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Lipotoxicity in Nonalcoholic Fatty Liver Disease: Not All Lipids Are Created Equal

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Summary

Nonalcoholic Fatty Liver Disease (NAFLD) is currently the most common form of chronic liver disease affecting both adults and children in the United States and many other parts of the world. NAFLD encompasses a wide spectrum of conditions associated with over-accumulation of lipids in the liver ranging from steatosis to nonalcoholic steatohepatitis or NASH, to cirrhosis and its fear complications of portal hypertension, liver failure and hepatocellular carcinoma. In this review, we will focus on the growing evidence linking changes in hepatic lipid metabolism and accumulation of specific lipid types in the liver with hepatocellular damage, inflammation and apoptosis resulting in disease progression to the more serious forms of this condition.

Keywords

Lipotoxicity; apoptosis; nonalcoholic fatty liver disease; steatohepatitis; free fatty acids.

Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) a common form of chronic liver disease affecting both adults and children [1-3]. NAFLD encompasses a wide spectrum of conditions associated with over-accumulation of lipids in the liver ranging from steatosis to nonalcoholic steatohepatitis or NASH characterized by the accumulation of fat in the liver along with evidence of liver cell damage, inflammation and different degrees of scarring or fibrosis [3]. Although most patients with NAFLD tend to have a benign non-progressive clinical course, some may have a progressive disease with significant associated risk of developing cirrhosis and its complications. A central question that is the center of intense investigation is why most patients with hepatic steatosis have a benign non-progressive disease while others develop steatohepatitis and end stage liver disease. A net retention of lipids in hepatocytes is a prerequisite for the development of NAFLD. The mechanisms of steatosis development as well as the potential links between steatosis and liver damage resulting in disease progression to NASH and NASH-cirrhosis remain incompletely understood but are of significant biomedical importance as identification of these processes may help to identify novel therapeutic targets to treat this disease.

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Altered Lipid Metabolism in NAFLD

Although the majority of hepatic lipids in NAFLD are stored in the form of triglycerides (TG) [4], several other lipid metabolites such as different free fatty acids (FFA), diacylglycerol, free cholesterol (FC), cholesterol ester (CE), ceramide, and phospholipids also accumulate [5]. In patients with NAFLD hepatic lipid loading appears to be mainly determined by the availability of FFA from circulation [6] Other potentially important mechanisms include oxidation of FFA mainly at the level of the mitochondria, de-novo lipogenesis from glucose, and the export of TG in the form of very-low density lipoproteins (VLDL) (Fig. 1) [6]. A growing body of evidence mainly from experimental studies suggests that lipid compartmentalization in hepatocytes and in particular the type or "quality" as opposed to the "quantity" of lipids accumulating may play a central role in the risk for progressive disease [7]. This novel concept has significant implications when considering potential alternative treatment strategies for patients with NAFLD. The following paragraphs focus specially on the current evidence of the potential role of some lipid metabolites stored in hepatocytes during NAFLD development.

Hepatic TG: The "Good" Fat

TG accumulation in the liver in response to lipid overloading has been historically considered the "first hit" in NAFLD development [8]. The additional storage of fatty acids within the TG pool and its expansion is one characteristic of NAFLD and has been associated with features of insulin resistance. In this way, TG accumulation in NAFLD have been postulated to contribute to the development of hepatic and systemic insulin resistance through mechanisms involving interference of insulin signaling pathways [1-3]. However, a recent study clearly demonstrated that hepatic TG accumulation is insufficient to cause insulin resistance. They generated mice that selectively overexpress diacylglycerol acyltransferase (DGAT) 2, an enzyme that catalysis the final step in TG formation, in the liver. These mice displayed hepatic steatosis with increased amounts of TG, however they had no abnormalities in plasma glucose and insulin levels and no evidence for either hepatic or systemic insulin resistance [9]. Moreover, considerable data now indicate that TG accumulation *per se* is not harmful to hepatocytes and may represent in fact a protective mechanism against FFA-induced lipotoxicity [7]. In various experimental systems the exposure of cultured cells to unsaturated FFA resulted in significant increase in TG content without a decrease in cell viability. In contrast, cells incubated with saturated fatty acids (SFA) showed significant increase in apoptotic death in conjunction with absence of TG accumulation [10].

