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Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease

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ABSTRACT

The steady rise in Western obesity rates has been closely linked to significant increases in a multitude of accompanying health problems including non-alcoholic fatty liver disease (NAFLD). NAFLD severity ranges from simple steatosis to acute steatohepatitis, but the molecular mechanisms controlling progression of this disease are poorly understood. Recent literature suggests that elevated free fatty acids (FFAs), especially saturated FFAs, may play an important role in lipotoxic mechanisms, both in experimental models and in NAFLD patients. This review highlights important cellular pathways involved in hepatic lipotoxicity and how the degree of intrahepatic lipid saturation controls cell fate in response to an elevated FFA load. Relevant cellular processes that have been causally linked to lipid-induced apoptosis, known as lipoapoptosis, include endoplasmic reticulum (ER) stress, oxidative stress, mitochondrial dysfunction, and Jun N-terminal kinase (JNK) signaling. In contrast, increased triglyceride synthesis has been shown to have a protective effect against lipotoxicity, despite being one of the hallmark traits of NAFLD. Developing a more nuanced understanding of the molecular mechanisms underlying NAFLD progression will lead to more targeted and effective therapeutics for this increasingly prevalent disease, which to date has no proven pharmacologic treatment to prevent or reverse its course.

Contents

Abbreviations: NAFLD, non-alcoholic fatty liver disease; FFA, free fatty acid; ER, endoplasmic reticulum; JNK, Jun N-terminal kinase; NASH, non-alcoholic steatohepatitis; NAS, NASH activity score; MCD, methionine and choline deficient; HFD, high fat diet; SFA, saturated fatty acid; ROS, reactive oxygen species; UPR, unfolded protein response; PC, phosphatidylcholine; SCD-1, steroyl-CoA desaturase-1; LPC, lysophosphatidylcholine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DGAT2, diacylglycerol acyltrasferase 2; TG, triglyceride.

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1. Introduction

As the global waistline continues to expand, the incidences of obesity and type II diabetes are reaching epidemic proportions. Insulin resistance and obesity are often associated with a cluster of other metabolic abnormalities including hypertension, dyslipidemia, and hyperinsulinemia, collectively termed the ''metabolic syndrome''. The pathogenesis of this disease state is hypothesized to begin with abnormal accumulation of lipids in non-adipose tissues (steatosis) due to increased export of free fatty acids from adipose tissue. The hepatic manifestation of metabolic syndrome is known as non-alcoholic fatty liver disease (NAFLD), a chronic condition affecting approximately one-third of the US population [1]. Because of its growing prevalence in Western society, NAFLD is currently the leading cause of referrals to hepatology clinics in the US. An estimated 10% of NAFLD patients will progress to a more severe condition known as nonalcoholic steatohepatitis (NASH), which involves liver inflammation and apoptotic cell death and can eventually result in cirrhosis and/or liver failure [2]. Despite its prevalence and potential serious complications, the underlying molecular mechanisms that regulate NAFLD progression remain poorly understood, and further investigation is needed to identify targeted therapeutic approaches for prevention and treatment of this disease. Lack of such knowledge represents an important problem, because it limits the ability of biomedical researchers to develop novel nutritional and/or pharmacologic interventions to combat the effects of NAFLD and to prevent its progression toward NASH [3].

2. NASH models and the role of fatty acids

Identifying persons with NASH for experimental studies and clinical trials relies on biopsies and plasma samples as markers of disease severity. Using liver biopsies, NASH progression is based upon the NAFLD Activity Score (NAS) and fibrosis. NAS was designed by the Pathology Committee of the NASH Clinical Research Network [4] as a scoring system of 14 histological features, including lobular inflammation, the level of steatosis, and hepatocellular ballooning, which is used as a guideline for differentiating severity of NAFLD (NAS $\geqslant$ 5 diagnosed as ''NASH'', $\leqslant$3 ''Not NASH''). Plasma indicators of acute liver injury include elevated levels of alanine aminotransferase and aspartate aminotransferase. Although these enzymes are reliable biomarkers of liver injury, their presence is not specific to NASH. Therefore, they remain somewhat equivocal when used as the sole metric for assessing the state of NAFLD progression.

Due to the lack of definitive biomarkers for non-invasive monitoring of NAFLD in humans, as well as the limited scope of interventions that can be applied in clinical studies, animal models provide an important research tool that enables mechanistic studies of NASH development. There are many different diet-induced and genetic models of steatosis/steatohepatitis, each having its own advantages, disadvantages, and idiosyncrasies. (Larter et al. [5] have provided an excellent review on the various animal models in steatohepatitis.) For example, common genetically obese mouse models such as the leptin resistant db/db or leptin deficient ob/ob do not spontaneously develop NASH despite pronounced hepatic lipid accumulation [6,7]. In order to model NASH in these animals it is necessary to provide a seconday 'hit' or insult to initiate liver inflammation and fibrosis. In *ob/ob* models, it is possible to use lipopolysaccharide to initiate acute liver damage [8]. Alternatively, it is possible to model NASH in *db/db* mice by feeding a methionine and choline deficient (MCD) diet [9]. Even wild-type animals fed a MCD diet will rapidly develop hepatic steatosis, inflammation, and liver fibrosis [10–12]. Additionally, the liver damage in non-genetically obese mice occurs independently of insulin resistance, providing a model that is free from the confounding effects of dysregulated insulin signaling [13]. This model of NAFLD/NASH may prove to be particularly important in light of recent data which indicate a disconnection between hepatic lipid accumulation and insulin resistance in studies on hypobetalipoproteinaemic human patients [14,15]. However, because of obvious differences in etiology between MCD-diet-induced NASH and human NASH, questions still remain as to whether the MCD mouse model provides a relevant *in vivo* recapitulation of human disease [16]. Instead of the MCD diet, rats and mice fed high-fat diets (HFD) may provide a more accurate model of human steatohepatitis since these models mimic the overnutrition that is typical of obesity. In fact, analysis of liver tissues from both high-fat fed mice and human NAFLD patients reveal similar trends of lipid alterations and histological changes [17].

