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Molecular Mechanisms of Lipotoxicity and Glucotoxicity in Nonalcoholic Fatty Liver Disease

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Abstract

The exposure of hepatocytes to high concentrations of lipids and carbohydrates and the ensuing hepatocellular injury are termed lipotoxicity and glucotoxicity, respectively. A common denominator is metabolic derangement, especially in regards to intracellular energy homeostasis, which is brought on by glucose intolerance and insulin resistance in tissues. In this review, we highlight the lipids and carbohydrates that provoke hepatocyte injury and the mechanisms involved in lipotoxicity and glucotoxicity, including endoplasmic reticulum stress, oxidative stress and mitochondrial impairment. Through upregulation of proteins involved in various pathways including PKR-like ER kinase (PERK), CCAAT/enhancer-binding homologous protein (CHOP), c-Jun NH2-terminal kinase-1 (JNK), Bcl-2 interacting mediator (BIM), p53 upregulated modulator of apoptosis (PUMA), and eventually caspases, hepatocytes in lipotoxic states ultimately undergo apoptosis. The protective role of certain lipids and possible targets for pharmacological therapy are explored. Finally, we discuss the role of high fructose and glucose diets in contributing to organelle impairment and poor glucose transport mechanisms, which perpetuate hyperglycemia and hyperlipidemia by shunting of excess carbohydrates into lipogenesis.

Keywords

Hepatic lipid; NASH; ER stress; CHOP; JNK; BH3-only proteins; Death receptors; Oxidative stress; Inflammation; Fibrosis

1. INTRODUCTION

Lipotoxicity refers to the harmful effects of high concentrations of lipids and lipid derivatives to cells. Hyper-alimentation with diets rich in lipids and carbohydrates is associated with the development of one of two clinical-histopathological phenotypes of liver fatty accumulation: non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). Clinically, NASH is associated with obesity, metabolic syndrome, insulin resistance and dyslipidemia. Histological features of NASH include hepatic macrovesicular lipid accumulation, chronic inflammation, hepatocyte ballooning, interstitial fibrosis and

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necro-apoptosis [1]. The mechanisms involved in lipotoxicity include endoplasmic reticulum (ER) stress, c-Jun NH2-terminal kinase (JNK)-induced toxicity and BH3-only protein-induced mitochondrial and lysosomal dysfunction [2-6].

Glucotoxicity refers to the toxic effects of hyperglycemia and excess carbohydrate intake on cells and tissues. Glucotoxicity is intrinsically linked to insulin resistance, which facilitates hyperglycemia. Excess carbohydrates can be converted into free fatty acids (FFA) and triglycerides (TG), and subsequently hepatotoxic lipids such as lysophosphatidyl choline (LPC), ceramides, free cholesterol and bile acids (BA) may accumulate. High carbohydrate diets activate several lipogenic enzymes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) or SCD-1, inducing lipogenesis and steatosis. Recent data indicate that glucotoxicity can be injurious to liver cells by inducing ER stress and hepatocyte cell death. In this review, we explore some of the organelle dysfunction and molecular pathways activated by excessive consumption of carbohydrates and lipids fats, leading to hepatotoxicity.

2. HEPATOTOXIC LIPIDS

Several members of the lipid family have been shown to mediate hepatic lipotoxicity. These include free fatty acids (FFA), triglycerides (TG), lysophosphatidyl choline (LPC) and ceramides, free cholesterol (FC), and bile acids (BA).

2.1 Free fatty acid (FFA) and triglycerides (TG)

2.1.1 Free Fatty acid—FFA with double bonds are referred to as “unsaturated” while those without double bonds are called “saturated”. Palmitate (PA; C16:0), the most common saturated FFA found in animals and plants, is ingested as part of the diet or can be produced by *de novo* lipid synthesis from excess carbohydrate consumption. Oleate (OA; C18:1), an unsaturated FFA, is commonly present in the Western diet. Several in vitro studies have demonstrated the toxic effects of unsaturated FFA such as PA or stearate on liver cells by inducing apoptosis (*vide infra*).

Hepatocytes exposed to high circulating FFAs increase uptake in order to clear the FFAs in the blood. FFAs entering the liver are mostly derived from lipolysis of adipose tissue triglyceride in the fasting state (constituting 60% of liver FFAs in NAFLD subjects), *de novo* lipogenesis (26%) and from hydrolysis of dietary triglycerides (15%) [1]. Although the precise mechanisms regulating increased hepatic FFA uptake are unclear, it seems to involve a tetrameric plasma membrane protein complex that comprises plasma membrane fatty acid-binding protein (FABP), caveolin-1, fatty acid translocase (FAT/CD36) and calcium independent membrane phospholipase A2 (iPLA2β) [2]. FAT/CD36-mediated incorporation of circulating FFA into hepatocyte vacuoles causes these hepatocytes to resemble adipocytes [3]. In the liver of mice and humans with insulin resistance, steatosis and NASH, CD36 is overexpressed via transcriptional regulation by the transcription factor PPARγ. Hepatocytes exposed to PA and OA display increased expression of FAT/CD36 and fatty acid transport protein (FATP)-2 leading to accumulation of diacylglycerol (DAG) or ceramides into the cells [4].

2.1.2 Triglycerides—Triglycerides (TG) are composed of a glycerol molecule with three free fatty acids and represent a form of energy storage. Studies performed in humans have shown that accumulation in excess of hepatic TGs is mainly the result of increased delivery of adipose-derived FFAs to the liver and enhanced *de novo* lipid synthesis in the liver [1]. In contrast hepatic steatosis is only modestly affected by lipid disposal via β-oxidation or very low density lipoproteins (VLDL) export [5]or directly by increased dietary lipids [1]. Also, consumption of large amounts of carbohydrates can contribute to hepatic steatosis by facilitating lipogenesis and lipid storage as TG. Indeed, rat pups fed a 60% fructose rich diet showed altered lipid profile with increased TG, cholesterol, VLDL and low density lipoproteins (LDL) which was reversed when fed a standard diet [6].

2.1.3 Unlike TG, saturated FFA are toxic to hepatocytes—Treatment of hepatocyte cell lines with OA versus PA showed that while OA generated more hepatocyte steatosis, PA was responsible for higher rates of apoptosis. PA was associated with PPARα activation and impairment of insulin signaling. In addition, PA triggered cell death via JNK-dependent mitochondrial dysfunction and caspase activation [7](Table 1). OA, on the other hand, generated higher formation of TG [7]. It appears that TG represent a defense system against the pro-apoptotic effects of large loads of FFA in cells [7,8]. Yamaguchi *et al.* confirmed the protective role of TG by showing that inhibition of dyacylglycerol acetyltransferase 2 (DAGT2), the final catalyst in hepatocyte TG synthesis, generated increased necro-inflammation, increased peroxidation and oxidative stress [9] (Table 1).

