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The Unfolded Protein Response and Hepatic Lipid Metabolism in Non-alcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease is a major public health burden. Although many features of nonalcoholic fatty liver disease pathogenesis are known, the specific mechanisms and susceptibilities that determine an individual's risk of developing nonalcoholic steatohepatitis versus isolated steatosis are not well delineated. The predominant and defining histologic and imaging characteristic of nonalcoholic fatty liver disease is the accumulation of lipids. Dysregulation of lipid homeostasis in hepatocytes leads to transient generation or accumulation of toxic lipids that result in endoplasmic reticulum (ER) stress with inflammation, hepatocellular damage, and apoptosis. ER stress activates the unfolded protein response (UPR) which is classically viewed as an adaptive pathway to maintain protein folding homeostasis. Recent studies have uncovered the contribution of the UPR sensors in the regulation of hepatic steatosis and in the cellular response to lipotoxic stress. Interestingly, the UPR sensors can be directly activated by toxic lipids, independently of the accumulation of misfolded proteins, termed lipotoxic and proteotoxic stress, respectively. The dual function of the UPR sensors in protein and lipid homeostasis suggests that these two types of stress are interconnected likely due to the central role of the ER in protein folding and trafficking and lipid biosynthesis and trafficking, such that perturbations in either impact the function of the ER and activate the UPR sensors in an effort to restore homeostasis. The precise molecular similarities and differences between proteotoxic and lipotoxic ER stress are beginning to be understood. Herein, we provide an overview of the mechanisms involved in the activation and cross-talk between the UPR sensors, hepatic lipid metabolism, and lipotoxic stress, and discuss the possible therapeutic potential of targeting the UPR in nonalcoholic fatty liver disease.

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Conflict of interest statement

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Keywords

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is currently the leading cause of chronic liver disease and estimated to affect approximately 30% of the U.S population (Satapathy & Sanyal, 2015; Younossi, et al., 2018). NAFLD is a heterogeneous disease ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) with or without hepatic fibrosis. Its prevalence in specific groups, such as patients with type 2 diabetes or morbid obesity is even higher, occurring in roughly 60 and 80% of these individuals, respectively (Amor & Perea, 2019; Blond, et al., 2017; Younossi, et al., 2018). NAFLD is characterized by the accumulation of various lipid species within hepatocytes (Marra & Svegliati-Baroni, 2018). The lipids that can activate and induce progressive liver injury are defined as toxic lipids in NASH pathogenesis. In general, the saturated fatty acids (SFAs) such as palmitate, ceramides, lysophosphatidylcholine (LPC), and free cholesterol are directly cytotoxic, whereas the monounsaturated free fatty acids, such as oleate and palmitoleate, may protect from SFA-induced toxicity (Akazawa, et al., 2010; Hirsova, Ibrahim, Gores, & Malhi, 2016; Malhi, Barreyro, Isomoto, Bronk, & Gores, 2007; Malhi, Bronk, Werneburg, & Gores, 2006). The consequent hepatocellular damage characterized by inflammation and hepatocellular apoptosis is linked to dysfunction of the endoplasmic reticulum (ER), resulting from toxic lipids termed as lipotoxic ER stress or lipid bilayer stress (Pagliassotti, 2012; Volmer, van der Ploeg, & Ron, 2013). Lipotoxic ER stress appears to follow a continuum that progresses from non-lethal or sub-lethal responses to ER stress-induced cell death. Lipotoxic ER stress may also be proinflammatory as we have recently demonstrated that lipotoxic sublethal ER stress response leads to the release of proinflammatory extracellular vesicles (Kakazu, Mauer, Yin, & Malhi, 2016). Steatosis and ER stress are also features of alcoholic hepatitis and ER stress is now accepted as an important mechanism in alcoholic hepatitis pathogenesis and progression (Joshi-Barve, Kirpich, Cave, Marsano, & McClain, 2015; Masouminia, et al., 2016). The role of ER stress in alcoholic hepatitis is reviewed elsewhere in detail (Ji, 2014; Joshi-Barve, et al., 2015; Masouminia, et al., 2016). This review focuses on the UPR in lipid metabolism and NAFLD. We review the signaling pathways mediated by the canonical UPR, how the UPR sensors contribute to and respond to perturbation in hepatic lipid homeostasis under ER stress or independently, and lipotoxic ER stress. Lastly, we discuss the therapeutic potential of targeting the UPR in NAFLD.

2. Unfolded Protein Response

The ER is an essential subcellular compartment responsible for the synthesis and folding of proteins that traffic through the secretory pathway in the cell. Protein folding is sensitive to alterations in ER homeostasis including Ca^{2+} levels, energy and nutrient availability, as well as the protein-folding load in the ER (S. Wang & Kaufman, 2014) (Walter & Ron, 2011). Perturbations in these pathways interfere with protein folding in the ER leading to proteotoxic ER stress, which in turn activates the UPR. The UPR is initially an adaptive

signaling pathway which aims to elicit global cellular changes such as attenuation of translation and activate specific pathways of protein folding and degradation to restore ER homeostasis (S. Wang & Kaufman, 2012). When ER homeostasis cannot be restored ER stress-induced apoptosis occurs. Canonically, the UPR is activated via the luminal domains of three principal transmembrane sensors: inositol-requiring enzyme (IRE)-1α, protein kinase RNA-like ER kinase (PERK), and activating transcription factor (ATF)-6α.

A. IRE1α pathway

The IRE1α pathway is the most conserved arm of the UPR. IRE1α is a ubiquitously expressed transmembrane protein that possesses dual enzymatic activities of serine/threonine kinase and endoribonuclease (RNase) on its cytosolic domain (Lebeaupin, Vallee, Hazari, et al., 2018). In the physiologic state, the luminal domain of IRE1α is bound by the ER chaperone binding immunoglobulin protein (BiP) and remains inactive. In response to the accumulation of unfolded proteins, BiP releases IRE1α allowing its activation by dimerization and autophosphorylation via kinase activity, inducing a conformational change that activates the RNase activity. Alternatively, unfolded proteins may directly bind to the luminal domain of IRE1α, inducing allosteric changes that trigger its activation (Karagoz, et al., 2017). Activated IRE1α induces unconventional (cytosolic) excision of 26 nucleotides from the mRNA encoding X-box binding protein 1 (XBP1) to generate spliced XBP1. Spliced XBP1 is a transcription factor which upregulates the expression of several ER chaperones to increase protein folding capacity and also ER-associated degradation (ERAD). In irremediable ER stress, IRE1α RNase activity becomes promiscuous, leading to degradation of many other ER-bound mRNAs and microRNAs through regulated IRE1α-dependent decay (RIDD) (D. Han, et al., 2009; Hollien, et al., 2009; Hollien & Weissman, 2006). Enhanced RIDD leads to pro-apoptotic signaling by rapid decay of select micro-RNAs that normally repress the pro-apoptotic pathway (Upton, et al., 2012). Activated IRE1α also recruits tumor necrosis factor receptor-associated factor 2 leading to activation of the c-Jun N-terminal kinase (JNK) via apoptosis-signal-regulating kinase 1 and the activation of nuclear factor κ B (NFκB) (Hu, Han, Couvillon, Kaufman, & Exton, 2006; Tam, Mercado, Hoffmann, & Niwa, 2012; Urano, et al., 2000). JNK promotes apoptosis via Bcl-2 homology (BH) 3-only proteins and the Bax/Bak-dependent mitochondrial apoptotic machinery.

