic lipogenesis.

6.4.3. Transcription factor 6 (ATF6)

ATF6 and SREBP are both ER membrane bound transcription factors, and nuclear ATF6 interacts with nuclear SREBP 2, antagonizing the SREBP2- regulated transcription of lipogenic genes and preventing lipid accumulation in cultures of liver cell [153]. Moreover, ATF2 α -knockout mice develop hepatic steatosis in response to ER stress, due to reduced fatty acid oxidation and decreased VLDL secretion [154].

Thus, taken together, all three proximal UPR sensors including PERK, IRE1 α and ATF6 α , regulate lipid stores in liver but the degree to which the UPR contributes to hepatic steatosis may depend on activation of three proximal UPR sensors relative to each other, coupled with appropriate downstream protein-protein and/or protein-DNA interaction [120].

6.5. Endoplasmic reticulum stress and progression towards NASH

Multiple factors, including but not limited to, insulin action, oxidative stress, cytokine mediated signaling, inflammation, bacterial endotoxin, and excess fatty acids function in concert and interact with UPR to provoke disease progression of NAFLD towards NASH.

6.5.1. ER stress and hepatic inflammation

Several Signaling pathways connect ER stress to hepatic inflammation:

- Reactive oxygen species (ROS)

Protein folding in ER is intimately linked to generation of ROS, such that each disulfide bond formation during oxidative protein folding leads to production of 1 ROS [155]. An elevated protein folding load, as in ER stress, leads to accumulation of ROS, which may lead to inflammation. In turn, the oxidative stress from ROS can disrupt ER homeostasis and induce ER stress [156].

However, in an unsurprising adaptive pathway, UPR activates an antioxidant program via transcription factor *Nrf2* (nuclear factor erythroid-derived 2-related factor 2) to prevent accumulation of ROS [157]. *Nrf2* is activated after phosphorylation by PERK pathway of UPR and it regulates the inducible expression of anti-oxidant response element-containing genes [157]. Importantly, *Nrf2* deletion results in rapid onset and progression of steatohepatitis in mice provided a methionine choline-deficient (MCD) diet [158]. ATF4, one of the other terminal player of PERK pathway, has also been an important transcription factor in maintenance of adequate Glutathione levels in cells [159]. Thus, PERK arm of UPR and its downstream players are directly related to regulation of anti-oxidant effects. In a recent study, IRE1 α -XBP1 branch of UPR was also found linked to anti-oxidant effect, where XBP1 deficiency leads to reduced catalase expression [160].

- NF- κ B and JNK

In response to ER stress, IRE1 α binds to adaptor protein tumor-necrosis factor α (TNF- α) receptor-associated factor 2 (TRAF2). IRE1 α -TRAF2 complex activates NF- κ B and JNK, both of which induce production of Pro-inflammatory cytokines, such as C-reactive protein (CRP), amyloid P-component, fibrinogen, and interleukin-6 (IL-6) [161].

The UPR-mediated signaling can lead to activation of NF- κ B not only via IRE1 α but also via PERK [162] and/or ATF6 pathway [163]. Activation of NF- κ B has been detected in steatohepatitis induced by MCD diet, however, the exact mechanism about how ER stress-induced signaling involving NF- κ B and JNK might regulate inflammation, cell survival and apoptosis in NAFLD is still unknown.

- PKR (double-stranded RNA-activated Protein Kinase)

PKR is an interferon-induced Serine/threonine protein kinase, activated by dsRNA, and is capable of activating NF- κ B and eIF2 α in response to dsRNA and oxidative stress, respectively [164]. PKR activity is increased in adipose tissue and liver of murine model of obesity [165]. With its ability to respond to pathogens, nutrients and organelle stress, PKR appears to be core component of inflammatory and immune pathways. However, depending on which key factor is activated downstream, that is, either NF- κ B (pro-apoptotic) or eIF2 α (anti-apoptotic), PKR may “serve as molecular clock to time the sequential events of survival and death” [166]. In summary, PKR affirms the complexity of UPR signaling and its downstream outcomes [120].