2.2 Lysophosphatidyl choline (LPC)

LPC is a class of lipids derived from phosphatidylcholine by partial hydrolysis through the phospholipase A2 (PLA2). LPCs have been implicated in phagocyte chemotaxis and are released secondary to activation of calcium independent PLA2 by caspase 3 when cells undergo apoptosis [10]. LPC levels are increased in the liver or plasma of both human NASH patients [11] and animal models of NASH [12,13]. Furthermore, treatment of liver cells with PA results in increased intracellular LPC concentration and cell toxicity [14]. LPC appears to be a key instigator of lipotoxicity by triggering an ER stress and inducing apoptotic pathways downstream of the activation of JNK or glycogen synthase kinase 3 (GSK3) and the induction of the transcription factor CCAAT/enhancer-binding homologous protein (CHOP), all leading to the upregulation of pro-death proteins, *e.g* p53 upregulated modulator of apoptosis (PUMA). LPC toxicity was attenuated by inhibition of JNK or GSK3 activity and by knockdown of *CHOP* [14]. In fact, it seems that LPC-induced lipotoxic mechanisms are largely indistinguishable from those of PA, suggesting that FFAs could exert cytotoxicity through generation of LPC [14] (Figure 1).

2.3 Ceramides

Ceramides and their derivative sphingolipids are cell membrane components and biologically active lipids that have been implicated in insulin resistance, oxidative stress and inflammation [15]. Increased circulating concentration of ceramides were seen in peripheral blood samples of obese patients with NASH [16]. Ceramides can be produced *de novo* after oxidative stress from palmitoyl co-A and serine through the action of serine palmitoyl co-A transferase (SPT), which is the rate limiting enzyme. Another pathway for generation of

ceramides is through the action of neutral sphingomyelinase (N-SMase) hydrolysis of cell membrane sphingomyelin. N-SMase is upregulated by inflammation [17] (Table 1).

Increased pro-inflammatory cytokines such as interleukin (IL)-1 and IL-6 are present in NASH and correlate with augmented ceramide levels [17]. Indeed, in the liver, ceramides interacts with TNFα and promotes the release of reactive oxygen species (ROS) by hepatic mitochondria, resulting in apoptosis and worse hepatic inflammation [17,18]. Similarly, mice fed a high fat diet with subsequent steatosis displayed an increase in sphyngomyelinase activity [19]and in hepatic long chain ceramides (C16 and C18) which were associated with hepatocyte apoptosis [20]. Also, increased liver ceramide levels were also associated with the development of hepatic insulin resistance [4,21]. The role of ceramides in NAFLD and lipotoxicity is still not fully understood and seems to be cell-type specific. Indeed, saturated FFAs-induced hepatocyte apoptosis was found to be ceramide-independent [22,23], whereas in pancreatic β-cells, ceramides are important mediators of cellular failure and apoptosis and may work as second messenger in cross-talk between the ER and mitochondria [24].

Inhibition of ceramide synthesis, using inhibitors such as myriocin or imipramine, reduced liver injury, steatosis, and improved insulin sensitivity in target hepatic tissue in rodents fed a high fat diet [25,25,26].

2.4 Free Cholesterol (FC) and Bile Acids (BA)

In NAFLD, hepatic free cholesterol (FC) accumulates as a result of enhanced synthesis, increased cholesterol de-esterification and decreased cholesterol export and BA synthesis. Abundant intracellular FC stimulates Kupffer cells and hepatic stellate cells (HSCs) which mediate inflammation and fibrosis as well as mitochondrial dysfunction, generation of ROS, activation of unfolded protein response (UPR) and downstream effects that culminate in hepatocyte apoptosis [27].

In the liver, disruption of cholesterol homeostasis contribute to its accumulation in liver cells and within organelles. Sterol regulatory-element binding protein (SREBP)-2 is the principal regulatory enzyme of hydroxymethylglutaryl-CoA reductase (HMGCoA), which is the key cholesterol synthesis enzyme. SREBP-2 levels are increased in NASH, correlating with increased levels of FC and steroidogenic acute regulatory protein (StAR), a mitochondrial cholesterol transporter [28]. Feeding animals with high levels of FC results in accumulation of toxic hepatic oxysterols which contributes to mitochondrial dysfunction and liver injury [29, 30] (Table 1). In another study, mitochondrial FC accumulation led to apoptosis through a toll like receptor 4 (TLR4)-regulated JNK1 pathway that activated the inflammatory mediators high mobility group box 1 protein (HMGB1) [31](Table 1). The key role that cholesterol plays in the development of steatohepatitis has prompted research into the use of utilizing cholesterol lowering medications to protect subjects from developing NASH. Indeed, this was demonstrated recently in a multicenter cohort of 1201 patients where statins were protective against liver damage [32]. *In vitro* and *in vivo*, fluvastatin attenuated HSC activation and protected against liver fibrosis, inflammation and oxidative stress [33].

BA are end-products of the degradation of cholesterol. Hydrophobic bile acids, including deoxycholic acid (DCA), can trigger hepatic apoptosis and are increased in NASH through a JNK1/p53/sirtuin 1 (SIRT1) pathway [34]. Farnesoid X receptors (FXR) are bile acid receptors that inhibits BA synthesis, and FXR agonists can protect from steatohepatitis and improve insulin resistance and hyperlipidemia in obese patients [35,36].

Different lipid families are toxic to hepatocytes and induce lipoapoptosis. Among them, some of the most prominent offenders to the liver are unsaturated FFA, arising from TGs lipolysis in adipose tissue and excess release of FFA into the circulation. Saturated FFA (such as palmitate and stearate) or their end products (LPC) exert their toxicity by triggering several apoptotic processes implicating the activation of ER stress- and JNK-dependent pathways. In addition, the role of ceramides in the liver is still being defined, but it exerts an important effect on insulin resistance. Other lipids, such as FC and BA, contribute to liver inflammation, fibrosis, mitochondrial dysfunctions and apoptosis in the fatty liver disease spectrum.