B. PERK pathway

PERK is a transmembrane protein with an N-terminal stress-sensing domain and a cytosolic kinase domain. Similar to IRE1α, the luminal domain of inactive PERK is bound to BiP (Bertolotti, Zhang, Hendershot, Harding, & Ron, 2000). PERK activation relies on dimerization and tetramerization mediated via its luminal domain leading to auto-phosphorylation upon the sensing of unfolded proteins (Carrara, Prischi, Nowak, & Ali, 2015). After activation, PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) (N. Donnelly, Gorman, Gupta, & Samali, 2013). To attenuate overall protein overload, phosphorylated eIF2α holds back mRNA translation preventing 80S ribosome assembly, except for select mRNAs including the transcription factor ATF4 (Blais, et al., 2004; Harding, Zhang, & Ron, 1999). ATF4 then activates the transcription of specific UPR target genes, such as CCAAT-enhancer-binding protein homologous protein (CHOP) and the

growth arrest and DNA damage-inducible protein (GADD34). CHOP is a transcription factor that triggers cell death by inducing genes involved in apoptosis in the presence of ER stress (Nishitoh, 2012) and may also promote ER stress-induced cell death by premature translational recovery (J. Han, et al., 2013). In contrast, GADD34 forms a feedback loop by dephosphorylation of eIF2α and recovery from translational inhibition in the UPR (Novoa, Zeng, Harding, & Ron, 2001).

C. ATF6α pathway

ATF6α is a type II ER transmembrane protein with a cytosolic bZip transcription factor domain and specialized in the regulation of ER quality control proteins (Adachi, et al., 2008). Upon proteotoxic ER stress, ATF6α is packed into coat protein complex II vesicles and transported to the Golgi (Schindler & Schekman, 2009). In the Golgi apparatus it is cleaved by site-1 protease (S1P) and site-2 protease (S2P), releasing a 50 kDa cytosolic fragment that migrates to the nucleus, and is referred to as ATF6N or ATF6F. Though the activation of ATF6α in the Golgi is similar to SREBPs (discussed below), the proximal pathways that mediate translocation of ATF6α or SREBPs to the Golgi are distinct. ATF6α is activated by the accumulation of unfolded proteins and SREBPs are regulated by sterols (Haze, Yoshida, Yanagi, Yura, & Mori, 1999; Horton, Goldstein, & Brown, 2002). Cleaved ATF6α activates UPR target genes including ERAD, phospholipid synthesis, and cooperatively the transcription of XBP1 target genes via direct binding to the ER stress response element (Bommiasamy, et al., 2009; Ho, Xu, & Thibault, 2018; Wu, et al., 2007; Yoshida, Matsui, Yamamoto, Okada, & Mori, 2001). Enforced expression of cleaved ATF6α can also upregulate cholesterol synthesis (Maruyama, Kamoshida, Shimizu, Inoue, & Sato, 2013).

3. Hepatic lipid Metabolism

Lipids are part of the cell structure, and are involved in essential functions such as cellular homeostasis, cell-to-cell communication and regulation of inflammation. The liver plays a predominant role in regulating lipid homeostasis via the major lipid metabolic pathways, including de novo lipogenesis which includes fatty acid (FA) synthesis, triglyceride (TG) synthesis and storage, synthesis of complex lipids such as cholesterol, ceramide and phospholipid, lipoprotein synthesis and secretion, lipolysis and FA oxidation (Simha & Garg, 2006; S. Wang & Kaufman, 2014). Many of these functions are housed in the ER, especially *de novo* ceramide biosynthesis, cholesterol synthesis, lipid droplet formation and export of TG as very low density lipoprotein (VLDL) (Fagone & Jackowski, 2009).

A. De Novo Lipogenesis

Hepatic de novo lipogenesis is regulated by ER membrane-bound transcription factors, the sterol regulatory element binding proteins (SREBPs), SREBP-1a, SREBP-1c, and SREBP-2, of which SREBP-1c and SREBP-2 are predominantly expressed in the liver (Brown & Goldstein, 1997; Eberle, Hegarty, Bossard, Ferre, & Foufelle, 2004; Horton, et al., 2002). SREBP-1c controls FA and TG biosynthesis, while SREBP-2 controls cholesterol metabolism and low density lipoprotein receptor expression (Hinz, Giebel, & Campos-Ortega, 1994; Yokoyama, et al., 1993; Zhou & Liu, 2014). SREBP-1c and SREBP-2

expression can be regulated transcriptionally and post-transcriptionally, comprehensively reviewed elsewhere (Horton, et al., 2002). Post-transcriptional processing of SREBPs is suppressed by sterols. SREBP-1c is embedded in the ER membranes bound to the protein SREBP cleavage-activating protein (SCAP). SCAP interacts with the insulin-induced gene (INSIG) protein which retains the complex SREBP-1c/SCAP in the ER. Similar to canonical regulation by sterols and insulin, under ER stress, SCAP dissociates from INSIG and the complex SCAP/SREBP-1c is transferred to the Golgi apparatus in coat protein II vesicles (Kammoun, et al., 2009). SREBP-1c undergoes regulated intramembrane proteolysis by S1P and S2P to release a cytosolic transcription factor that traffics to the nucleus to induce the expression of genes involved in de novo FA synthesis including acetyl-CoA carboxylase (ACC), FA synthase, the long-chain elongase and stearolyl-CoA desaturase (SCD) (Brown & Goldstein, 1997; Foufelle & Ferre, 2002; Postic & Girard, 2008; S. Wang & Kaufman, 2014). Cleavage of SREBP-2 occurs in a manner similar to SREBP-1c via dissociation of INSIG from SREBP-2/SCAP leading to transcriptional upregulation of cholesterol biosynthetic genes (Dong, Tang, & Chen, 2012; Sakakura, et al., 2001). SREBP-1c and SREBP-2

B. Hepatic lipid storage in the form of TGs

The hallmark of NAFLD is the accumulation of fat in the hepatocytes, in the form of lipid droplets containing TG (Marra & Svegliati-Baroni, 2018). TGs are synthesized from FAs and glycerol by a group of ER-localized acyltransferase enzymes including glycerol-3-phosphate acyltransferase, acylglycerolphosphate acyltransferase, monoacylglycerol acyltransferase or diacylcglycerol acyltransferase (Gimeno & Cao, 2008; Q. Liu, Siloto, Lehner, Stone, & Weselake, 2012). The majority of TG produced by the ER further drives the synthesis of lipid droplets, which originate from the ER membrane and form phospholipid monolayer enclosed droplet structures containing a high abundance of hydrophobic TGs, cholesterol ester and droplet structural proteins. Lipid droplets composed of TGs and cholesterol esters serve as a storage reservoir to prevent toxic accumulation of free FAs and cholesterol (Olofsson, et al., 2008; Reue, 2011). In addition, membrane phospholipids including phosphatidylcholine or phosphatidylethanolamine are synthesized via ER-localized enzymes, including the diacylglycerol choline/ethanolaminephosphotransferases (Fagone & Jackowski, 2009; Lagace & Ridgway, 2013).