- CREBH

CREBH is a transcription factor belonging to CREB/ATF family of transcription factor and is required for liver synthesis of Amyloid P-component and CRP [167]. CREBH is activated via RIP process (Regulated Intramembrane Proteolysis: release and transport of ER resident protein from the ER membrane to Golgi for processing) upon ER stress. Other than ER stress, $\text{TNF}\alpha$, Interleukin 6 (IL6) and lipoprotein LPS also induce expression of CREBH [168]. This makes room for another revelation: ER stress in the liver may be linked to systemic inflammation via the RIP- mediated mobilization of CREBH [120].

6.5.2. ER stress and apoptosis

Apoptosis is an important component of disease progression in NAFLD [169] and is positively correlated with disease severity in NASH [24]. The failure of UPR in mitigating the ER stress leads to cell death via several mechanisms (**Figure 4**).

CHOP is one of the best characterized UPR-regulated pro-apoptotic protein [120]. CHOP is an ER-derived transcription factor activated downstream from PKR- and ATF6-pathway of UPR. Significance of CHOP in inducing apoptosis can be emphasized from results of study where silencing CHOP lead to decreased hepatocyte apoptosis in alcohol-induced liver disease [130] and attenuated cholestasis-induced liver fibrosis [170]. However, the role of CHOP is paradoxical in NAFLD, as demonstrated in study where CHOP deletion can reduce palmitate-induced apoptosis in hepatocyte cell line, whereas MCD diet-induced apoptosis was not reduced in CHOP knockout mice [171, 172].

CHOP has been described as an unstable protein compared to other protein chaperons like GRP78 [120]. Above described paradoxical role of CHOP in NAFLD makes way for observation: role of CHOP as a pro-apoptotic protein may be dependent on level of CHOP expression, the presence of factors which increase stability and/or protein-protein interactions that direct cell specific effects [173, 174]. Hence, future studies regarding role of CHOP in mice model are needed to elicit exact contributions of CHOP towards disease progression in NAFLD and NASH.

Furthermore, The IRE1 branch of the UPR, via its activation of JNK and Caspase 12 [175], and its interaction with Bax and Bak (two pro-apoptotic Bcl2 family members) [176], can also activate path towards apoptosis.

Additionally, another mechanism proposed for hepatic cell apoptosis is dysregulation in ER calcium flux. The ER calcium flux is regulated by ER stress, ER-localized protein and BCL-2 proteins interacting with other ER-localized proteins [177, 178]. The $\text{ERO1}\alpha$ -mediated activation of IP_3 , as mentioned earlier, can lead to disruption of ER calcium homeostasis [132]. This disruption inhibits sarco-endoplasmic reticulum Ca^{2+} -ATPase uptake pump, decreasing the folding capacity of ER, and hence, can induce ER stress and apoptosis [179]. Truncated variants of sarco-endoplasmic reticulum Ca^{2+} -ATPase have also been implicated in dysregulation