3. MOLECULAR MECHANISMS OF HEPATOCYTE LIPOTOXICITY

Failure of hepatocytes to dispose of excess FFA results in lipoapoptosis, a cardinal feature of NASH. In hepatocytes, apoptosis can occur via an intrinsic pathway activated by intracellular stress such as oxidative stress or by organelle dysfunctions including ER stress and mitochondrial permeabilization. Hepatocyte apoptosis can also occur via an extrinsic mechanism initiated by binding of death ligands, such as Fas or TRAIL, to their respective receptors. Both intrinsic and extrinsic pathways converge on effector caspases to mediate apoptosis.

3.1 Endoplasmic reticulum (ER) stress

The progression of liver disease from steatosis to NASH is characterized by endoplasmic reticulum (ER) stress[37]. The ER supports many vital cellular processes, including synthesis, maturation, folding and transport of protein, lipid synthesis and packaging and regulation of calcium homeostasis. Disturbances of any of these processes will initiate an ER stress. The response to an ER stress is initially mediated by the combination of several signaling pathways termed the unfolded protein response (UPR), which serves at first to re-establish ER homeostasis and promote survival by directing unfolded or misfolded proteins towards degradation [38]. However, a prolonged activation of the UPR will trigger apoptotic pathways causing cell death. In addition, recent work has shown that lipid saturation of the ER membrane can activate UPR independently of unfolded proteins, supporting a direct role of lipids in ER stress response [39].

The three main UPR-mediated transmembrane proteins activated in ER stress are IRE1/X-box binding protein 1 (XBP1), PRKR-like endoplasmic reticulum kinase (PERK)/eukaryotic translation initiation factor 2α (eIF2α), and activating transcription factor-6 (ATF6) [40]. During normal cell function, the transmembrane proteins are bound to the intraluminal chaperone binding immunoglobulin protein (Bip/GRP78). Under stressful conditions induced by accumulation of misfolded or unfolded proteins, depletion of ER-calcium levels or increased free cholesterol in the ER lumen, Bip/GRP78 is released from the UPR

transmembrane proteins, resulting in the activation of the IRE1-, PERK- or ATF6-mediated signaling pathways [38]. These pathways regulate the expression of genes involved in UPR and ER-assisted degradation (ERAD) with the aim of restoring ER homeostasis. However, these pathways also regulate expression of genes that mediate apoptosis. IRE1 may mediate cell apoptosis by recruiting TNF receptor-associated factor 2 (TRAF2) and activating JNK [41]. PERK-mediated apoptosis involves activation of downstream gene *GADD153* (also known as CHOP [CCAAT-enhancer-binding protein homologous protein]), while activated ATF6 promotes the expression of XBP1 and CHOP [38,42].

All three ER stress sensing pathways (ATF6α, PERK/eIF2α, IRE1α/XBP1) can regulate the development of microvesicular steatosis in the liver [43]. Of note, both XBP1 and eIF2α have been shown to participate in basal and/or diet-induced regulation of lipid metabolism [44,45]; and prolonged expression of CHOP can inhibit CCAAT/enhancer-binding protein alpha (C/EBPα) leading to the suppression of genes involved in lipid homeostasis and possibly driving the development of steatosis [43] . It appears that once the liver crosses the threshold from fatty accumulation to NASH, genes involved in apoptosis are upregulated, while ER stress/UPR gene sets are downregulated [46]. Liver specific depletion of *PERK* reduces the transcriptional and translational phases of the UPR with consequent disruption of lipid metabolism and enhanced apoptosis [47].

Upregulation of the transcription factor CHOP, which has low levels of expression under non-stress conditions, plays a critical role in FFA-mediated ER stress-related liver cell death [48,49]. Treatment of Huh-7 hepatocytes with the FFA palmitate led to increased interaction between CHOP and the other transcription factor c-Jun which bind to the activator protein-1 (AP-1) binding site within the *p53 upregulated modulator of apoptosis (PUMA)* promoter. This results in upregulation of the BH3-only protein PUMA, with subsequent Bax activation and cell death [50](Figure 1). Hepatic cells with knockdown of *CHOP* failed to upregulate PUMA in response to PA insult [50]. CHOP has also been implicated in the upregulation of the TRAIL receptor death receptor 5 (DR5) and the other BH3-only protein BIM (Figure 1), both inducing cell death [51, 52].

3.2 JNK-dependent toxicity

Several articles have explored the relationship between free fatty acids and JNK activation, since the JNK pathway is one of the key mediators of insulin resistance and fatty acid-induced hepatotoxicity [50, 53]. The JNK pathway is known to be stimulated by both oxidative stress and ER stress. Malhi *et al.* demonstrated that hepatocyte apoptosis induced by saturated FFAs occurred via a JNK-dependent mechanism involving upregulation of BIM and Bax activation, leading to caspase release and mitochondrial dysfunction and culminating in cell death [54]. Hepatocytes contain two JNK genes, *JNK1* and *JNK2*. The isoform JNK1 plays a central role in apoptosis induction by regulating the expression of pro-apoptotic B-cell lymphoma protein 2 (Bcl-2) family BH3-only proteins [50]. It is known that JNK1 can phosphorylate c-Jun, which in turn can be integrated into the pro-apoptotic AP-1 complex. It has been demonstrated that saturated FFA, through the activation of JNK1, can upregulate PUMA in both murine and human cellular models [50,55](Table 1). PUMA works cooperatively with BIM to mediate FFA-induced hepatocyte injury (Figure 1). PUMA also

activates Bax, which mediates the mitochondrial pathway of apoptosis [55]. Non selective inhibition of JNK by SP600125 *in vitro* led to attenuation of PA-induced PUMA upregulation, resulting in decreased lipoapoptosis [54-56].

3.3 BH3-only proteins

The Bcl-2 family proteins regulate the permeabilization of the mitochondrial membrane, allowing cytochrome *c* leakage from the mitochondria. The Bcl-2 proteins contain one or more Bcl-2 homology (BH) domains. Anti-apoptotic members of the Bcl-2 family contain all four BH domains, while the proapoptotic members are divided among the multi BH domain members including Bak, Bax and Bok, and the BH3-only proteins including Bad, Bid, BIM, and PUMA [42]. Once Bid, a BH3-only protein, is cleaved by caspase-8, it can activate multi-domain members Bak and Bax to form pores in the outer mitochondrial membrane and allow for cytochrome *c* leakage. Caspase-8 activation is mediated by binding of Fas ligand to its receptor Fas, a death receptor that has been found to be overexpressed in patients with hepatitis C, NASH and other chronic hepatocyte inflammatory conditions [57].