C. Export of TG as VLDL

TGs are secreted from the liver in the form of VLDL through the fusion of apolipoprotein B-100 (Cohen & Fisher, 2013). VLDL synthesis is initiated by co-translational translocation of apolipoprotein B (ApoB) into the ER lumen for assembly with bulk neutral lipids, especially TGs (Tiwari & Siddiqi, 2012). The microsomal TG transfer protein (MTP) protein complex, composed of MTP and protein disulfide isomerase 1, is thought to play a critical role in facilitating the accretion of TGs into the ER lumen for ApoB lipidation and prevent intrahepatic TG accumulation (Hussain, Shi, & Dreizen, 2003). VLDL assembly and secretion are complex processes involving an array of protein and lipid factors (Rutledge, Su, & Adeli, 2010). A significant portion of ER chaperone proteins were found to be associated with ApoB, which could either promote ApoB folding and lipidation or mediate ApoB co-translational or post-translational degradation (Olofsson & Boren, 2012).

Overproduction of hepatic VLDL particles can cause dyslipidemia, which is frequently observed in NAFLD and tightly associated with an increased risk of cardiovascular disease in obese patients with type 2 diabetes and NAFLD (Hassing, et al., 2012). Thus, the ER in hepatocytes not only plays an important role in intrahepatic lipid homeostasis, but is also tightly linked to circulating lipid homeostasis.

4. Unfolded protein response and hepatic lipid metabolism

Protein misfolding in the ER with associated ER stress causes hepatic steatosis, which is observed in mice with genetic ablation of the UPR sensors (Rutkowski, et al., 2008). ER stress-induced hepatic steatosis is multifactorial and can occur via the regulation of lipogenesis, VLDL secretion, and FA oxidation. Not only is steatosis observed in response to ER stress, recent studies have demonstrated that the UPR sensors directly play a role in the regulation of lipid metabolism, and lipotoxicity can activate an ER stress response, as discussed below. Overall, the interplay between UPR sensors and hepatic lipid metabolism is complex and likely only partially understood.

A. IRE1α/XBP1 pathway

IRE1α is a key regulator of hepatic lipid homeostasis through repressing hepatic lipid accumulation and maintaining lipoprotein secretion. Under ER stress with chemical inducing agent, mice with hepatocyte-specific deletion of IRE1α developed hepatic steatosis and increased expression of several transcriptional genes involved in lipid metabolism, including *CCAAT/enhancer-binding protein (C/EBP) β, C/EBPδ, peroxisome proliferator-activated receptor (PPAR) γ*, and enzymes involved in TG biosynthesis pointing to increased de novo lipogenesis (Zhang, et al., 2011). On the other hand, in the genetic absence of *Ire1α* in hepatocytes, under basal conditions there was reduced protein disulfide isomerase, which acts with MTP to promote the delivery of neutral lipids to the smooth ER lumen for VLDL assembly (S. Wang, et al., 2012). Thus, hepatocyte-specific *Ire1α* deletion did not alter lipogenesis, but rather, specifically impaired VLDL assembly and secretion, leading to steatosis (S. Wang, et al., 2012; Zhang, et al., 2011). In addition, experimental manipulation of XBP1, a downstream transcription factor of IRE1α, highlighted a critical role for IRE1α in lipid metabolism. Hepatic deletion of *Xbp1* decreased de novo hepatic lipogenesis, leading to reduced serum TG, cholesterol, and free FAs by regulation of lipogenic genes including *diacylglycerol acyltransferase 2, Scd1, and Acc2* (Lee, Scapa, Cohen, & Glimcher, 2008). Later studies demonstrated that *Xbp1* deletion triggers feedback hyperactivation of IRE1α, inducing RIDD of cytosolic mRNAs encoding lipid metabolism functions, thereby reducing plasma TG and cholesterol in these mice (So, et al., 2012). IRE1α is also a target for inflammatory signaling as recently demonstrated via inducible nitric oxide synthase mediated nitrosylation of IRE1α which results in a reduction in IRE1α mediated *Xbp1* splicing and impaired glucose homeostasis (L. Yang, et al., 2015), and impaired degradation of microRNAs which repress PPARα and the deacetylase sirtuin 1, thus decreasing FA oxidation and lipolysis (J. M. Wang, et al., 2018). Elevated plasma VLDL is a component of the dyslipidemia commonly observed in obesity-associated NAFLD. It is possible that IRE1α activation leads to increased rates of VLDL secretion in obesity, and thus contributes

to dyslipidemia. These studies place IRE1α in a central position in mitigating hepatic steatosis via repression of lipolysis and export of VLDL.

On the other hand, there is evidence that constraining IRE1α activity in hepatocytes and in mice impairs inflammasome activation, liver injury, inflammation and hepatocyte apoptosis (Lebeaupin, Vallee, Rousseau, et al., 2018). Recently, it has been demonstrated that IRE1α activation leads to the release of proinflammatory extracellular vesicles from hepatocytes (Kakazu, et al., 2016). Thus inhibition of proinflammatory extracellular vesicle release may be a possible explanation for why inhibition of IRE1α activation impairs hepatic inflammation. Altogether, observations on the role of IRE1α in regulation of lipid metabolism point toward IRE1α-mediated mitigation of steatosis which would suggest that IRE1α activation might improve hepatic steatosis. However, IRE1α also promotes inflammation. We propose that the predominant output of IRE1α activation in the subset of NAFLD that develop progressive hepatic injury and inflammation (NASH) is proinflammatory. The determinants of IRE1α fates suggest a role for Bax Inhibitor 1 (BI-1), a negative regulator of IRE1α activation, and the magnitude of RIDD. For example, absence of BI-1 worsens NASH, potentially by hyperactivation of IRE1α with resultant persistent XBP1 processing and greater RIDD, with activation of the inflammasome, inflammation, and liver injury.