As an alternative to human and animal studies, *in vitro* experiments using hepatic cell lines and primary hepatocytes have provided detailed insight into the molecular mechanisms that regulate lipotoxicity under conditions that mimic the *in vivo* disease state. In particular, obesity and insulin resistance are associated with elevated plasma levels of free fatty acids and triglycerides (TGs) [18]. *In vitro* experiments in a diverse range of cell types have demonstrated that saturated fatty acid (SFA) overexposure promotes the expression of pro-inflammatory cytokines, impairs insulin signaling, and stimulates apoptosis characterized by both ER impairments and oxidative stress [19–23]. In contrast, monounsaturated fatty acids induce significant steatotic triglyceride formation but do not initiate apoptosis [18,21]. However, an accepted mechanism explaining how SFAs trigger apoptotic signaling or promote the progression from NAFLD to NASH has yet to be determined conclusively [24]. Several putative signaling mechanisms including the accumulation of reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, and increased ceramide synthesis have been hypothesized to explain how SFAs initiate apoptosis in hepatic cells. In particular, ceramide signaling has been hypothesized as an initiator of hepatic lipoapoptosis due to the fact that ceramides are synthesized *de novo* from palmitate and serine and have been shown to promote apoptosis in myocytes [25]. However, studies using both pharmacologic and genetic interventions have revealed that SFAs can induce apoptosis independently of ceramide synthesis in a variety of cell types including CHO [26,27], breast cancer cells [19], and H4IIEC3 hepatomas [28], suggesting that other mechanisms involving ER stress and ROS accumulation may predominate in these tissues.

3. SFAs promote cellular dysfunction by activating ER stress pathways

The ER is a specialized organelle that is integral in many cellular functions, particularly disulfide bond formation, proper protein folding, and synthesis and secretion of several critical biomolecules including steroids, cholesterol, and lipids [29]. The ER also is the most important regulator of intracellular calcium as a result of its large Ca^{2+} stores and Ca^{2+} ATPases, which are necessary for proper functioning of Ca^{2+}-dependent chaperones that stabilize protein folding. Very small changes in cellular redox state [30] or abnormal accumulation of unfolded proteins and/or toxic lipid species [31] can result in activation of compensatory response pathways, which comprise the unfolded protein response (UPR) [29,32,33]. The UPR stress signaling pathway is initiated by three main ER transmembrane proteins, protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring 1 (IRE-1), and activating transcription factor 6 (ATF6), which together promote

transcription of genes designed to increase protein folding and degradation. Markers that are often assessed in order to demonstrate cellular ER stress include phosphorylation of the three aforementioned transmembrane proteins as well as the splicing of X-box binding protein, initiated by IRE1 signaling, and CHOP, a pro-apoptotic protein downstream of PERK activation. The UPR initially serves a protective role to increase protein folding capacity and degrade any misfolded proteins already synthesized. However, excessive and/or prolonged stress can trigger apoptosis via JNK signaling and release of ER Ca^{2+} stores [34] (see [33] for a comprehensive review of general ER stress pathways). The released Ca^{2+} is readily taken up by the mitochondria adjacent to ER Ca^{2+}-release channels [35]. Acute Ca^{2+} overload results in changes in mitochondrial potential and opening of the permeability transition pore (PTP) [36], invoking a potent cellular death signal [30].

Increasing empirical evidence points towards endoplasmic reticulum (ER) stress as an upstream signal in SFA-induced cellular dysfunction and apoptosis (Table 1). Clinical studies of patients suffering from metabolic syndrome disorders, such as NAFLD, have increased levels of ER stress markers in the liver and other tissues [37–39]. *In vivo* data from male Wistar rats indicate that diets high in saturated, but not unsaturated, fat results in induction of hepatic ER stress and liver damage [40]. SFAs have also demonstrated a potent ability to induce ER stress in a multitude of cell types *in vitro*, including hepatocytes [40–42], pancreatic β-cells [43,44], adipocytes [45] and CHO cells [46]. Therefore, prior studies have sought to define the role of ER stress in mediating SFA-induced lipotoxicity. Pfaffenbach et al. [47] investigated the role of ER stress in palmitate (PA)-treated H4IIEC3 cells and found that although CHOP expression was increased in PA-treated cells, siRNA knockdown of CHOP did not attenuate apoptosis, demonstrating that CHOP was not critical for lipoapoptosis to occur. Additionally they showed that $CHOP^{-/-}$ mice and their wild-type counterparts displayed comparable levels of liver injury when both were fed a MCD diet, as assessed by alanine and aspartate aminotransferase levels. Similarly, another study found that CHOP inhibition via siRNA in PA-treated HepG2 cells had no effect on apoptosis [48]. These results confirm that palmitate alters ER function, but does not fully define how ER stress contributes to apoptosis, implying that alternate downstream targets of ER stress could mediate lipotoxicity.