Both BIM and PUMA have been implicated as important contributors of FFA-induced hepatocyte toxicity [50,58]; and knockdown of *BIM* or *PUMA* in liver cells partially protects against FFA-induced Bax activation, mitochondrial dysfunctions, caspase-3 activation and apoptosis. As discussed in previous above sections, increase in PUMA cellular levels during the lipotoxic insult involves both JNK1 and CHOP-dependent transcriptional regulation of the protein [50]. In the context of lipotoxicity, BIM transcriptional upregulation is dependent on the transcription factor Forkhead box O3a (FoxO3a), activated upon dephosphorylation by the protein phosphatase 2A (PP2A) [58]. Cellular levels of anti-apoptotic Bcl-2 proteins, such as Bcl-XL and Mcl-1 are also decreased during FFA-induced lipotoxicity [23,59]. Decreased expression of Bcl-XL or increased proteasomal degradation of Mcl-1 both contribute to the apoptotic phenotype. [50] Thus, preventing the decrease of Bcl-xL or Mcl-1 by either overexpressing Bcl-XL or a non-degradable form of Mcl-1 partially reduced the lipotoxic insult in liver cells. Whereas BIM and PUMA directly or indirectly activate Bax; the pro-survival proteins Mcl-1 and Bcl-xL inhibit Bax function at the mitochondria by direct interaction with Bax or indirectly by sequestration of activator BH3-only proteins [60]. Following Bax activation and permeabilization of mitochondrial membranes, proteins that participate in the execution phase of apoptosis, such as caspases 3, 6 and 7, are activated and trigger DNA damage and cell death. NAFLD severity is reflected by an increase of serum caspase-cleaved cytokeratin–fragments [61]; and caspase 3 knockout mice are protected against liver injury induced by a high fat diet [62]. Thus, BH3-only proteins can be activated by multiple mediators with the end result of hepatocyte apoptosis [63].

The pan-caspase inhibitor Z-VAD-fmk was found to attenuate saturated FFA-induced apoptosis in mouse hepatocytes [54]. Despite a recent phase 2 clinical trial accessing tolerability and efficacy of caspase inhibitor GS9450 in adults with NASH, a longer trial was stopped as a result of drug toxicity [64]. The utility of other caspase inhibitors in NASH may need to be investigated.

3.4 Death receptors

The tumor necrosis factor related apoptosis-inducing ligand (TRAIL) receptor 2 or DR5, has been implicated as one of the death receptor signaling pathways in lipotoxicity [52](Figure 1). Exposure to PA induced an increase in *DR5* mRNA and protein expression in Huh-7 cells, leading to DR5 clustering into lipid rafts, recruitment of caspase-8, and cell death through activation of Bax [52]. In another study, oleic acid (OA) sensitized Huh-7 cells and primary mouse hepatocytes to TRAIL-induced toxicity, via JNK mediated upregulation of DR5 [65].

3.5 Mitochondria and oxidative stress

The features of hepatic steatosis include insulin resistance, increased FA beta-oxidation and oxidative stress, while NASH patients show evidence of mitochondrial dysfunctions and structural defects [66]. Koliaki *et al.* showed by using high-resolution respirometry that mitochondrias work harder in obese humans with or without NASH (4.3-5.0 max respiration rate). However, NASH livers had a higher mass of mitochondrias with 30-40% lower respiration rates, with associated insulin resistance, mitochondrial uncoupling, and leaking activity [67]. OA exposure of Chang liver cells caused mitochondrial swelling and reduced mitochondrial calcium concentration, leading to generation of reactive oxygen species (ROS), alteration of cell cycle and decreased cell viability. Cells were rescued when pre-treated with the p53 inhibitor pifithrin-alpha (PTA), suggesting a role for p53 in OA-associated oxidative stress [68]. The voltage dependent anion channel (VDAC) of mitochondria's outer membrane can act as an early sensor for lipotoxicity. When exposed to lipid accumulation states, VDAC exhibits less phosphorylation which allows great influx of water and calcium into the organelle. Ultimately, this edema leads to cytochrome *c* release and cell death [69]. Moreover, free cholesterol in livers of diabetic mice with NASH accumulates in the hepatocyte plasma membrane, mitochondria and ER, resulting in mitochondrial permeability transition pore, generation of oxidative stress and apoptosis in a JNK1-dependent manner. At the molecular level, Sab (SH3BP5), a mitochondrial outer membrane JNK docking protein and substrate, undergoes JNK phosphorylation with subsequent impairment of mitochondrial function and apoptosis. These findings were dependent on the PA concentration to which hepatocytes were exposed [70].

A recent study by Gariani et *al.,* demonstrated that high-fat high-sucrose fed mice display lower hepatic NAD+ levels causing reductions in hepatic mitochondrial functions in parallel to increase hepatic weight, steatosis and lipid peroxidation. Enhanced NAD^+ salvage may represent a prerequisite for the upregulation of mitochondrial metabolism as an adaptive mechanism in response to chronic hepatic lipid accumulation and glucose intolerance [71]. In support of that concept, NAD^+ repletion with the NAD^+ precursor nicotinamide riboside (NR) prevented or reversed NAFLD in mice fed a high fat-high sucrose diet. NAD^+ protective effects were dependent on the induction of the aging-associated histone deacetylases, SIRT1 and SIRT3-mediated mitochondrial unfolded protein response (UPR^{mt}) [72], triggering an adaptive pathway to increase hepatic beta-oxidation and mitochondrial complex content and activity. Thus, NAD^+-induced SIRT1 activation regulates various cellular processes including energy metabolism, stress response and mitochondrial functions [68].

Lysosomal permeabilization and release of cathepsin B, a major lysosomal cysteine protease, into the cytosol is observed in human liver tissues from patients with NAFLD [73] and could also contribute to mitochondrial dysfunctions and caspases activation associated with the disease. Treatment of Huh7 cells with PA activates the lysosomal pathway of apoptosis through Bax-dependent permeabilization of lysosomes and release of cathepsin B in the cytosol; and genetic or chemical inhibition of cathepsin B activity partially reduced PA-induced mitochondrial dysfunction and liver cell death [74].