B. PERK-eIF2α pathway

The PERK pathway also regulates hepatic lipid metabolism. Antipsychotic drug-induced PERK and eIF2α phosphorylation results in increased hepatic steatosis through activation of SREBP-1c and SREBP-2 (Lauressergues, et al., 2012). The eIF2α phosphorylation resistant knockin mutant mice also showed exacerbation of lipid droplet coat proteins in response to pharmacologic ER stress (Rutkowski, et al., 2008). Transgenic mice with compromised eIF2α phosphorylation by overexpressing GADD34, eIF2α specific phosphatase, are protected from high-fat diet (HFD)-induced hepatic steatosis (Oyadomari, Harding, Zhang, Oyadomari, & Ron, 2008). ATF4, the downstream effector of the PERK/eIF2α pathway, is involved in lipid metabolism. *Atf4*$^{-/-}$ mice displayed high-carbohydrate diet induced suppression of SCD1 expression which was protective for hepatic steatosis (H. Li, et al., 2011). *Atf4* depletion also attenuated lipid accumulation accompanied by a significant reduction in hepatic expression of SREBP-1c, ACC, and FA synthase (G. Xiao, et al., 2013).

CHOP is involved in the disruption of FA oxidation and lipoprotein secretion through suppression of C/EBPα (Rutkowski, et al., 2008). Under pharmacologic ER stress, deletion of *Chop* in mice showed less suppressed expression of transcriptional regulators, including C/EBPα, PPARα, SREBP-1, and PPAR gamma coactivator 1α, compared with wild-type mice. However, though *Chop*$^{-/-}$ mice exhibit greater adiposity basally, they showed reduced expression of lipogenic genes with less lipid accumulation than wild-type mice upon human immunodeficiency virus protease inhibitor (Y. Wang, et al., 2013). Therefore, it is necessary to elucidate the exact molecular and cellular mechanisms underlying CHOP-mediated dysregulation of hepatic lipid metabolism.

C. ATF6α pathway

Transcriptional induction of mammalian ER quality control proteins is mediated by combined action of ATF6α and XBP1 (Yamamoto, et al., 2007). ATF6α stimulates hepatic FA oxidation possibly through interaction with PPARα. The PPARα/retinoid X receptor heterodimer may serve as the key functional regulator transducing ATF6α signaling to the transcription of genes involving hepatic FA oxidation, such as carnitine palmitoyltransferase 1A, medium-chain acyl-coenzyme A dehydrogenase, and fibroblast growth factor 21(FGF21), via the PPAR response element sequence (Chen, et al., 2016). Cleaved ATF6α binds to sterol regulatory response element-bound SREBP-2 and recruits histone deacetylase 1, thus inhibiting sterol regulatory response element-mediated transcriptional activation and activate genes involved in UPR (Zeng, et al., 2004). *Atf6α* knockout mice accumulated lipids in the liver, due to blockage of β-oxidation of FAs and the suppression of VLDL formation (DeZwaan-McCabe, et al., 2017; Yamamoto, et al., 2010). When fed a HFD, $Atf6\alpha^{-/-}$ mice developed hepatic steatosis and glucose intolerance in association with increased expression of SREBP-1c (Usui, et al., 2012).

5. Lipotoxic ER stress

Lipotoxicity is defined as dysregulation of the lipid environment and/or intracellular composition leading to the accumulation or transient generation of toxic lipids, which may result in hepatocyte injury or death (Malhi & Gores, 2008). Toxic lipid classes include the following; the SFA palmitate, sphingolipids including C16:0 ceramide, the phospholipid LPC, and free cholesterol (Musso, Cassader, Paschetta, & Gambino, 2018). ER stress is one manifestation of lipotoxicity due to these lipid classes, whereas the monounsaturated free FAs, such as oleate and palmitoleate, may protect from SFA-induced toxicity (Fig 1) (Akazawa, et al., 2010; Malhi, et al., 2007; Malhi, et al., 2006).

A. Palmitate

Palmitate in hepatocytes is converted to palmitoyl-CoA and under excess conditions can be incorporated in to various lipids (Figure 2). Palmitate can induce the accumulation of ceramide from de novo synthesis at the ER, increase LPC formation via phospholipase A2 (PLA2) action on phosphatidylcholine (PC), which in turn is derived from diacylglycerol (Hirsova, et al., 2016). Excess palmitate can also lead to the accumulation of di-saturated glycerolipids in the ER which trigger sustained IRE1α and PERK activation (Ariyama, Kono, Matsuda, Inoue, & Arai, 2010; Volmer, et al., 2013). The increased availability of palmitate by upregulating ER localized glycerol-3-phosphate acyltransferase can incorporate this FA into membrane lipids (Piccolis, et al., 2019). Additionally, in hepatocytes, palmitate can increase the de novo biosynthesis of saturated phospholipids contributing to palmitate-induced lipotoxic ER stress (Leamy, et al., 2014). Therefore, homeostatic pathways that maintain membrane lipid saturation during palmitate stress are important to prevent lipotoxicity. Palmitate-induced ER stress can result in apoptosis, such that knockdown of *PERK* gene expression significantly inhibited palmitate-induced apoptosis (Cao, et al., 2012). Attenuation of ER stress by BiP overexpression, suppressed palmitate-induced cell death through regulation of IRE1α, phosphorylated eIF2α and CHOP (Gu, et al., 2010). Downstream of persistent UPR activation, palmitate-mediated hepatocyte lipoapoptosis was

triggered by JNK activation and CHOP- and JNK-dependent upregulation of the potent proapoptotic BH3-only protein p53 upregulated modulator of apoptosis (PUMA) (Cazanave, et al., 2010; Kakisaka, et al., 2012).

B. Ceramides

Ceramides are members of the sphingolipid family including sphingosine and sphingosine 1-phosphate (**SP1P**) and are integral to the structure of the lipid bilayer that makes up cell membranes (Hannun & Obeid, 2008; Pagadala, Kasumov, McCullough, Zein, & Kirwan, 2012). Ceramides can be synthesized *de novo* from serine and palmitate by the sequential action of 3 ER resident enzymes (serine palmitoyltransferase, ceramide synthase, and dihydroceramide desaturase) or generated from hydrolysis of plasma membrane sphingomyelin into ceramide and phosphocholine by the enzyme sphingomyelinase (Musso, et al., 2018). Sphingolipids are necessary to maintain lipid homeostasis and prevent ER stress, although it is unclear how alterations in sphingolipid biosynthesis are sensed by the IRE1α and PERK (Breslow, 2013). ATF6α can be activated by specific sphingolipids which are recognized by a motif in its transmembrane domain (Tam, et al., 2018). The deletion of Orm1 or Orm2 in yeast, which negatively regulate serine palmitoyltransferase complex in sphingolipid synthesis, led to lipid-mediated UPR activation (Futerman & Riezman, 2005; S. Han, Lone, Schneiter, & Chang, 2010; Ho, et al., 2018; Jonikas, et al., 2009; M. Liu, Huang, Polu, Schneiter, & Chang, 2012). In addition, the accumulation of C16 ceramide induced ER stress by the disturbance in Ca^{2+} homeostasis and led to cell death through the activation of the PERK/ATF4 and ATF6α arms of the UPR, resulting in the induction of CHOP expression (Aflaki, et al., 2012; Epstein, et al., 2012; Pettus, Chalfant, & Hannun, 2002).