4. Molecular mechanisms of SFA-induced ER stress

Although there is no definitive mechanism explaining how SFAs induce ER stress, increasing evidence points to disordered phospholipid metabolism as one initiating factor. Unsaturated fatty acids are readily incorporated into inert TGs, but excess SFAs remain largely unesterified [28]. Recent literature suggests that these free SFAs are rapidly assembled into saturated phospholipid species that are subsequently integrated into ER membrane bilayers [46]. The degree of saturation in membrane phospholipids plays an important role in many membrane-associated functions. Abnormal incorporation of saturated phospholipid species can result in detrimental stiffening of cellular membranes and loss of functionality [49]. The composition of the ER membrane is unique and typically contains unsaturated phosphatidylcholine (PC) as its major phospholipid component [46,50,51]. This allows the ER to maintain a high degree of fluidity in order to carry out its critical role in maintaining proper protein folding and trafficking. Relatively small changes in ER homeostasis can result in the induction of UPR [32,50]. Therefore, even limited incorporation of saturated phospholipid species could be detrimental to the ER and lead to the increased UPR signaling observed in response to SFA overexposure.

Borradaile et al. [46] published a comprehensive study investigating the mechanism leading to ER stress in palmitate-treated

Table 1
The role of ER stress in SFA-induced lipotoxicity. Abbreviations: endoplasmic reticulum (ER); saturated fatty acid (SFA); unfolded protein response (UPR); steroyl-CoA desaturase-1 (SCD-1); phosphatidylcholine (PC); lysophosphatidylcholine (LPC); non-alcoholic steatohepatitis (NASH); Jun N-terminal kinase (JNK).

Type	Model system	Key findings	References
In vitro/*in vivo*	H4IIEC3 rat hepatomas/ $CHOP^{-/-}$ mice	Palmitate induced significant ER stress as assessed by CHOP expression, but CHOP knockdown did not attenuate apoptosis *in vitro*. Knockout of CHOP in C57BL/6 J mice ($CHOP^{-/-}$) did not reduce liver injury compared to wild-type mice when fed a MCD diet	[42]
In vivo	Male Wistar rats	Hepatic steatosis was associated with elevated SFAs, but not unsaturated fatty acids, and was characterized by increased liver injury and markers of ER stress	[40]
In vitro	CHO-K1	In lipotoxic conditions, palmitate was rapidly incorporated into microsomal membrane lipid components resulting in compromised ER membrane integrity and subsequent UPR stress signaling	[46]
In vitro	HeLa	Knockdown of SCD-1 resulted in increased SFA content in phospholipids and expression of ER stress signaling chaperones. Synergistic knockdown of SCD-1 and lysophosphatidyl acyltransferase 3, an enzyme associated with preferential incorporation of polyunsaturated fatty acids into phospholipids, further exacerbated UPR	[53]
In vivo	*Saccharomyces cervisiae*	SFA incorporation into phospholipids increased the order of membranes due to the characteristic suboptimal shape they create within the bilayer. The detrimental effects can be relieved by incorporation of unsaturated fatty acids with a more optimized shape that restores membrane disorder	[50]
In vivo/*in vitro*	Chang cells, ICR mice, human biopsy	LPC is the death effector in cellular apoptosis resulting from SFA overexposure. SFA toxicity can be attenuated by the addition of phospholipase A_2 inhibitors which prevent the generation of LPC species and ER stress. Direct administration of LPC *in vivo* induced significant hepatitis. LPC content was also found to be significantly increased in liver specimens from NASH patients	[55]
In vitro	Huh-7, mouse/human primary hepatocytes	LPC content increased linearly with increasing exogenous SFA concentration. Substitution of LPC for SFA in models of lipotoxicty resulted in induction of ER stress, glycogen synthase kinase-3/JNK dependent apoptosis and p53 upregulated modulator of apoptosis upregulation	[56]

CHO cells. Following exposure to deuterated palmitate for 1 hour, the cells displayed a 1.5-fold and 3.0-fold increase in the saturation of membrane-bound PC and TG, respectively, in the rough microsomal fraction. Accompanying the increased saturation were markers of ER stress including the dissociation of protein-folding chaperones protein disulfide isomerase (PDI) and GRP78 from the membrane into the cytosol and presentation of a drastically dilated rough ER morphology, indicating severely impaired ER function. Similar effects were not observed in response to H_2O_2 treatment alone, demonstrating that ER stress induced by SFA overexposure was not solely the result of increased oxidative stress. The study also reported a rapid depletion of ER calcium stores in response to palmitate treatment, pointing to impairment of calcium ATPase activity associated with the ER membrane, a phenomenon that has been previously linked to increased saturation of phospholipid fatty acyl chains [52].

Another recent study explored the implications of increasing saturation of membrane phospholipids in HeLa cells [53]. Specifically, they investigated the effects of inhibiting steroyl-CoA desaturase 1 (SCD-1), the enzyme responsible for the desaturation of saturated fatty acids for lipid biosynthesis, and lysophosphatidyl acyltransferase 3, an enzyme that preferentially incorporates polyunsaturated fatty acids into PC. Even without SFA supplementation, both the SCD-1 knockdown and lysophosphatidyl acyltransferase 3 knockdown cells demonstrated a significant increase in phospholipid saturation and UPR activation indicated by increased X-box binding protein splicing and PERK phosphorylation. These results were exacerbated by the simultaneous lysophosphatidyl acyltransferase 3/SCD-1 knockdown and further enhanced by palmitate supplementation. Overall, these experiments indicate that proper incorporation of unsaturated fatty acids into phospholipids is of critical importance for maintaining normal ER membrane functionality, and that SFA overexposure may disrupt this process. Further investigation into the mechanism and effects of SFAs in controlling the phospholipid composition of cellular membranes will be important in formulating a complete picture of lipotoxic cell death.