There is crosstalk between the ER and mitochondria. Exposure of primary hepatocytes and H4IIEC3 cells to PA resulted in efflux of calcium from the ER, leading to mitochondrial dysfunction and oxidative stress [75]. One of the key mediators of mitochondrial dysfunction is generation of ROS during oxidative stress (Figure 1). CYP2E1 is implicated in hepatocyte injury and progression to NASH by promoting oxidative stress, inflammation, protein modification and insulin resistance [76]. A significant amount of CYP2E1 is found in the mitochondria. This enzyme hydrolyzes various small molecules such as FA and ethanol into byproducts (toxic superoxide anion) which alter mitochondrial respiratory chain and damage mitochondrial constituents [77]. It has been suggested that the accumulation of ROS occurs downstream of the inner membrane mitochondrial oxidative metabolism, since blocking of cytochrome complex I was associated with no accumulation of ROS in H4IIEC3 rat hepatoma cells exposed to PA [75]. Another way by which FFA-mediated oxidative stress induces toxicity is through the Keap1-Nrf2 pathway. Kelch-like ECH-associated protein (Keap1) works as an adaptor for E3 ligase and initiates proteasomal degradation of several proteins. Treatment of liver cells with PA resulted in decreased Keap1 cellular levels as the result of an increased Keap1 degradation through autophagy by a p62 dependent mechanism [78, 79]. In addition, short hairpin RNA-mediated knockdown of *Keap1* resulted in upregulation of BIM and PUMA, leading to hepatocyte apoptosis [80] (Table 1).

Reduction of oxidative stress is a potential therapeutic strategy for patients with NASH. In the PIVENS trial, vitamin E improved histological and serum biomarkers of liver injury in adults with NASH, with no significant effect on fibrosis over the 2-year study period [81]. In the subsequent TONIC trial, NASH resolution was greater in children treated with vitamin E when compared to metformin [82]. Also, inhibition of cathepsin B could also represents another potential therapeutic strategy for NAFLD patients. Pharmacological cathepsin B inhibition using cathepsin inhibitors CA07 and E-64 preserved mitochondrial function, reduced oxidative stress, and protected against hepatic steatosis, liver injury, and insulin resistance [74] .

Many molecular mechanisms contribute to lipoapoptosis. This review focused on how organelle dysfunction (*i.e.* ER stress and mitochondrial impairment) contribute to liver cell injury. Indeed, prolonged ER stress and exhaustion of the UPR mechanisms results in the activation of CHOP and downstream upregulation of the death mediators PUMA, BIM and DR5. Also, ER stress-dependent and -independent activation of JNK is an important pathway activated by FFA, resulting in BIM and PUMA-mediated activation of Bax with subsequent mitochondrial dysfunctions, increased caspase activity and cellular demise. Finally, oxidative stress downstream of mitochondrial impairment also contributes to hepatocyte apoptosis.

4. PROTECTIVE ROLE OF CERTAIN LIPIDS

Despite the hepatotoxic effect of most lipids, there is increasing awareness of the beneficial effects of certain lipids. At present, studies on beneficial lipids are few and far between, calling for more studies in this arena.

4.1 Monounsaturated Fatty acids (MUFAs)

Exposing human and murine hepatocytes to MUFAs generated lipid accumulation, but preserved cell viability. This was thought to be dependent on stearoyl-CoA desaturase-1 (SCD-1), and enzyme that converts the transformation of saturated FA into MUFAs. Thus, MUFAs are a safe form of excess lipid storage [83,84].

Palmitoleate (PO) is a monounsaturated FFA which works as a lipokine in adipose tissue. Recent studies have shown that it has a protective role against apoptosis in Huh-7 cells and primary hepatocyte [85]. However, PO did not protect against steatosis, but rather accentuated the lipid accumulation induced by PA in Huh-7 cells. PO inhibited ER stress responses by blocking upregulation of BIM and PUMA, thus protecting hepatocytes against downstream death mediator Bax [85].

4.2 Polyunsaturated Fatty Acids (PUFAs)

PUFAs have recently been heralded as a potential therapy for prevention of NASH. The availability of n-3 long chain PUFAs to the liver is crucial in fat removal from hepatocytes [86]. The beneficial effect of n-3 PUFA was suggested by the finding that NASH patients had decreased levels of n-3 PUFA eicosapentaenoic and docosahexaenoic acids [87], which may contribute to the progression of steatosis to steatohepatitis [88]. Several clinical trials have demonstrated the beneficial effect of n3-PUFAs eicosapentaenoic acid supplementation in NAFLD patients resulting in improvement in the major histological features of disease activity (steatosis, necro-inflammation, ballooning, fibrosis) [89,90] , suggesting a potential medical application of PUFAs in inhibiting disease progression. Moreover improvement in NASH activity scores correlated with increase in plasma levels of the n3-PUFA alpha-linolenic (ALA) [89]. Similar protective effects of PUFAs have been described *in vitro* using Huh7 cells. Indeed, treatment with the n-6 PUFAs linoleate can prevent PA-induced apoptosis and inflammation in liver cells by inhibiting JNK and nuclear factor Kappa beta (NFκβ) activation and by suppressing the production of the pro-inflammatory cytokine IL-8 [91]. Another study found that alpha-linolenic acid played a protective role to primary rat hepatocytes exposed to stearic acid through decrease of ER stress markers GRP78, CHOP and GRP94 [92]. However, the beneficial role of n3-PUFAs in NAFLD remains controversial. Indeed, in a larger double-blind trial (performed in 37 sites in North America) of NAFLD patients receiving ethy-leicosapentanoic acid supplementation versus placebo, there was no significant difference in NASH activity scores, insulin resistance, liver enzymes and other inflammatory markers between groups [93].

As mentioned prior, some lipids work as safe fat storage and even have a protective role against toxic FFAs. Of note, MUFAs are relatively non-toxic to liver cells and can protect hepatocytes against saturated FFA-induced lipotoxicity by redirecting these toxic FFAs into

TGs storage. PUFAs, commonly present in fish oil, may also have a protective potential, but further studies are needed to better elucidate this effect.

5) HEPATOTOXIC CARBOHYDRATES AND GLUCOTOXICITY

Epidemiological studies indicate a correlation between high-carbohydrate diets and NAFLD as reviewed by Basaranoglu M. *et al.* [94]. Indeed, high-carbohydrate diets induce lipogenesis and steatosis through the activation of several lipogenic enzymes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) or SCD-1. The abundant carbohydrate consumption and resultant increased levels of sugars in the blood also have deleterious effects on liver cells, a phenomenon termed glucotoxicity. This concept is intrinsically linked to insulin resistance, which allows these elevated glycemic levels. Insulin resistance is considered the first step in the path toward the development of type II diabetes mellitus (T2DM). Because abundant levels of plasma FFA can also lead to insulin resistance, it is difficult to distinguish between the effects of glucotoxicity and lipotoxicity *in vivo*. However, *in vitro* studies have demonstrated that chronic hyperglycemia affects both insulin secretion and action as a consequence of the generation of ROS and the activation of an oxidative stress. Most of the studies in the literature have focused on the deleterious effect of glucotoxicity on pancreatic β-cells, but recent data indicate that glucotoxicity can also be injurious to liver cells and induce an ER stress and hepatocyte cell death.