Ceramides also play a critical role in insulin resistance and regulation of hepatic steatosis. Although the mechanism by which ceramides induce hepatic insulin resistance is not completely understood, ceramide-mediated insulin resistance may be induced through atypical protein kinase C activation and associated lipogenic and lipid uptake processes, while simultaneously impairing Akt-mediated regulation of hepatic glucose output (Ribaux & Iynedjian, 2003; Summers, 2006; G. Yang, et al., 2009). Recent studies show that inhibition of dihydroceramide desaturase 1 in the liver or the adipose tissue was sufficient to restore insulin sensitivity in high fat fed mice. These studies not only identified a potential therapeutic target for the treatment of obesity-associated insulin resistance, but also suggest that ceramides may mediate organ-to-organ crosstalk as deletion of the enzyme in liver alone or adipose tissue alone restored insulin sensitivity (Chaurasia, et al., 2019). Experimental models show that ceramide promotes protein kinase C ζ activation as a mediator of SREBP-1c driven lipogenesis and CD36-mediated lipid uptake in the liver (Xia, et al., 2015). In HFD mouse, overexpression of sphingosine kinase 1 which promotes the conversion of ceramide to SP1P ameliorates insulin resistance (Bruce, et al., 2012). The accumulation of SP1P upon depletion of SP1P-phosphohydrolase triggered ER stress and autophagy (Lepine, et al., 2011). Hepatic ceramide accumulation is increased in NAFLD and correlated with disease progression (Gorden, et al., 2015). Furthermore, blocking de novo ceramide synthesis reduced fat accumulation and hepatic TG content (G. Yang, et al., 2009). Ceramides may also promote inflammation in NASH as palmitate-induced the release of pro-inflammatory extracellular vesicles in an IRE1α/XBP1-dependent manner via the

transcriptional activation of the *de novo* ceramide synthesis pathway (Kakazu, et al., 2016). Though ceramide accumulation in the ER is implicated in pancreatic β-cell death (J. Han & Kaufman, 2016), inhibition of de novo ceramide synthesis did not prevent palmitate-induced ER stress and apoptosis in hepatocytes (Wei, Wang, Topczewski, & Pagliassotti, 2006). Altogether, these data suggest a role for sphingolipid metabolism in maintenance of ER homeostasis and in insulin resistance, hepatic steatosis as well as formation of lipotoxic extracellular vesicles.

C. Lysophosphatidylcholine

LPC is a major component of cell membrane bilayers, lipid droplet envelop monolayers, and VLDL (Neuschwander-Tetri, 2010; Wiesner, Leidl, Boettcher, Schmitz, & Liebisch, 2009). LPC is another important phospholipid mediator of SFA induced lipotoxicity in NASH (M. S. Han, et al., 2008). LPC is generated from PC intracellularly by PLA2 or extracellularly by plasma lecithin-cholesterol acyltransferase (Musso, Gambino, & Cassader, 2009). PLA2 inhibition decreased intracellular LPC and palmitate-induced apoptosis (K. L. Donnelly, et al., 2005; Kakisaka, et al., 2012). An additional important mechanism of lipotoxicity is the depletion of membrane PC caused by PLA2 activation (Z. Li, et al., 2006). PC is the most abundant phospholipid and is essential for cell membrane integrity, and is an important feedback inhibitor of SREBP-1c-mediated lipogenesis (Walker, et al., 2011). Hepatocytes have a high demand for PC, which is used for the production and secretion of VLDL. Hepatic PC depletion and perturbation of hepatocyte membrane integrity result in extracellular release of lipotoxic lipids, inflammation, and hepatocyte apoptosis. Similar to palmitate, LPC induces ER stress including phosphorylation of eIF2α and subsequent increased CHOP expression and JNK activation, resulting in the induction of the BH3-only protein PUMA. Increased PUMA causes subsequent Bax activation, caspase 3/7 activation, and apoptosis (Kakisaka, et al., 2012).

D. Interconnectedness of lipotoxic and proteotoxic ER stress

The dual function of the ER in protein and lipid homeostasis suggests that proteotoxic and lipotoxic stress are interconnected. ER stress triggered by disequilibrium in the lipid bilayer is evidently distinct from proteotoxic ER stress, which is triggered by the accumulation of unfolded or misfolded proteins in the ER lumen. Furthermore, activation of the UPR sensors in lipotoxic stress relies on their transmembrane domains whereas misfolded proteins are sensed by their luminal domains. Both IRE1α and PERK can be activated via their transmembrane domains upon increases in membrane lipid saturation (Volmer, et al., 2013). Interestingly, ATF6α can be activated by specific sphingolipids, dihydroceramide and dihydrosphingosine, which are recognized by a motif in its transmembrane domain (Tam, et al., 2018). However, in spite of these differences, the relationship between lipotoxic ER stress and proteotoxic ER stress is likely bidirectional. SFA accumulation not only disrupts phospholipid homeostasis at the ER membrane, but also impacts on the morphology and integrity of the ER, thereby leading to disturbed ER proteostasis (Borradaile, et al., 2006). In addition, treatment with the chemical chaperone 4-phenylbutyric acid (4-PBA), which binds ER misfolded proteins, stabilized lipid-induced UPR, suggesting that lipotoxicity induced proteotoxic stress (Pineau, et al., 2009). Thus, it is likely that the impairment in protoestasis secondary to lipotoxic ER stress is a consequence of the disruption of normal ER structure

and function. Furthermore, lipotoxic UPR induced proteome-remodeling mitigated the detrimental effects of lipotoxic lipid bilayer stress (Thibault, et al., 2012). Conversely, proteotoxic UPR activates ER expansion which requires lipid biosynthesis and ER stress impacts cellular lipid homeostasis, as discussed above. The comprehensive signaling outputs of the UPR retain some similarities but also demonstrate some differences between lipotoxic and proteotoxic ER stress. For example, sphingolipid-induced activation of ATF6α preferentially activated ER lipid biosynthetic genes over ER chaperones and protein-folding genes (Tam, et al., 2018). In *Caenorhabditis elegans* models lipid disequilibrium from impaired phosphatidylcholine synthesis resulted in activation of an ER stress response with a transcriptional profile quite distinct from proteotoxic ER stress (Fun & Thibault, 2019). One distinct pathway is autophagy which is activated in an IRE1α/XBP-1 dependent manner and may be protective against lipotoxic ER stress (Koh, Wang, Beaudoin-Chabot, & Thibault, 2018). Lipotoxic ER stress may also contribute to proteotoxic ER stress by degradation of ER proteins, as recently demonstrated in a *Saccharomyces cerevisiae* lipotoxic ER stress model (Shyu, et al., 2019). Thus lipotoxic and proteotoxic ER stress are interconnected and further studies are needed to understand this relationship especially in hepatocytes in the context of fatty liver disease.