Generation of lysophospholipid species, and lysophosphotidylcholine (LPC) in particular, has also been implicated as an important mediator in the apoptotic response to SFAs. LPC is generated from phosphatidylcholine through cleavage of the fatty acid from the sn-2 position by the enzyme phospholipase A_2. In two small-scale biopsy studies, concentrations of LPC in hepatic tissue samples were determined to be greater in livers of NASH patients than healthy controls [54,55]. Direct injection of LPC into the tail vein of ICR mice significantly increased *in vivo* AST/ALT levels, lobular hepatitis, and apoptosis without any evidence of steatosis [55]. Using a variety of *in vitro* models, including Huh-7, Chang, and primary mouse and human hepatocytes, SFA lipotoxicity was shown to be significantly attenuated in the presence of the phospholipase inhibitors bromoenol lactone (BEL) and palmityl trifluoromethyl ketone ($PACOCF_3$). This indicates that action of the phospholipase A_2 enzyme may play an important role in the lipotoxic phenotype [55,56]. LPC content was also shown to be linearly correlated with the concentration of exogenous SFA supplied [56]. Data from these same *in vitro* models also demonstrate that addition of exogenous LPC produces a similar lipotoxic phenotype as SFA overexposure, including markers of ER stress, caspase activation and apoptosis [55,56]. Kakisaka et al. [56] demonstrated that LPC-dependent lipoapoptosis is dependent on activation of caspase and glycogen synthase kinase-3/JNK signaling, and is associated with p53 upregulated modulator of apoptosis activation. Identifying the role of LPC accumulation in SFA-induced lipotoxicity presents an interesting new avenue of investigation, but further work is needed to define if and how the two events are directly connected.

5. ROS accumulation and oxidative stress

An elevated level of ROS, or oxidative stress, has been proposed as a possible companion to ER stress in promoting NASH development. Although normally present at low levels, ROS accumulate in response to cellular stress, mitochondrial dysfunction, or decreased antioxidant defenses. The electrons lost from complexes I and III of the electron transport chain combine with oxygen to generate ROS, which includes superoxide ions, hydroxyl radicals, and hydrogen peroxide [57]. In addition to their role in mediating signaling pathways, some ROS can be powerful oxidizing agents and indiscriminately damage many important components of the cell including DNA, lipid membranes, and proteins [58]. Evidence for oxidative stress in NASH patients and animal models includes the accumulation of oxidized lipids such as malodialdehyde [59]. NASH-associated oxidative stress has also been attributed to a variety of mechanisms including upregulated levels of cytochrome P450 2E1 [60], NADPH oxidase [61], and changes in mitochondrial function such as increased beta-oxidation [62–64].

Although hepatic oxidation of free fatty acids, as measured by positron emission tomography, is elevated in obese individuals [65], the role of beta-oxidation in promoting lipotoxic ROS accumulation is unclear. *In vitro* studies using H4IIEC3 rat hepatoma cells appear to be in disagreement on this point. One study used etomoxir, a specific inhibitor of the rate-limiting enzyme carnitine palmitoyltransferase-1 [66], to prevent the transport of fatty acyl CoAs into the mitochondria. After combined treatment with palmitate and etomoxir, the authors measured a decrease in ROS levels in comparison to treatment with palmitate alone [62]. Conversely, another study also in the same cell line found ROS accumulation to be independent of beta-oxidation [67]. While both studies concluded that palmitate exerts toxic effects through ROS accumulation, only one indicated that beta-oxidation may be the source of increased ROS production. Alternatively, a recent *in vivo* study demonstrated that increased beta-oxidation may actually protect against NASH by enhancing lipid disposal. Administering PPAR-α agonists to mice on a MCD diet resulted in attenuated scores of liver damage while simultaneously increasing peroxisomal beta-oxidation [68]. Recent studies in pancreatic β-cells [69] and breast cancer cells [19] agree with these results. Both studies used etomoxir to reduce beta-oxidation. Reducing beta-oxidation had no positive effect on palmitate-induced apoptosis and even increased cell death in response to excessive SFA treatment [19,69]. Taken together, these studies indicate that lipids may play a more complex role in promoting hepatic ROS accumulation other than simply providing fuel substrates for increased oxidative metabolism. This could involve indirect effects to dysregulate normal mitochondrial function.

6. Dysregulation of mitochondrial metabolism

Several recent studies highlight a role of accelerated mitochondrial metabolism in lipotoxicity and NAFLD (Table 2). For example, [U-$^{13}C_3$] propionate and D_2O isotope tracers were administered to patients with high and low levels of intrahepatic triglyceride content to study the impact of liver fat levels on *in vivo* mitochondrial metabolism [70]. Examination of ^{13}C incorporation patterns in blood glucose revealed that mitochondrial oxidative metabolism was approximately 2-fold greater in NAFLD patients. This significant increase in mitochondrial activity in NAFLD patients coincided with elevation of both systemic lipolysis and gluconeogenesis by 50% and 30%, respectively. It was hypothesized that increased mitochondrial TCA cycle activity satisfied the energy demand for elevated gluconeogenesis. However, the increase in gluconeogenic flux alone is unlikely to account for the almost twofold increase in

Table 2
Metabolic studies that indicate upregulated mitochondrial activity in models of NAFLD.

Type	Model system	Treatment	Metabolic analysis	Key findings	References
In vitro	H4IIEC3 rat hepatomas	Palmitate	Metabolic flux analysis using [U-$^{13}C_5$] glutamine	Palmitate-treated cells exhibited enhanced oxidative metabolism fueled by glutamine. Altering media amino acid levels modulated oxidative stress and apoptosis	[67]
In vivo	C57Bl/6 mice	High fat diet (HFD)	Analysis of D_2O and [U-$^{13}C_3$] propionate incorporation into glucose	Chronic HFD-fed mice exhibited elevated TCA cycle activity corresponding to the onset of markers of oxidative stress	[71]
In vivo	NAFLD patients	Patients separated into two groups based upon intrahepatic lipid content (IHTG)	Analysis of D_2O and [U-$^{13}C_3$] propionate incorporation into glucose	Under gluconeogenic conditions, persons with high IHTG exhibited elevated mitochondrial metabolism, possibly linking IHTG and oxidative stress	[70]