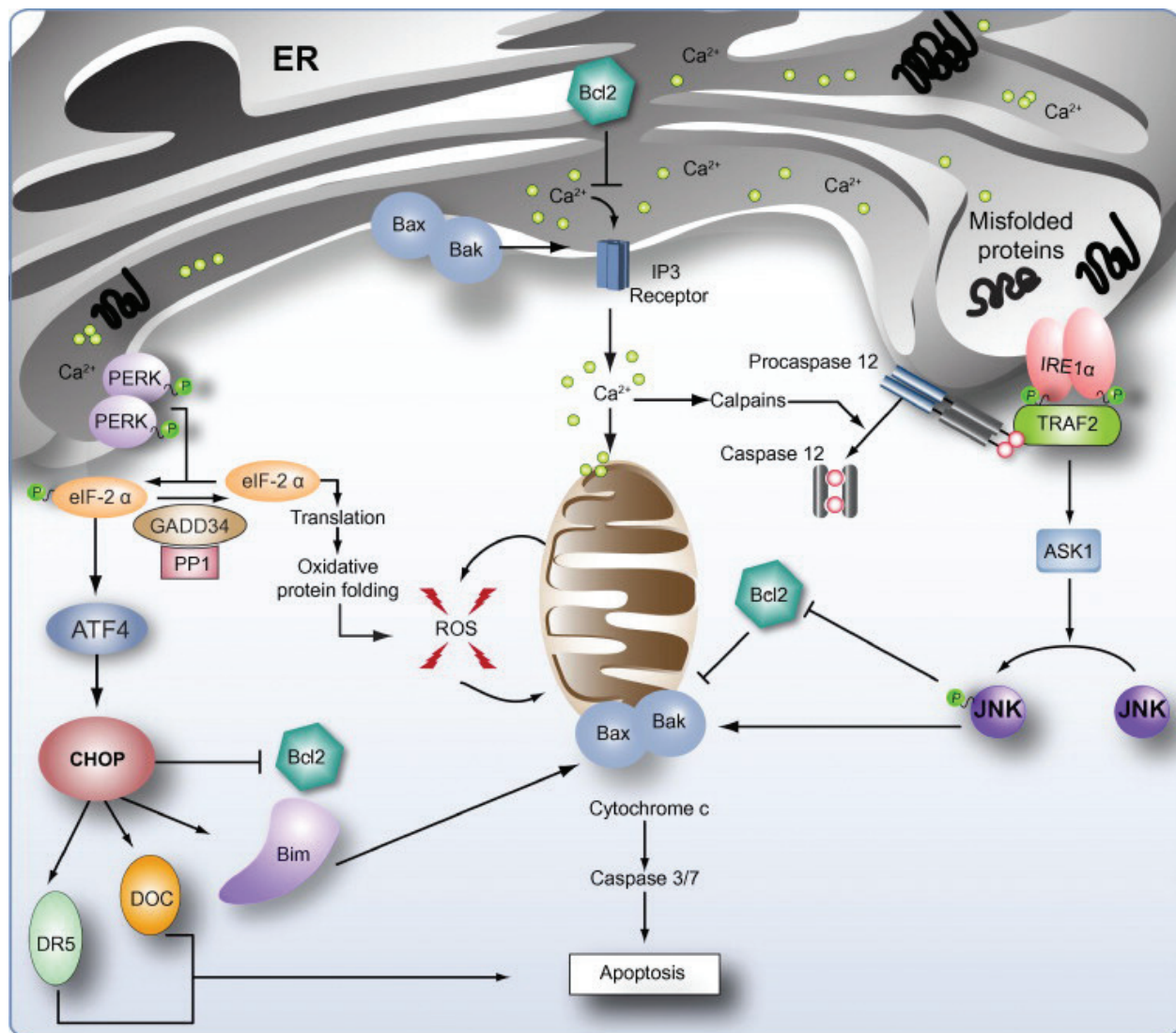


Figure 4. Different mechanisms for ER stress-induced apoptosis [135] (<https://doi.org/10.1016/j.jhep.2010.11.005>) (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

of Calcium flux [180]. Subsequently, the release of ER calcium and its uptake in mitochondria leads to mitochondrial membrane permeabilization, and activation of intrinsic apoptotic pathway. Recent studies also suggest that Smooth and Rough ER may be physically and functionally interacting with mitochondria via tethers and reduction in lengths of these tethers in response to pro-apoptotic agents might be one mechanism for apoptosis [181, 182].

7. Genetic factors

A possible explanation for observed inter-individual variability in susceptibility to NAFLD and progressive NASH is provided by genetics. *Patatin-like phospholipase domain-containing*

3 (PNPLA3), also called adiponutrin, is a protein expressed on endoplasmic reticulum, and on lipid droplets in hepatocytes and adipocytes [183]. It is activated after feeding and is the master regulator of lipogenesis by SREBP-1c. The 148 M variant of PNPLA3 is associated with increased expression of lipogenic transcription factor SREBP1c and alters the lipid catabolism [184]. The 148 M variant is associated with increased severity of NAFLD. In another study, patients with 148 M variant in genotype developed increased steatosis, with augmented lobular inflammation, hepatocellular ballooning and NASH [185].