6. Therapeutic potential targeting ER stress and UPR in NAFLD

ER stress is a pathogenic feature of NAFLD (Bechmann, et al., 2012; Malhi & Kaufman, 2011); thus targeting the specific UPR signaling pathways to attenuate ER stress and UPR activation may provide opportunities for developing new therapeutic strategies to treat NAFLD. Several compounds have been considered for the treatment of various diseases via their effects on the UPR signaling pathway (Table 1). However, further preclinical development and clinical trials are needed to validate their therapeutic potential in NAFLD.

A. PERK-eIF2α-ATF4 Signaling

PERK signaling reduces protein translation by phosphorylated eIF2α and attenuates ER stress levels by reducing protein misfolding overload. Salubrinal is a small molecule that inhibits dephosphorylation of eIF2α and protected cells from ER stress-induced apoptosis (Boyce, et al., 2005; Hetz, Chevet, & Harding, 2013). It also inhibited free FA-induced-apoptosis and NFκB activation (Kuo, et al., 2012). The PERK inhibitor, GSK2606414, was shown to attenuate palmitic acid–induced JNK activation and cell death in primary mouse hepatocytes (Win, et al., 2015). FGF21 was recently identified to play important roles in UPR signaling (Lin, et al., 2015; Shimizu, Morimoto, Maruyama, Inoue, & Sato, 2015). Administration of recombinant FGF21 alleviated tunicamycin-induced hepatic steatosis, in parallel with reduction of eIF2α-ATF4-CHOP signaling (Jiang, et al., 2014). Guanabenz is a α2-adrenergic receptor agonist approved for hypertension. It also binds to GADD34 and selectively inhibits GADD34-mediated dephosphorylation of eIF2α and exerted a cytoprotective effect against ER stress by prolonging UPR signaling (Tsaytler, Harding, Ron, & Bertolotti, 2011). As optimal eIF2α phosphorylation is protective in several metabolic pathologies, including β cell failure, guanabenz may be a promising candidate for the treatment of NAFLD.

B. IRE1α Signaling

Activated IRE1α induces processing of XBP1 and RNA degradation through RIDD via its RNAse activity. Salicylaldimine analogs, such as STF-083010 and 4μ8C were shown to covalently attach to a lysine residue and to inhibit the RNase activity of IRE1α (Mimura, et al., 2012; Papandreou, et al., 2011; Sanches, et al., 2014; Volkmann, et al., 2011). Many such salicylaldehyde derivatives block both XBP1 processing and RIDD without affecting the kinase activity of IRE1α (Cross, et al., 2012; Sanches, et al., 2014; Volkmann, et al., 2011). STF-083010 or 4μ8C showed improvement of NAFLD in HFD-fed mice (Lebeaupin, Vallee, Rousseau, et al., 2018). Toyocamycin was recently reported to attenuate the activation of XBP1, possibly by inducing a conformational change in IRE1α (Ri, et al., 2012). Toyocamycin ameliorated hepatic steatosis and liver injury caused by HFD-induced NASH (Takahara, et al., 2017).

C. Chemical chaperons

Chemical chaperones modulate ER stress by attenuating protein misfolding and aggregation and stabilizing folding intermediates. Chemical chaperones reduced ER stress, improved insulin sensitivity and glucose homeostasis, and reversed leptin resistance in the liver of an obese mouse model (Hetz, et al., 2013; L. Ozcan, et al., 2009; U. Ozcan, et al., 2006). Tauroursodeoxycholic acid (TUDCA) and 4-PBA are chemical chaperones that have been shown to reduce ER stress by facilitating proper protein folding and trafficking (Henkel, 2018; Vilatoba, et al., 2005; Xie, et al., 2002). Treatment with 4-PBA improved glucose tolerance in insulin-resistant patients and TUDCA partially restored insulin sensitivity in liver and muscle, but not adipose tissue in obese patients (Kars, et al., 2010; C. Xiao, Giacca, & Lewis, 2011). Mitigating ER stress using these chemical chaperones in mouse models with steatotic livers reduces hepatic lipid accumulation and hepatic fibrogenesis (Jimenez-Castro, et al., 2012; Namisaki, et al., 2016; U. Ozcan, et al., 2006). Unfortunately, the efficacy of these chaperones in NAFLD has been less impressive. Several randomized trials using ursodeoxycholic acid have failed to improve the overall histology in patients with NASH in comparison with placebo (Dufour, et al., 2006; Leuschner, et al., 2010; Lindor, et al., 2004). Whether other chemical chaperones may be effective remains to be determined.

D. Additional consideration on the therapeutic modulation of the UPR

BiP is an essential chaperone involved in the activation of PERK and IRE1α and translocation of ATF6α. BiP inducer X was identified as a modulator to induce BiP expression in the neural system and kidney (Nakanishi, et al., 2013; Prachasilchai, et al., 2009). Studies on the use of BiP inducer X in NAFLD remain to be explored. CHOP is another UPR target. Inhibitors of p38 mitogen-activated protein kinase inhibit the phosphorylation of CHOP, which is critical for its function as a transcription factor (X. Z. Wang & Ron, 1996). However, not only is there a shortage of CHOP-specific inhibitors, but there is a lack of research on the effectiveness of CHOP inhibitors in NAFLD. Glucagon-like peptide-1 analogues that are currently used in the treatment of type 2 diabetes reduce hepatic steatosis and insulin resistance in mouse models of fatty liver disease and reduce ER stress and fat accumulation in cultured human hepatocytes (Ding, Saxena, Lin, Gupta, & Anania, 2006; Sharma, Mells, Fu, Saxena, & Anania, 2011). The safety and efficacy of the long

acting glucagon-like peptide-1 analogue, liraglutide, has been reported in patients with NASH (Armstrong, et al., 2016). However, whether this improvement involves a reduction of ER stress remains to be proven.

7. Conclusions

NASH is a complex disease that is modulated by numerous mechanisms including metabolic, genetic, and environmental factors. Although steatosis is a fundamental characteristic of NAFLD, the specific signaling mechanisms that lead to inflammation and progressive injury in NASH are not completely delineated. ER stress is considered a key factor in steatosis, such that hepatic lipid accumulation is both a cause and a consequence of ER stress, thus creating a positive feedback loop which may promote the development of hepatic steatosis. We further propose that the selective lipotoxic activation or inactivation of UPR sensors and their downstream signaling molecules may play a role in determining the outcomes of isolated steatosis versus progressive NASH. This concept needs further experimental testing to identify which of these signaling pathways offers interesting targets to therapeutically inhibit NASH progression. Lastly, the inter-connectedness of proteotoxic and lipotoxic ER stress is beginning to be defined, as are their unique characteristics, which will have relevance to disease pathogenesis and therapeutic targeting.