5.1) Glucose

Glucose is unique as it is utilized by all body tissues to produce energy. After its consumption, approximately 20% of the glucose enters the hepatocyte via the glucose transporter (GLUT2) membrane transporter, where it is either stored as glycogen or undergoes glycolysis. Glucose stimulates insulin release from the pancreas. Indeed, the rest of the glucose (80%) contributes to the increase in glucose levels in the peripheral circulation; thus, in response to this hyperglycemia, insulin is released by the β-cells. Administration of a high-glucose diet to mice for 4 weeks resulted in elevated FA accumulation in the liver, increased insulin resistance and induction of hepatic oxidative stress, all major players in the development of diabetes [95].

5.2) Fructose

Fructose is also a lipogenic sugar, selectively metabolized in the liver where it is directed toward liver glycogen and triglyceride synthesis. Absorbed fructose is delivered to the liver via the portal vein and enters the hepatocyte via GLUT2, but it does not induce insulin release from pancreatic β-cells. The end-products of fructose metabolism in the glycolytic pathway of the liver are glucose, glycogen, lactate, and pyruvate. Many studies have shown that dietary fructose induces lipogenesis in humans, reinforcing the role of fructose in the obesity epidemic [96]. Also, administration of high-fructose corn syrup induces deposition of fat in the liver secondary to increase in fatty acyl coenzyme A, diacylglycerol (DAG) and TAG. Indeed, high fructose exposure also increases the lipogenic enzymes FAS, carbohydrate responsive element binding protein (ChREBP) and ACC-1 as well as higher amounts of the fat transporter CD36 and that even prior to any body weight changes in rats [97]; and high fructose diet-induced upregulation of FAS and SCD-1 results in higher

saturated FA liver content and increased hepatic gluconeogenesis inducing the steatotic phenotype in the liver [98]. The lipogenic and pro-inflammatory effects of fructose may be related to its rapid intracellular phosphorylation and release of calcium and uric acid.

Diet high in fructose also reduced insulin sensitivity associated with impaired hepatic insulin action and whole-body glucose disposal. Although high fat diet alone induces insulin resistance, obesity and steatosis with little inflammation and without fibrosis, the combination of a diet with high fat and fructose content exacerbates the liver injury with the presence of hepatic fibrosis, inflammation, endoplasmic reticulum stress and lipoapoptosis. Whether fructose consumption directly contributes to NAFLD pathogenesis is unknown and large prospective studies following subjects with high fructose consumption and the development of NAFLD will be necessary to ascertain fructose contribution to the disease [94].

The toxic effects of high levels of carbohydrates to cells are intrinsically related to insulin resistance and derangement of energy management within cells and tissues. Glucose cannot be properly stored or utilized in the absence of insulin in most tissues. High glucose diets can lead to steatotis in the liver and induction of oxidative stress. Fructose has also been shown to induce lipogenesis and liver steatosis through induction of fatty acyl CoA, DAG and TAG as well as FAS, ChREBP and ACC-1. This happens in addition to upregulation of fat transporter CD36. Prospective studies of fructose's contribution in NASH particularly, are still needed.

6) HARMFUL EFFECTS OF HEPATOTOXIC CARBOHYDRATES

Several studies indicate that exposure of liver cells to high glucose, fructose or sucrose content induce hepatic insulin resistance in rodent [99,100] which can be the consequence of decreased insulin receptor expression or increased phosphorylation of the insulin receptor substrate (IRS)1 therefore suppressing insulin receptor signaling [101,102]. The deleterious effects of chronic hyperglycemia on insulin sensitivity have been linked, among others, to oxidative stress, inflammation and ER stress [103].

Oxidative stress induced by high sugar consumption may contribute to the establishment of an insulin resistant status. Indeed, both fructose and glucose-sweetened liquid consumption upregulates thioredoxin-2 expression in mice, a key protein upregulated in response to mitochondrial oxidative stress [104]. Fructose-fed rats harbor liver dysfunctions, high transaminases and increased levels of oxidative markers including higher protein carbonyl and nitrosothiol content in liver [105] as well as diminished levels of total antioxidant status, reduced glutathione, catalase and cellular glutathione peroxidase (GPX1) activity in the liver [106].

Several kinases and phosphatases activated downstream of stress-induced pathways have been identified as important contributor of high carbohydrate diet-induced insulin resistance. First, recent studies suggests that JNK may contribute to fructose-induced antagonism of insulin signaling in the liver. Indeed, hyperactivation of JNK mediates insulin resistance by phosphorylating and inhibiting IRS-1, blocking downstream insulin signaling [107]. Thus,

hepatic insulin resistance induced by a sucrose-enriched diet in rats was associated with elevated hepatic JNK activity, and treatment of hepatocytes isolated from high sucrose diet-fed rats with a JNK inhibitor improved insulin-stimulated tyrosine phosphorylation of IRS proteins and insulin suppression of glucose release [107]. Also, a whole body of data indicate a central role for AMP-activated protein kinase (AMPK) in regulating insulin sensitivity, as sustained decreases in AMPK activity accompany insulin resistance, whereas AMPK activation increases insulin sensitivity. In HepG2 cells exposed to high glucose media, AMPK activity is decreased [108]; and decrease in AMPK activity can be mediated by the inhibition of Sirtuin 1 (SIRT1) [109], a redox-regulated NAD-dependent histone/protein deacetylase. Interestingly, heme-oxygenase 1 (HO-1), an endogenous anti-oxidant gene, can attenuate fructose-induced hepatic lipid accumulation and improve insulin sensitivity via the activation of SIRT1 gene expression [109,110]. Therefore, the identification of potent and specific AMPK activators may be beneficial for the prevention and treatment of metabolic syndrome–associated disorders. Futhermore, other studies also indicate that sucrose-induced hepatic insulin resistance is associated with increased expression of protein-tyrosine phosphatase 1B (PTP1B) [107]. PTP1B inhibits insulin signaling by dephosphorylating the insulin receptor and possibly IRS1; and liver-specific PTP1B-deficiency improves insulin sensitivity in animal model of NASH [111]. Thus, PTP1B could also represent an attractive therapeutic target for obesity, diabetes, and metabolic syndrome.