the rate of mitochondrial metabolism, suggesting that alternative endergonic mechanisms are active in the presence of excess lipids. The investigators proposed that the correlation between high levels of intrahepatic triglycerides, FFA delivery to the liver, and elevated TCA cycle fluxes could explain the induction of oxidative stress in NAFLD patients. Similar experiments using [U-$^{13}C_3$] propionate and D_2O isotope tracers performed in mice revealed that animals fed a high fat diet had higher rates of TCA cycle flux [71]. After 32 weeks of HFD feeding, mice exhibited elevated superoxide dismutase activity. It was hypothesized that these enzymes were elevated to counteract ROS accumulation due to heightened mitochondrial activity. These two *in vivo* isotope labeling studies reveal that activated hepatic mitochondrial metabolism is a common characteristic of NAFLD in both human subjects and animal models.

Alternatively, stable isotope-based metabolic flux analysis (MFA) has been performed to study how elevated SFAs impact central metabolism of hepatic cells cultured *in vitro* [67]. Detailed flux mapping with [U-$^{13}C_5$]glutamine revealed that palmitate treatment strongly increased TCA cycle fluxes relative to glycolytic fluxes in H4IIEC3 cells. Changes in intracellular metabolic fluxes coincided with the onset of ROS accumulation and preceded the appearance of apoptotic markers such as caspase 3/7 activation and DNA laddering. Glycolytic fluxes including glucose uptake and lactate secretion were significantly inhibited by palmitate, whereas TCA cycle and anaplerotic fluxes were significantly upregulated. The timing of these events suggests that palmitate-stimulated metabolic flux alterations were responsible for generating ROS and triggering apoptosis. Interestingly, increased glutamine uptake, rather fatty acid beta-oxidation, was reported to fuel the observed increase in TCA cycling. Additionally, by varying the concentration of various amino acids in the cell culture medium it was possible to modify the metabolic phenotype of palmitate-treated H4IIEC3 cells. These alterations were also reflected in changes to ROS accumulation and cell viability. Overall, these studies suggest that mitochondrial dysfunction arising from increased FFA availability plays a key role in both *in vitro* and *in vivo* lipotoxicity mechanisms. The ability of amino acids to simultaneously modulate mitochondrial metabolism and lipotoxic outcomes implies that nutritional interventions may provide one possible strategy to control NAFLD progression. However, the regulatory connections linking FFAs to altered mitochondrial function and fuel source selection are still undefined.

While these studies support a role for accelerated mitochondrial metabolism in NASH and lipotoxicity, other studies provide contrasting results. For example, one report found the activities of several electron transport chain (ETC) complexes to be significantly reduced in liver biopsies of NASH patients [72]. Similarly, mitochondria isolated from C57BL/6 mice with HFD-induced NASH had decreased state 3 respiration compared to control [73]. *Ob/ob* mice also demonstrated depressed ETC activity, possibly as a result of increased tyrosine nitration of key mitochondrial proteins [74]. Decreased ATP levels have also been reported in livers isolated from MCD-diet fed rats as a consequence of impaired ETC activity [75]. These conflicting hypotheses surrounding the role of mitochondria in NASH are quite intriguing and warrant further investigation.

7. JNK stress signaling

Since inhibition of CHOP expression does not prevent apoptosis in SFA-treated hepatocytes, other stress signaling pathways must contribute to apoptosis. Stimulation of the c-Jun N-terminal kinase (JNK) pathways has been hypothesized as a concurrent pro-apoptotic mechanism in NASH and lipotoxicity. JNK stress signaling pathways are stimulated by the same factors that have been demonstrated to contribute to NASH, including inflammation, oxidative stress, and ER stress. JNK activation has been observed in NASH patients as well as murine models of steatohepatitis [38,76]. Unlike CHOP, pharmacological inhibition of JNK attenuated SFA-dependent apoptosis in both hepatic cell lines and primary mouse hepatocytes [77]. Additionally, JNK activation appears to be a common component in both ER stress and oxidative stress signaling. In a model of lipid-induced ER stress, JNK activation was observed rapidly after exposure to lysophosphatidylcholine [56]. At high levels, ROS are known to activate pro-apoptotic pathways through JNK-dependent signaling, and studies using *in vitro* models have confirmed that SFA-induced oxidative stress is associated with JNK activation. Murine models of NASH display increased lipid peroxidation products as well as JNK activation [78] and elevated hepatic apoptosis. It has also been shown that co-treating hepatic cells with antioxidants reduces palmitate-induced JNK phosphorylation [62]. These observations demonstrate that JNK signaling is involved in mediating stress responses and promoting apoptosis in SFA-treated liver cell models.

The two liver-specific isoforms of JNK, JNK1 and JNK2, are both associated with obesity-related liver injury [79]. In particular, JNK1 activity is associated with elevated steatohepatitis and apoptosis [80]. This is due to JNK1's unique ability to phosphorylate c-Jun, which can then be integrated into the activator protein-1 (AP-1) complex, a pro-apoptotic transcription factor [81,82]. Knockdown of JNK1 in both murine models and primary hepatocytes resulted in reduced markers of steatohepatitis and lipotoxicity [76]. JNK1 mice fed a high-fat diet exhibited less steatosis, liver inflammation, and fewer apoptotic cells in comparison to wild-type controls. This same study found that antisense oligonucleotide knockdown of JNK1 in high-fat fed wild-type mice resulted in the attenuation of continued liver damage and a reduction in apoptotic cells. Sim-

ilarly, it has been shown that palmitate stimulates the expression of p53 upregulated modulator of apoptosis through a JNK1-dependent mechanism [81].