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Abbreviations

ACC	acetyl-CoA carboxylase
ATF	activating transcription factor
ApoB	apolipoprotein B
BI-I	Bax Inhibitor 1
BH	Bcl-2 homology
BiP	binding immunoglobulin protein
C/EBP	CCAAT/enhancer-binding protein
CHOP	CCAAT-enhancer-binding protein homologous protein
ER	endoplasmic reticulum
ERAD	ER-associated degradation
eIF2α	eukaryotic translation initiation factor 2α
FA	fatty acid
FGF21	fibroblast growth factor 21

GADD34	growth arrest and DNA damage-inducible protein
HFD	high fat diet
IRE	inositol-requiring enzyme
INSIG	insulin-induced gene
JNK	c-Jun N-terminal kinase
LPC	lysophosphatidylcholine
MTP	microsomal TG transfer protein
NAFLD	Nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
4-PBA	4-phenylbutyric acid
PC	phosphatidylcholine
PERK	protein kinase RNA-like ER kinase
PLA2	phospholipase A2
PPAR	peroxisome proliferator-activated receptor
PUMA	p53 upregulated modulator of apoptosis
RIDD	regulated IRE1α-dependent decay
RNase	endoribonuclease
SCAP	SREBP cleavage-activating protein
SCD	stearolyl-CoA desaturase
SFA	saturated fatty acid
S1P	site 1 protease
S2P	site 2 protease
SP1P	sphingosine 1-phosphate
SREBP	sterol regulatory element binding proteins
TG	triglyceride
TUDCA	tauroursodeoxycholic acid
UPR	unfolded protein response
VLDL	very low density lipoprotein
XBP1	X-box binding protein 1

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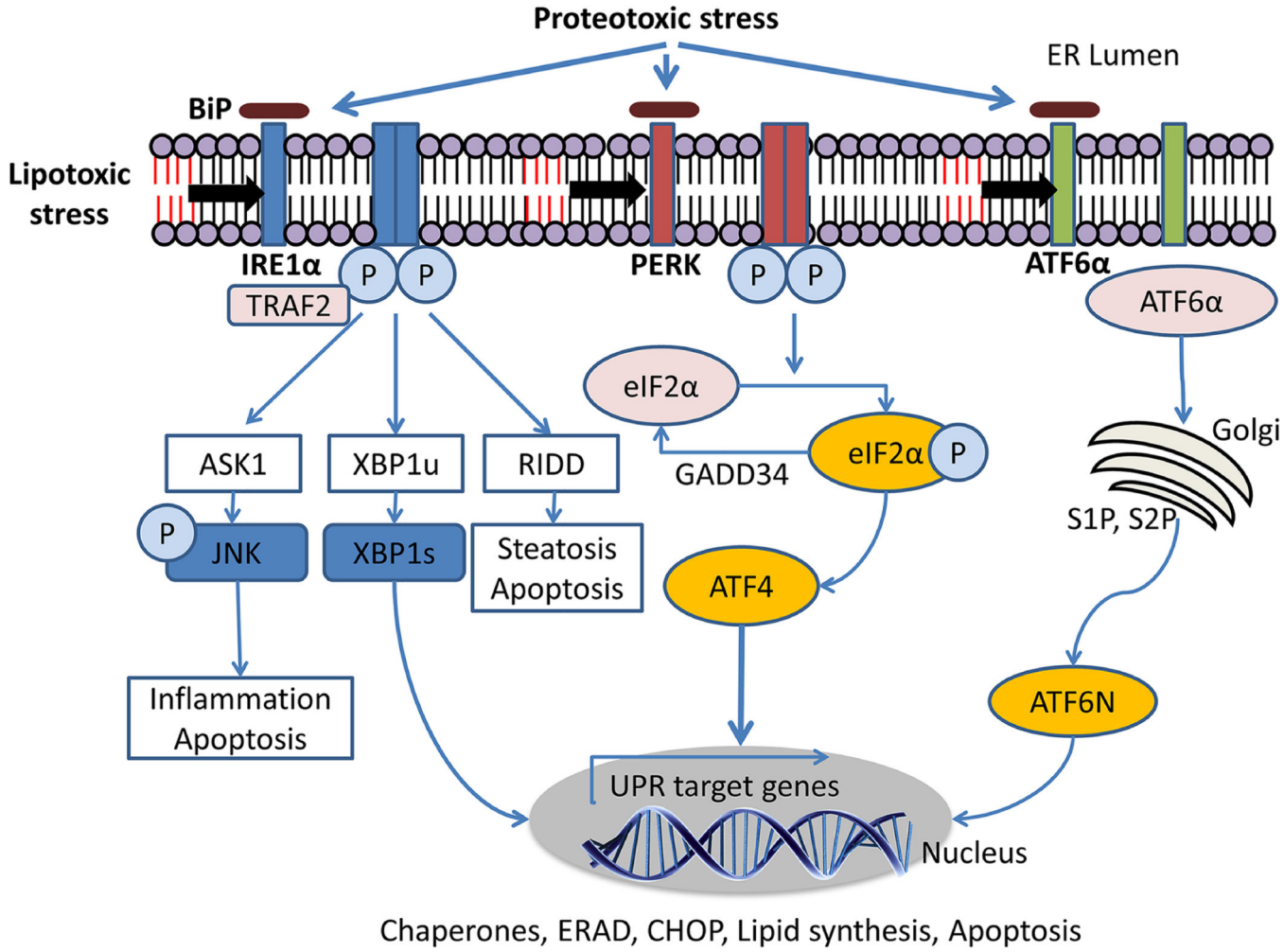

Figure 1. Proteotoxic and lipotoxic unfolded protein response signaling.

Proteotoxic stress due to the accumulation of misfolded or unfolded proteins activates the three transmembrane ER stress sensors (IRE1α, PERK, ATF6) by releasing them from BiP binding or by direct biding of the unfolded proteins to the luminal domain of the UPR sensors. Lipotoxic ER stress due to increased membrane saturation or sphingolipid accumulation is sensed by the transmembrane domains of the UPR sensors.Activated IRE1α induces splicing of XBP1. Spliced XBP1 transcriptionally upregulates genes that encode protein folding machinery, ERAD genes, and lipid synthesis pathways. PERK phosphorylates eIF2α which suppress mRNA translation leading to attenuation of protein synthesis. ATF4 is selectively translated and upregulates several transcriptional targets including CHOP. CHOP-dependent apoptosis occurs under unresolved ER stress. ATF6α translocates from the ER to the Golgi complex and cleaved by S1P and S2P. Cleaved ATF6α, termed ATF6N, transcriptionally upregulates UPR target genes. ***Abbreviation*:** IRE1α, inositol-requiring enzyme 1; PERK, PKR-like endoplasmic reticulum kinase; ATF6α, activating transcription factor 6; TRAF2, Tumor necrosis factor receptor-associated factor 2; ASK1, apoptosis-signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; XBP1u, X-box binding protein 1; XBP1s, spliced X-box binding protein 1; RIDD, regulated IRE1α-dependent decay; eIF2α, eukaryotic translation initiation factor 2α; GADD34,

growth arrest and DNA damage-inducible protein; ATF4, activating transcription factor 4; UPR, unfolded protein response; ERAD, endoplasmic reticulum-associated degradation; CHOP, CCAAT-enhancer-binding protein homologous protein; ATF6N, cleaved ATF6; BiP, binding immunoglobulin protein; S1P, site 1 protease; S2P, site 2 protease.