In vivo evidence of a potential protective role of TG accumulation was recently provided by Dr. Anna Mae Diehl laboratory [11]. They demonstrated that feeding the leptin-receptor deficient db/db mice, a genetic model of obesity and steatosis with the methionine- and choline-deficient (MCD) diet, results in hepatic steatosis, liver cell injury and apoptosis, increase reactive oxygen species (ROS) production and fibrosis. Interestingly, while the degree of liver damage worsened with the duration of the MCD diet consumption, the hepatic TG content tended to decline over time. Moreover, treatment of these mice with DGAT2 antisense oligonucleotides (ASO) to suppress DGAT2 expression produced an expected reduction in hepatic TG content. However, blocking TG synthesis by this approach resulted in a significant increase in hepatic FFA content which was associated with a significant increase in oxidative stress, hepatocellular apoptosis, and worsening of hepatic inflammatory activity and fibrosis.

Hepatic FFA: The "Bad" Fat

A surplus of FFA in non-adipose cells may enter deleterious pathways leading to cell dysfunction (lipotoxicity) and apoptotic cell death (lipoapoptosis) [10,12]. FFA can induce these effects through several mechanisms that may differ across different cell types. We have recently uncovered some key aspects linking hepatocyte FFA overloading, to hepatocellular

apoptosis and liver injury[13]. The results demonstrated that the ratio of monounsaturated fatty acids (MUFA) to saturated fatty acids (SFA) determines whether liver cells are damaged by the flux of exogenous FFA, and thus the nature rather than the quantity of FFA determines hepatic stress. By using both in vivo dietary models of NAFLD and in vitro cell models of lipid overloading we evaluated the role of stearoyl-CoA desaturase-1 (SCD1), the enzyme that converts SFA to MUFA, in hepatic lipid partitioning in NAFLD. We found that genetic or pharmacological inhibition of SCD1 sensitized hepatocytes to apoptosis induced by SFA. When we placed SCD1 knock out mice on the MCD diet, the mice accumulated less TG compared to wild-type animals but had an increase in hepatocellular apoptosis and liver injury. The results support a model (Fig. 2) in which during the development of NAFLD, overflow of FFA to the liver is associated with an increased in SCD1 expression and activity resulting in a tilt of the balance towards MUFA formation, triglyceride storage, liver adaptation and development of isolated hepatic steatosis. In contrast SCD1 deficiency results in hepatic over-accumulation of SFA triggering hepatocellular apoptosis, liver damage and development of steatohepatitis

FFA and Death Receptors

Hepatocyte apoptosis is a prominent morphologic and pathogenic feature of NASH [14]. Apoptosis can be executed by two fundamental pathways: 1) the extrinsic pathway mediated by death receptors on the cellular surface and 2) the intrinsic pathway which is organelle based [15,16]. Death receptors that can initiate the extrinsic pathway include Fas, tumor necrosis factor receptor 1 (TNF-R1), and tumor necrosis factor related apoptosis inducing ligand receptors (TRAIL-Rs). When engaged by their natural ligands, these receptors trigger intracellular cascades that activate death-inducing proteolytic enzymes, especially caspases. FFA may induce up-regulation of death receptors such as Fas and TRAIL receptor 5 (DR5) (Fig. 3) [17,18]. Thus, enhanced expression of these receptors is one potential mechanism by FFA may sensitize liver cells to apoptotic cell death. Both Fas and DR5 expression have been shown to be increased in liver of patients with NASH [14,18]. While studies in mice showed that high carbohydrate feeding, which results in steatosis as well as several features of the human metabolic syndrome, also up-regulates Fas expression in hepatocytes resulting in increase sensitivity to Fas-mediated apoptosis and liver injury [17].