7.1. Epigenetic modifications

Epigenetic modifications, mainly including microRNA, DNA methylation, histone modification and ubiquitination, refers to phenotypic changes irrespective of changes to underlying DNA. "miRNA" are small single stranded RNA molecules regulating mRNA degradation or translation inhibition, subsequently altering protein expression of target genes [186]. The miR-122, which accounts for 70% of all miRNA in the liver, is significantly under-expressed in NASH subjects compared to normal subjects [187]. Inhibition of miR-122 via antisense oligonucleotide in diet-induced obesity mouse models resulted in decreased mRNA expression of acetyl-CoA carboxylase-2, fatty acid synthase, SREBP1c, Stearoyl-CoA desaturase, all of which are key lipogenic factors in human NASH, and the histology showed marked improvement in liver steatosis [188]. In another study in mice, the plasma cholesterol level, hepatic fatty acid and cholesterol synthesis rate as well as HMG CoA reductase level were all significantly reduced after silencing miR-122 [189]. These findings strongly suggest the significance miR-122 in the regulation of lipid metabolism. Besides miR-122, miR-34a and miR-146b were shown to be significantly over-expressed in human NASH [187].

Similarly, aberrant methylation patterns of genomic DNA have been linked to NAFLD. A recent study found positive correlation between NAFLD and hepatic DNA methylation of GpC in PPAR- δ and mitochondrial transcription factor A (TFAM), with methylation being higher in NAFLD liver as compared to control [190]. In conclusion, genetic and epigenetic factors interact with other determinants to produce NAFLD phenotype and determine the rate of its progression [187].

8. Conclusion

The above laborious and detailed discussion on complex molecular mechanisms associated with disease progression in NAFLD does point towards the fact: The pathogenesis and progression of disease in NAFLD is complex interplay of different hormonal, immunological, metabolic, genetic and environmental components. Each component can act on its own or act in concert with other culprits to causes augmented damage to liver. However, more studies are needed to uncover the still unknown players, and to understand the interactions between the different players of multiple-hit model.

Appendix

ACC	acetyl-CoA carboxylase
AhR	aryl hydrocarbon receptor
AMPK	cyclin AMP dependent protein kinase
AP-1	activating protein-1
ASK1	apoptotic signal kinase-1
ATF6	activating transcription factor 6
CDAA diet	choline-deficient, amino acid defined diet
CHOP	CCAAT-enhancer-binding protein homologous protein
ChREBP	carbohydrate response element-binding protein
CREB	cAMP response element-binding protein
DAMP	danger associated molecular pattern
DGAT	diacylglycerol transferase
DR5	death receptor 5
eIF-2 α	eukaryotic initiation factor-2 α
ER	endoplasmic reticulum
Ero 1 α	endoplasmic reticulum oxidoreductase 1
Fiaf	fasting-induced adipocyte factor
FXR	farnesoid X receptor
GADD	growth arrest and DNA damage
HNE	4-hydroxy-2-noneal
IKKb	Inhibitor of nuclear factor kappa-B kinase subunit beta
Insigs	insulin regulated proteins
IRE 1	inositol-requiring ER-to-nucleus signaling protein1
IRS	insulin receptor substrate
JNK	Jun N-terminal Kinase

LPS	lipopolysaccharide
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
mTOR	mechanistic target of rapamycin
NAFLD	nonalcoholic fatty liver disease
NALP	NACHT, LRR and PYD domains-containing protein 3
NF- κ B	nuclear factor κ beta
NLR	nod-like receptor
NLRP	NLR family, pyrin domain-containing 3 inflammasomes
Nrf2	nuclear factor-erythroid-derived 2-related factor 2
PERK	protein kinase RNA- like endoplasmic reticulum kinase
PKC- γ	protein kinase C-gamma
PKR	RNA activated protein kinase
PNPLA3	patatin-like phospholipase domain-containing 3
PPAR	peroxisome proliferator activated receptor
ROS	reactive oxygen species
SCAP	SREBP-cleavage activating proteins
SCD1	stearoyl-CoA desaturase-1
SCFA	short chain fatty acid
SOCS	suppressor of cytokine signaling
SREBP	sterol regulatory element-binding protein
TBARS	thiobarbituric acid reacting substrate
TFAM	mitochondrial transcription factor A
TGF- β 1	transforming growth factor- β 1
TLR	toll-like receptor
TNF	tumor necrosis factor

TNFR	tumor necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
UPR	unfolded protein response
XBP1	X-box binding protein-1

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