Chronic hyperglycemia can also provoke metabolic perturbations in the liver promoting a low-grade inflammation which contributes to the insulin-resistant state. Thus, high fructose consumption in rats results in hepatic increase of TNFα mRNA expression via NFκβ-dependent transcriptional upregulation [112] . TNFα induces serine phosphorylation of IRS-1 through inhibition of serine phosphatases (such as protein phosphatase 2A or PP2A) or activation of serine kinases (such as JNK or protein kinase C theta) leading to disruption of insulin signaling in the liver [113]. Also, suppressors of cytokine signaling (SOCS) are inflammatory mediators that regulate insulin signaling and *de novo* lipogenesis in the liver. SOCS-1 and 3 are induced by pro-inflammatory cytokines and can inhibit insulin signaling by interfering with IRS-1 tyrosine phosphorylation. SOCS proteins markedly induce *de novo* fatty acid synthesis in the liver by inhibiting the janus tyrosine kinase (JAK)/signal transduction and activator of transcription proteins (STAT) 3 resulting in the upregulation and activation of SREBP-1c [114]. In rats, high-fructose diet increases the amount of SOCS-3 [115], and downregulation of SOCS-3 can have beneficial effects on insulin-resistant rat hepatocytes by increasing their insulin sensitivity [116].

More recent data indicate that high-fructose-induced insulin resistance and *de novo* lipogenesis in the liver of mice were associated with the dual activation of the IRE1/XBP1 and PERK/eIF2α pathways [117,118]. Fructose-induced ER stress activation has been linked to the activation of SREBP-1c, associated with the upregulation of lipogenic enzymes genes, with downstream effect on insulin sensitivity [92,119] . High-fructose or sucrose diet can also results in CHOP upregulation and JNK activation [120,121], which further induce cellular apoptosis subsequent to BH3-only proteins BIM and PUMA-mediated mitochondrial dysfunction and caspase 3 activation [122] and glucose can further amplify FA-induced ER stress [123](Table 1).

Thus, chronic hyperglycemia can induce liver toxicity by activating oxidative stress, inflammation and ER stress responses leading to insulin resistance, steatosis and cellular demise (Figure 1). Whether chronic hyperglycemia directly contributes to the insulin resistance, T2DM and obesity characterizing the NAFLD phenotype remains to be clarified.

7. CONCLUSION

NASH represents a growing epidemic worldwide and is predicted to emerge as the main cause of liver cirrhosis, surpassing alcohol and viral hepatitis. Therefore, understanding the molecular mechanisms behind the hepatocellular injury is paramount in efforts to deter this epidemic. Metabolic syndrome, which is intrinsically linked to a diet rich in saturated fats and refined sugars (i.e. fructose and/or glucose), is characterized by glucose intolerance and insulin resistance. This abundance of FFA and carbohydrates generates cytotoxicity to tissues through deregulation of energy storage homeostasis. Several lipotoxic lipids have been described in the literature, including FFA, LPC, ceramides, FC, and BA. The pathways involved in lipotoxicity are related to organelle damage, mainly the mitochondria and endoplasmic reticulum, where, through activation of JNK and BH3-only family proteins, caspases become activated resulting in apoptosis. In contrast, other lipids like MUFAs or PUFAs (e.g., fish oil) can protect the liver from lipotoxicity, although the exact mechanisms by which they exert their hepatoprotective effects are still being delineated.

Several drugs are being studied as potential therapies for NASH. Among these are BA agonists (such as ursodeoxycholic acid), vitamin E, caspase inhibitors, JNK inhibitors, TRAIL antagonists, PUFAs, lipid lowering drugs (statins), drugs that sensitize tissues to glucose (metformin) and AMPK inhibitors. Further research is necessary to continue to explore this intricately woven metabolic web and determine future directions of therapy in the metabolic syndrome spectrum of diseases, including NASH.

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Abbreviations

ALA	alpha-linolenic
ACC	acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
ATF	activating transcription factor
AP-1	activator protein-1
BAs	bile acids
BH	Bcl-2 homology

BIM	Bcl-2 interacting mediator
CHOP	CCAAT/enhancer-binding homologous protein
ChREBP	carbohydrate responsive element binding protein
CYP2E1	cytochrome P450 2E1
DAG	diacylglycerides
DR	death receptor
ER	endoplasmic reticulum
ERAD	ER-assisted degradation
FAS	fatty acid synthase
FAT	fatty acid translocase
FATP	fatty acid transport protein
FC	free cholesterol
FFAs	free fatty acids
FXR	farnesoid X receptor
GLUT2	glucose transporter 2
HMGB1	high mobility group box 1 protein
HMGCo-A	hydroxymethylglutaryl-Co-A
HSCs	hepatic stellate cells
GSK3	glycogen synthase kinase 3
IRE1α	inositol-requiring enzyme-1α
IRS–1	insulin receptor substrate-1
JNK	c-Jun NH2-terminal kinase
Keap1	Kelch-like ECH-associated protein
LPC	lysophosphatidyl Choline
LDL	low density lipoprotein
MUFA	monounsaturated fatty acid
NF-kB	nuclear factor-kappaB
MCD	methionine choline deficient
NAFLD	Nonalcoholic fatty liver disease

NASH	Nonalcoholic steatohepatitis
N-SMase	neutral sphingomyelinasePA, palmitate
PERK	PKR-like ER kinase
PLA2	phospholipase A2
PO	palmitoleate
PTA	p53 inhibitor pifithrin-alpha
PTP1B	protein-tyrosine phosphatase 1B
PUFA	polyunsaturated fatty acid
PUMA	p53 upregulated modulator of apoptosis
OA	oleate
ROS	reactive oxygen species
SA	stearate
SCD-1	stearoyl-CoA desaturase-1
SOCS	suppressor of cytokine signaling
SREBP-2	sterol regulatory-element binding protein 2
StAR	steroidogenic acute regulatory protein
STAT5	signal transducer and activator of transcription 5
SIRT1	JNK-1/p-53/miR 34a sirtuin 1
T2DM	type 2 diabetes mellitus
TAG	triacylglycerides
TG	triglycerides
TGF beta	transforming growth factor beta
TLR4	toll like receptor 4
TNF	tumor necrosis factor
TRAIL	TNFα-related apoptosis-inducing ligand
UFA	unsaturated fatty acid
UPR	unfolded protein response
VDAC	voltage dependent anion channel
VLDL	very low-density lipoprotein particles