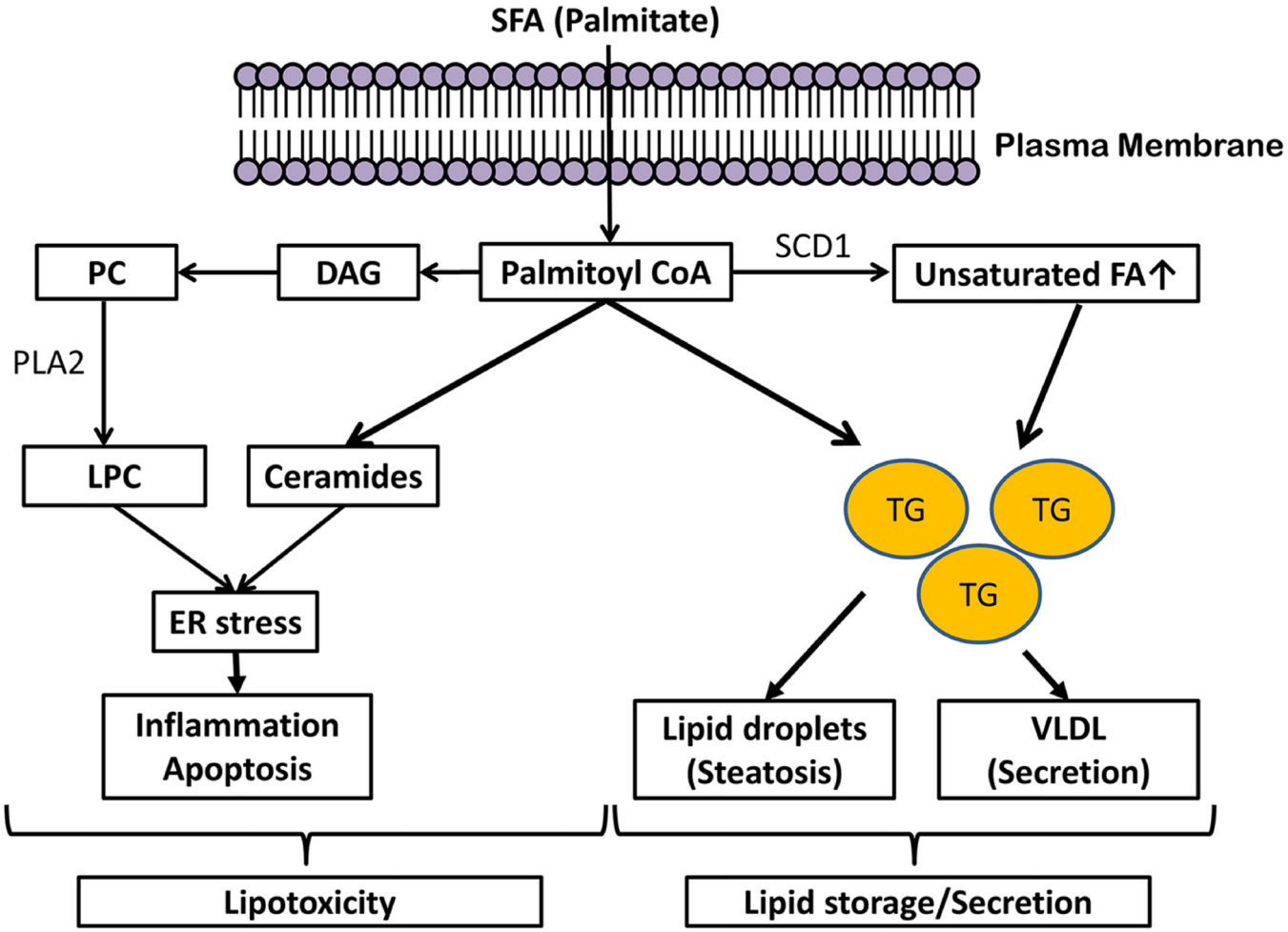

Figure 2. Palmitate-induced lipotoxicity.

Excess palmitate availability to the hepatocyte, derived predominantly from adipose tissue lipolysis in insulin resistance, leads to lipid accumulation and palmitate lipotoxicity. To maintain lipid homeostasis, fatty acid disposal in the liver occurs through the formation of triglyceride which is then stored temporarily as lipid droplets (steatosis) or secreted as VLDL. In hepatocytes, conversion of free fatty acids to triglycerides protects the cells from lipotoxicity; whereas higher levels of SFAs such as palmitate, are lipotoxic. Palmitate can be directly or indirectly metabolized into other lipid classes including ceramides and LPC which contribute to palmitate-induced ER stress. The generation of lipotoxic metabolites of fatty acids typically occurs in parallel with the accumulation of triglyceride droplets (steatosis), resulting in the hallmark features recognized as nonalcoholic steatohepatitis where steatosis and hepatocellular injury are present together. ***Abbreviation*:** SFA, saturated fatty acid; DAG, diacylglycerol; PC, phosphatidylcholine; PLA2, phospholipase A2; LPC, lysophosphatidyl choline; SCD1, stearolyl-CoA desaturase; TG, triglyceride; VLDL, very low density protein; ER, endoplasmic reticulum.

Table 1

Targeting the UPR in NAFLD

Molecule	Target	Mechanism	Ref.
4-PBA	Chemical chaperone	Stabilize misfolded proteins	(U. Ozcan, et al., 2006)
TUDCA	Chemical chaperone	Stabilize misfolded proteins	(Lebeaupin, et al., 2015)
Salubrinal	PERK	Prevent eIF2α dephosphorylation	(Kuo, et al., 2012)
GSK2606414	PERK	PERK-induced JNK activation	(Win, et al., 2015)
FGF21	PERK	Reduce eIF2α-ATF4-CHOP signaling	(Jiang, et al., 2014)
STF-083010	IRE1α	Inhibits RNase activity	(Lebeaupin, Vallee, Rousseau, et al., 2018)
4u8C	IRE1α	Inhibits RNase activity	(Tufanli, et al., 2017)
Toyocamycin	IRE1α	Block XBP1 processing	(Takahara, et al., 2017)