While JNK1 activation appears to be directly related to increased apoptosis and liver injury, the role of JNK2 is less clear. One *in vitro* study using primary hepatocytes isolated from JNK2$^{-/-}$ mice demonstrated decreased apoptosis in the presence of saturated fatty acids [77]. In this model of lipotoxicity, JNK phosphorylation was associated with elevated Bim-dependent Bax activation. Conversely, a separate study indicated that JNK2 had no effect on apoptosis and liver injury, as JNK2$^{-/-}$ mice had similar inflammation grades and increased apoptosis as compared to wild-type mice on the same high-fat diet [76]. Bim overexpression was also associated with JNK2 deficiency with this model, suggesting JNK2 regulates apoptosis possibly through the repression of Bim. A separate study also found *Jnk2*$^{-/-}$ mice had no change in histology compared to control animals on a MCD diet and no change in serum ALT levels [80]. These studies demonstrate that JNK2 may be involved in lipid toxicity, but may not contribute directly to NASH.

8. Triglyceride synthesis is a protective mechanism to prevent lipotoxicity

The most widely regarded theory in NAFLD progression is the so-called "two hit hypothesis" [83]. The first hit is a product of imbalanced fatty acid metabolism leading to hepatic triglyceride (TG) accumulation that is characteristic of NAFLD. The second hit is thought to involve oxidative and/or metabolic stress as the liver attempts to compensate for prolonged alterations in lipid metabolism, which culminates in hepatic inflammation and cell death [83]. However, questions remain as to whether the accumulation of intrahepatic TGs is actually the initial cause of progressive liver disease or merely an early adaptive response to increased lipid load. Recent literature points to the latter, suggesting that while TG synthesis is symptomatic of hepatic lipid overload, it is actually serving a protective role by providing a route to inertly dispose of excess fatty acids and prevent the formation of more toxic lipid intermediates [84].

Data from *in vitro* experiments provide some important clues as to the role of TGs in hepatic lipotoxicity. For example, experiments with H4IIEC3 rat hepatomas demonstrate that palmitate-induced ROS production and subsequent cell death occur in the absence of excess TG formation. Conversely, treatment with oleate causes significant TG formation with little ROS production or cell death [28]. Interestingly, when palmitate is co-supplemented with oleate, the lipotoxic effects of palmitate are completely abolished with a coincident increase in lipid droplet formation. These experiments suggest that the increased toxicity of SFAs may be in large part attributable to the fact that they are less efficiently esterified into TGs. When the supply of unsaturated fatty acids becomes limited or there is insufficient capacity to dispose of SFAs by either TG synthesis or beta-oxidation, the resulting accumulation of saturated phospholipids and other toxic lipid intermediates may subsequently promote cellular dysfunction and apoptotsis. Several different cell types including β-cells [44,69], cultured rat islets [85], Chinese hamster ovary (CHO) cells [27], breast cancer cells [19] and rat liver cells [28,86,87] have shown similar results; acute SFA lipotoxicity is inversely correlated with triglyceride synthesis.

SCD-1 is the enzyme responsible for catalyzing the endogenous desaturation of SFAs, such as palmitate (16:0) and sterate (18:0), into their monounsaturated counterparts, palmitoleate (16:1) and oleate (18:1), respectively [88,89]. Altering the cellular capacity for fatty acid saturation can dramatically change the potential lipotoxic effects of SFA supplementation. Hepatic models with SCD-1 inhibition have previously been shown to display significant reductions in triglyceride content [90]. Both primary C57BL/6 hepatocytes and HepG2 cells with pharmacological and genetic siRNA inhibition of SCD-1 showed significantly increased sensitivity to palmitate-mediated apoptosis compared to controls treated with palmitate alone [87]. Alternatively, CHO cells overexpressing SCD-1 displayed significant increases in TG synthesis and labeled SFA incorporation into TG species and were almost completely resistant to SFA-induced apoptosis [27].

The beneficial effects of partitioning FFAs into TGs demonstrated by *in vitro* models also translates to *in vivo* rodent models. Mice expressing SCD-1 (SCD-1$^{+/+}$) had significantly increased levels of TG accumulation in the liver compared to both high fat diet (HFD) controls and SCD knockout mice (SCD-1$^{-/-}$), but also significantly reduced markers of hepatic apoptosis and inflammation compared to SCD-1$^{-/-}$ [87]. Interestingly, *in vivo* rodent models of progressive liver disease have demonstrated a highly suppressed expression level of SCD-1 [87,91], suggesting a possible impairment in SFA esterification into TG, thereby implicating compromised and/or reduced TG synthesis as an instigator in the progressive pathology of NAFLD.

Methods for overexpressing or inhibiting enzymes responsible for TG synthesis have also been utilized in order to investigate the relationship between hepatic TG synthesis and progressive liver disease. Yamaguchi et al. [92] studied male *db/db* mice on a MCD diet. These animals have a genetically defective leptin receptor and provide a useful model of severe obesity and type II diabetes, and develop NASH when placed on an MCD diet [7,93]. Their study sought to investigate whether inhibiting TG synthesis with a liver-specific knockdown of diacylglycerol acyltrasferase 2 (DGAT2) would prevent hepatic steatosis and protect the liver from further progression to NAFLD. DGAT2 is responsible for catalyzing the final step in hepatic TG biosynthesis [94]; therefore, the antisense oligonucleotide suppression of this enzyme was successful in preventing hepatic steatosis resulting from MCD-induced accumulation of TGs. However, contrary to the investigators' expectations, reduction of hepatic steatosis with DGAT2-ASO did not protect the MCD-diet fed mice from hepatic inflammation and fibrosis. In fact, these mice experienced increased markers of inflammation and disease associated with NASH, including increased levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lobular inflammation, FFA levels and oxidative damage as assessed by TBAR and 4-HNE levels, compared to MCD-diet controls [92]. An alternate study by Monetti et al. [95] investigated the link between hepatic steatosis and insulin resistance in mice overexpressing the DGAT2 gene in the liver (liv-DGAT2). Finding DGAT-mediated steatosis in the liver to be insufficient to cause insulin resistance in the tissue, they investigated markers of inflammation and ER stress previously shown to promote insulin resistance. Mice expressing a 2-fold increase in hepatic DGAT2 mRNA (Liv-DGAT2-low) displayed a ~5-fold increase in hepatic TG content, but interestingly had reduced levels of phosphorylated JNK, nuclear factor kappa B (NF-κB), and PERK compared to the WT littermates when both groups were maintained on a high-fat diet [95]. Together, these findings suggest that TG synthesis provides a protective mechanism to buffer against toxic accumulation of FFAs in the liver.