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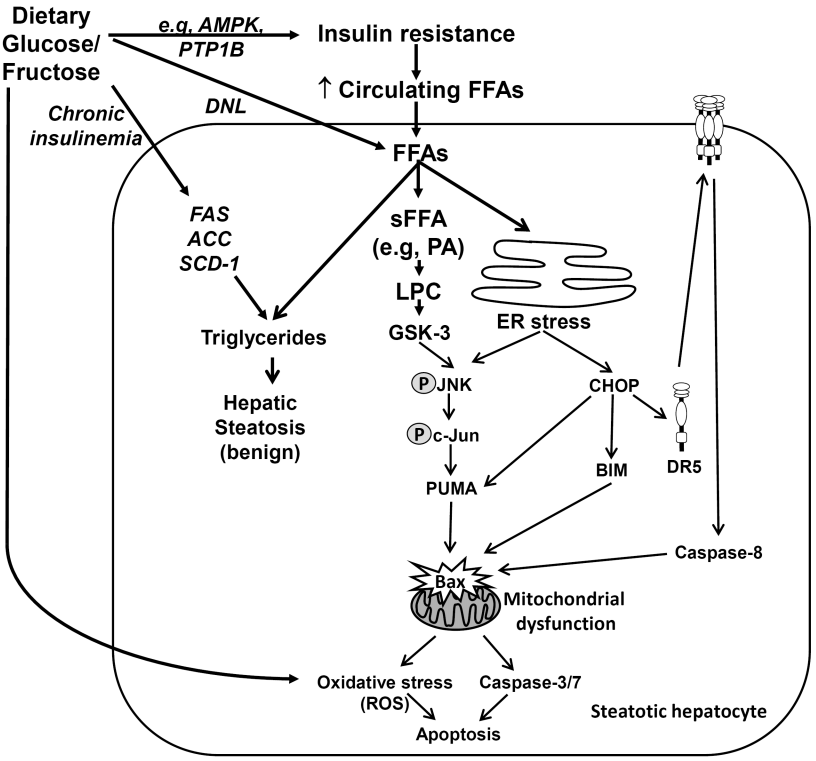

Figure 1. Molecular mechanisms of hepatocyte lipotoxicity and glutotoxicity

Insulin resistance, a hallmark of NAFLD, leads to an increase in serum concentration of circulating FFAs. FFAs are transported into the hepatocyte where they can be esterified into neutral triglycerides resulting in hepatic steatosis. Esterification of FFAs represents a buffering mechanism allowing cells to maintain viability in the face of excess non-esterified FFAs exposure. Saturated FFAs (sFFA) in excess are toxic to liver cells and they can accumulate in the endoplasmic reticulum (ER) and induce an ER stress, which in turn induce the transcription factor CHOP and induce JNK activity. Other hepatotoxic lipids such as palmitate (PA)-derived lysophosphatidyl choline (LPC) also mediate JNK-dependent hepatic toxicity downstream of glycogen synthase kinase (GSK)-3 signaling cascades and ER stress induction. Active JNK phosphorylates the transcription factor c-Jun, which cooperates with CHOP to upregulate the transcription of the pro-apoptotic BH3-only protein PUMA. CHOP also mediate the upregulation of another BH3-only protein BIM; and BIM and PUMA cooperate in activating the executioner proapoptotic protein Bax, causing mitochondrial dysfunction, activation of the effector caspases 3/7 and cellular apoptosis. CHOP also upregulates the expression of the death receptor DR5, resulting in increased DR5 cell surface expression which clustering of the receptor leading to recruitment and activation of the executioner caspase-8, which ultimately induces Bax. Saturated FFAs-induced mitochondrial dysfunctions is also mediated by a Bax-dependent permeabilization of lysosomes and release of cathepsin B in the cytosol. Mitochondrial dysfunction also results in generation of reactive oxygen species (ROS) which further induce cellular demise. In addition, excess dietary sugars, resulting in chronic hyperglycemia, can further induce liver toxicity or glucotoxicity by increasing hepatic steatosis via *de novo* lipogenesis (DNL) and exacerbating insulin resistance and cellular demise, through the activation of oxidative and

ER stress responses, and/or downstream the modulation of the activity of AMP-activated protein kinase (AMPK) and protein-tyrosine phosphatase 1B (PTP1B). Also, chronic hyperinsulinemia is associated with hepatic steatosis via the upregulation of hepatic lipogenic gene expression (ACC, FAS, SCD-1).

Table1

Lipid types and mechanisms of liver injury.

Lipid Types	Pathways mediating hepatic injury	References
Free Fatty Acids (FFA)/ Lysophosphatidyl choline (LPC)	– Activate PPARα resulting in impairment of insulin signaling	Ricchi, 2009
	– Phosphorylate and activate JNK which upregulates PUMA leading to Bax activation, mitochondrial dysfunction, caspases activation and apoptosis – Upregulate of DR5 in JNK-dependent manner and sensitize hepatocytes to TRAIL-induced apoptosis – Activate an ER stress and induce the expression of pro-apoptotic transcription factor CHOP which upregulates BIM and DR5	Cazanave, 2009; Malhi, 2006; Pagliassotti, 2007 Malhi, 2007 Cazanave, 2011
	– Induce p62-dependent degradation of Keap1, leading to upregulation of BIM and PUMA	Lee, 2012; Lin, 2013; Cazanave, 2014
Triglycerides (TG)	– Hydrolyzed into FFAs – Represent a safe fat storage for hepatocytes – Have a protective role against the pro-apoptotic effect of unsaturated FFA	Ricchi, 2009; Listenberger, 2003; Yamaguchi, 2007
Ceramides	– Interact with TNFα with release of ROS – Increase pro-inflammatory cytokines	Pagadala, 2012; Schwabe, 2006
Free Cholesterol (FC)	– Stimulates Kupffer cells and hepatic stellate cells to mediate fibrosis, mitochondrial dysfunction, ROS, and UPR culminating in apoptosis	Arguello, 2015; Tomita, 2014
	– induces mitochondrial dysfunctions and hepatocyte injury through accumulation of oxysterol, a toxic oxidative product of cholesterol	Musso, 2013
	– Activates HMGB1 through a JNK-1 mediated mechanism	Gan, 2014
Bile Acids	– Trigger hepatic apoptosis through JNK1/p53/SIRT1 pathway	Ferreira, 2014

c-Jun NH2 terminal kinase (JNK), p53 upregulated modulator of apoptosis (PUMA), death receptor 5 (DR5), TNFα related apoptosis inducing ligand (TRAIL), endoplasmic reticulum (ER), CAATT enhancer binding homologous protein (CHOP), Bcl-2 interacting mediator (BIM), Kelch like ECH associated protein 1 (Keap1), Reactive oxygen species (ROS), unfolded protein response (UPR), sirtuin 1 (SIRT1).