FFA and Mitochondrial-Lysosomal Pathway

Mitochondria are involved in many processes essential for liver cell survival, including energy production, redox control, calcium homeostasis, and certain metabolic and biosynthetic pathways [19]. In addition, mitochondria often play an essential role in cell death mechanisms [15,16]. Several lines of evidence suggest that impaired mitochondrial function is a central abnormality responsible for the progression from simple steatosis to steatohepatitis in NAFLD [20,21]. We demonstrated that incubation of human and murine hepatocytes with FFA results in a dose- and saturation-dependent mitochondrial dysfunction [22]. The saturated FFA palmitate at concentrations that mimic the levels of this FFA present in the circulation of humans with metabolic syndrome induced significant mitochondrial membrane permeabilization and increased ROS production. Another group showed that saturated FFA induce JNK-dependent hepatocyte lipoapoptosis by activating the pro-apoptotic proteins Bim and Bax which trigger the mitochondrial apoptotic pathway [23].

In addition to mitochondrial dysfunction, lysosomal permeabilization has an important role in apoptotic cell death. We initially reported that cathepsin B, a major lysosomal cysteine protease is released in the cytosol in response to FFA in vitro, and that the redistribution of cathepsin B in the cytoplasm is present in human liver tissues from patients with NAFLD [24]. Recently we have extended these observations by demonstrating that during FFA treatment of liver cells,

lysosome permeabilization and release of cathepsin B into the cytosol is an early event that occurs hours prior to mitochondrial depolarization and cytochrome c redistribution into the cytosol [25]. More importantly, cathepsin B silencing by siRNA, or chemical inhibition by two different selective pharmacological inhibitors significantly prevented FFA induced mitochondrial dysfunction, proving cathepsin B is essential for FFA-induced mitochondrial dysfunction.

FFA and Endoplasmic Reticulum (ER) Stress

A great deal of interest has been generated recently on the potential role of ER stress responses in NAFLD development. The ER is an intracellular membranous network where the vast majority of secreted and membrane proteins are folded. A variety of cellular perturbations can lead to the accumulation of unfolded proteins [26]. These proteins tend to form aggregates that activate a compensatory response, termed the unfolded protein response (UPR). The UPR is a coordinated response that includes cell cycle arrest, transient attenuation of global protein synthesis, induction of ER-localized chaperone proteins, folding catalysts, and activation of ER-associated protein degradation. Failure of the UPR to reestablish ER homeostasis can lead to apoptosis. Overaccumulation of SFA may result in ER stress and apoptosis [27,28]. Incubation of a rat hepatoma cell line with SFA (palmitic acid and stearic acid) resulted in a significant increase in ER stress response genes including CHOP, GADD34, and GRP78 followed by increase apoptotic cell death, which was mitochondrial-dependent. These changes were not observed in MUFA (oleic acid and linoleic acid) treated cells. In fact, palmitic acid induced ER stress was abrogated in oleic acid or linoleic acid treated cells. In vivo studies using dietary models of NAFLD resulting in different composition of hepatic FFA support this concept [29]. Feeding male Wistar rats either a high saturated fat diet or a high sucrose diet but not a high polyunsaturated fat diet resulted in significant increase in hepatic SFA as well as increased ER stress, caspase-3 activation and liver injury.

Puri and colleagues recently examined a potential role of ER stress in human NAFLD. They showed [30] a variable degree of UPR activation in liver biopsies from patients with NAFLD and NASH compared to subjects with the metabolic syndrome and normal liver histology. Human NASH was specifically associated with activation of C-jun N-terminal kinase (JNK) and failure to generate splicing X box protein-1 (sXBP-1), a key transcription factor involved in ER stress response.