Overall, recent literature points to a dissociation between hepatic triglyceride formation and progression towards severe fatty liver disease. While increased hepatic triglyceride formation is most certainly an early indicator of liver metabolic stress and disease, it does not appear to be the initiating factor in NASH, but instead serves as a protective metabolic mechanism to counter FFA overload [84]. Triglycerides may serve as inert storage species for diverting FFAs away from toxic pathways and thus protecting the cell from lipoapoptotic effects. The degree of FFA saturation has

also been demonstrated to have a strong effect on the tendency of the cell to store them as TGs, where unsaturated FFAs are more likely incorporated into TGs and their saturated counterparts are channeled towards other cellular fates. Once the ability of the cell to channel FFAs away from other metabolic pathways leading to lipotoxicity and into neutral TGs becomes diminished and/or overwhelmed, cellular FFA levels increase, resulting in a diseased phenotype characteristic of NASH.

9. From bench to bedside: clinical trials in NASH patients

Investigations into the molecular etiology of NASH have provided potential therapeutic targets to both ameliorate and prevent hepatic apoptosis and inflammation. For example, vitamin E was administered to insulin-tolerant patients to investigate antioxidants as a potential treatment for NASH [96]. Treatment with vitamin E resulted in reduced liver injury assessed by a reduction in serum alanine and aspartate aminotransferase levels. Although fibrosis scores were unimproved, other histologic features such as lobular inflammation and hepatocellular ballooning were improved. Similarly, pentoxifylline has been utilized successfully in mouse models [97] and human clinical trials [98] for treatment of NASH. Pentoxifylline is a non-selective phosphodiesterase inhibitor that has well known effects to suppress production of pro-inflammatory cytokines, such as TNF-alpha, as well as antioxidant properties that include reducing ROS production, scavenging free radicals [99,100], and preventing the depletion of glutathione stores [101]. In the clinical trial, 55 patients with confirmed NASH received three daily doses of pentoxifylline or placebo over a 1-year period. After the duration of treatment, analysis showed significantly more patients on pentoxifylline than placebo had NAS decrease by $\geqslant 2$ points. Steatosis, lobular inflammation and fibrosis also showed significant improvements although no change in hepatocellular ballooning was observed [98]. The ameliorating effects of antioxidant therapy highlight the central pathogenic role oxidative stress has in NASH disease progression.

Well-known antidiabetic drugs, such as metformin and thiazolidinedione derivatives, have also been frequently utilized as NASH treatments in diabetic and non-diabetic patients alike. Metformin is a first-line treatment for type II diabetes that inhibits hepatic gluconeogenesis [102] and alters fatty acid metabolism [103], often resulting in patient weight loss. In a clinical trial of NASH, metformin was shown to improve liver histology and ALT levels in a significant portion of patients (30%), which could not be attributed

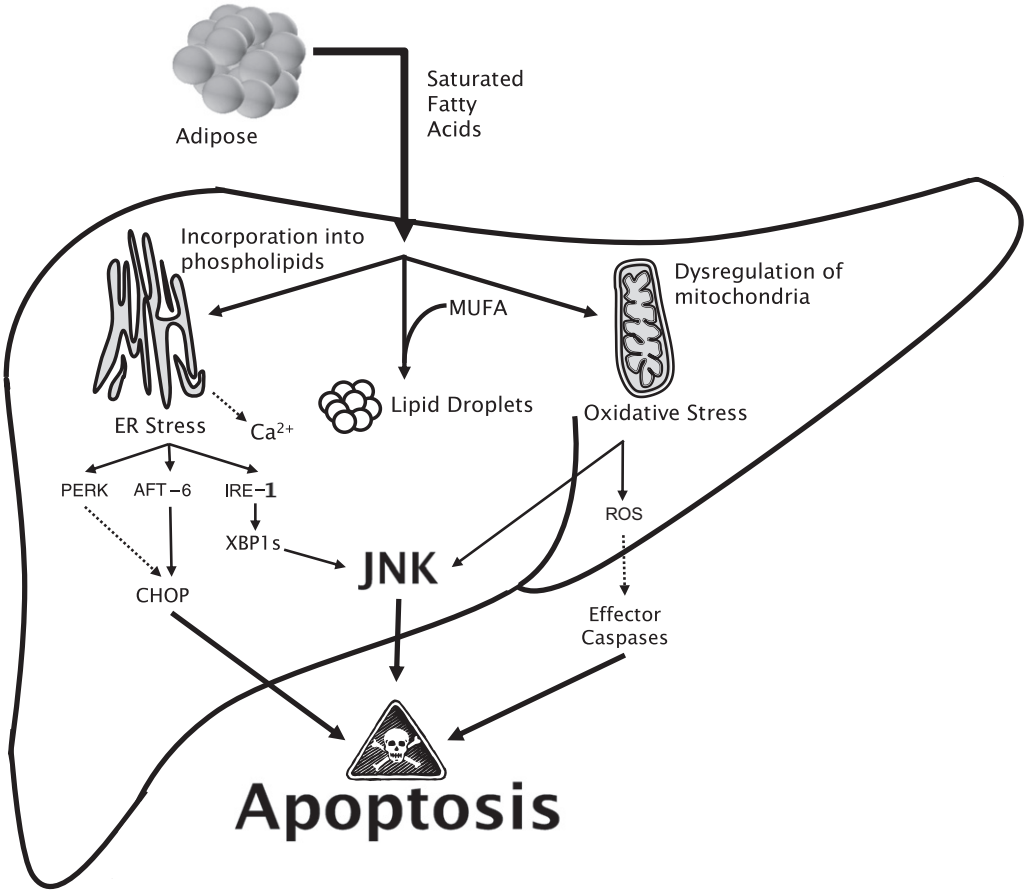

Fig. 1. Saturated fatty acids initiate cellular dysfunction in lipotoxicity/NAFLD. Lipolysis of subcutaneous and visceral adipose tissue gives rise to higher concentrations of free fatty acids in the blood resulting in ectopic fat storage within the liver. Upon entering the liver, FFAs are partitioned toward three main lipid disposal pathways: β-oxidation, TG synthesis, and phospholipid synthesis/remodeling. Mitochondrial β-oxidation gradually breaks down fatty acids into two-carbon acetyl-CoA units, which are subsequently used to fuel the TCA cycle. Esterification of FFAs into TG and phospholipids provides an alternate route for disposing of elevated FFAs. High concentrations of saturated fatty acids, however, avoid protective sequestration into triglyceride stores and/or β-oxidation. Instead, SFAs are channeled toward phospholipid incorporation. Increased saturation of ER phospholipids can compromise the integrity of the ER-membrane structure resulting in stimulation of UPR signaling pathways and disruption of mitochondrial function. Additionally, elevated SFAs deregulate TCA cycle metabolism leading to ROS accumulation. Both ER-stress pathways and mitochondrial dysfunction activate JNK stress signaling, thus leading to eventual apoptotic cell death. Abbreviations: monounsaturated fatty acid (MUFA); endoplasmic reticulum (ER); protein kinase RNA-like endoplasmic reticulum kinase (PERK); inositol-requiring protein–1 (IRE-1); Xbox binding protein-1 spliced (XBS-1s); activating transcription factor 6 (ATF-6); Jun N-terminal kinase (JNK); reactive oxygen species (ROS).

to increases in insulin sensitivity [104]. The beneficial effects on NASH appeared to be mediated partly by the ability of metformin to promote weight loss, where the most weight loss correlated with the most improvement in liver histology. In fact, reduction in body mass by itself has been described as an effective intervention in NALFD progression [105]. Results from clinical studies indicate that reductions in body weight of just 5% to >7% result in significant reductions in steatosis, NAS, and other laboratory abnormalities associated with progressive NAFLD in children [106] and adults with NASH [107,108], respectively. Studies [109–111] and reviews [112,113] on the impact of weight loss due to bariatric surgery have demonstrated similar improvements.

Pioglitazone is a derivative of the thiazolidinedione class of drugs used to treat type II diabetes mellitus due to its ability to reduce insulin resistance by activating PPAR-γ and controlling glucose and lipid metabolism in peripheral tissues [114]. A placebo-controlled clinical trial of pioglitazone in patients with NASH revealed encouraging results, where patients receiving pioglitazone displayed significant improvements in ALT and AST levels as well as hepatic histological markers such as reduced steatosis, ballooning, necrosis and inflammation despite a small weight gain [115]. However, fibrosis improvements were not significantly different than that of a placebo + dietary intervention group. The conclusions about the mediator of this marked NASH improvement in patients treated with pioglitazone were not attributable to a single factor, but speculation regarding several mechanisms was briefly discussed. Mechanisms postulated include the following two: (i) thiazolidinediones are known to increase insulin sensitivity and reduce peripheral lipolysis from adipose tissue [116], thus significantly decreasing the flux of free fatty acid substrates to the liver and (ii) pioglitazone has been shown to activate AMPK [116] and raise adiponectin levels in the liver [117], which may play an important role in mediating the effects of thiazolidinediones in the liver. The serum aminotransferase level and histological improvements demonstrated with some classic diabetes drugs in patients with NASH warrants further investigation into the metabolic mechanisms activated in order to develop more targeted therapies in the future.

10. Conclusion

Nonalcoholic steatohepatitis is the liver manifestation of obesity and the metabolic syndrome and is marked by lipid deposition, inflammation and apoptosis. While triglyceride accumulation is a hallmark of NAFLD, several *in vitro* and animal models suggest triglyceride accumulation is a protective mechanism to delay the toxic effects of excess lipids. Altered composition of intrahepatic free fatty acids and phospholipid metabolites have instead been implicated as major contributors to acute liver dysfunction and apoptosis. Many studies have demonstrated that saturated fatty acids are more toxic than their unsaturated counterparts, resulting in a progressive lipotoxic casacade (Fig. 1). SFAs have been shown to increase the saturation of membrane phospholipids, thus initiating UPR and leading to ER stress. SFAs also affect mitochondrial metabolism and promote ROS accumulation. Furthermore, hepatocyte apoptosis has been shown to be dependent on the activation of JNK stress signaling pathways that respond to prolonged ER and oxidative stress. Despite these common molecular features of lipotoxicity, a unifying mechanism is still unclear and has prevented the development of novel treatments for NASH. However, recent literature provides strong evidence that fatty acid saturation plays a critical role in determining cell fate under lipotoxic conditions. Continued research in understanding these molecular mechanisms will provide direction for more targeted therapeutics for NAFLD and NASH patients in the future.

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