Ceramide: The Dynamic Sphingolipid

Although sphingolipids have been considered mere structural components of biological membranes, ceramide has recently attracted significant attention due to its dynamic role in cell stress and death ligand-induced death [31]. Ceramide is the prototypic sphingolipid and its *de novo* synthesis occurs in the ER with the condensation of sphingosine and a fatty acid moiety, usually palmitoyl CoA. Ceramide synthesis rate depends on the availability of long chain saturated FA; therefore, obesity can be associated with excess ceramide production. In addition to *de novo* synthesis, ceramide can also be rapidly generated from sphingomyelin by sphingomyelinase [32,33]. The generation of ceramide through this pathway is quick and has been involved in apoptosis induced by death ligands such as TNF and Fas [34]. Ceramide also plays a role in insulin resistance by inhibiting insulin-induced glucose uptake, GLUT4 translocation and glycogen synthesis [34]. The dependence of ceramide synthesis on saturated fats and its role in insulin resistance makes this sphingolipid a candidate metabolite linking lipid oversupply to the development of MS and NAFLD. Nevertheless, the role of ceramide in the pathogenesis of NAFLD remains uncertain. While a recent study showed that genes involved in ceramide signaling and metabolism were positively associated with liver fat content

in patients with NAFLD [35], experimental studies have failed to demonstrate a role for ceramide in SFA-induced ER stress and apoptosis [28].

Free Cholesterol (FC): The New Suspect

Recent data suggest that FC accumulation sensitizes hepatocytes to TNF and Fas-induced apoptosis, which leads to progression from steatosis to NASH. Mari et al. fed rats a choline-deficient diet or a 2% cholesterol plus sodium cholate diet to increase either hepatic TG or cholesterol levels, respectively [36]. They examined the fate of TG or FC loaded hepatocytes in response to TNF and found that TNF treatment caused apoptosis, increased ROS formation and liver injury only in livers with increased cholesterol content. This observed sensitization to TNF was secondary at least in part to a reduction of glutathione content in the mitochondria. Depleting mitochondrial glutathione with a small molecule reiterated the effects of increased mitochondrial FC. Furthermore, treatment with atorvastatin (HMG-CoA reductase inhibitor) decreased mitochondrial free cholesterol and increased glutathione levels, suggesting a potential clinical implication for NASH treatment. A progressive increase in hepatic free cholesterol from controls with normal histology to patients with simple steatosis and NASH has been reported [37]. The mechanisms resulting in overloading of FC in NAFLD remain incompletely known but could be related to mitochondrial abnormalities and the relative depletion of n-3 FA which increases HMG-CoA reductase activity [38,39]. A recent study highlighted the emerging role of free cholesterol in the pathogenesis of human NAFLD by examining the expression of enzymes that regulate cholesterol homeostasis [40]. Both SREBP-2 (a transcriptional factor that plays an important role in cholesterol synthesis) and StAR (a transporter of FC from the external to the internal mitochondrial membranes) were overexpressed in patients with NASH compared to those with simple steatosis. These findings suggest a role of mitochondrial FC in disease progression from steatosis to steatohepatitis.

Toll-Like Receptors and the Innate Immune System

Toll-like receptors (TLR) are a family of pattern-recognition receptors that play a critical role in the innate immune system by activating proinflammatory-signaling pathways in response to microbial pathogens. TLR4 binds to lipopolysaccharides (LPS) of gram-negative bacterial cell walls which triggers a down stream signaling cascade, leading to activation of the NF-κB pathway and up regulation of its proinflammatory genes [41]. Suppression of TLR4 signaling in TLR4 mutant mice resulted in partial protection from the development of steatohepatitis [42], while TLR4 activation by a specific ligand results in increased liver injury and inflammatory cytokine induction in mice fed the MCD diet [43]. SFA including palmitate can act through TLR4 to induce NF-κB signaling, and liver samples from mice lacking TLR4 demonstrate the absence of inflammatory gene expression induction by a high fat diet [44]. These findings point to a potential link between SFA, and TLR4 activation in the pathogenesis of obesity-induced hepatic inflammation.

Expert commentary

Nonalcoholic Fatty Liver Disease (NAFLD) is a serious public health problem. In this review, we examined the consequences of altered lipid metabolism and lipid partitioning in the liver on activation of cytotoxic signaling pathways, cell death, and liver damage. We provided detailed descriptions on the experimental evidence supporting a role of specific lipid types in NAFLD development and their contributions to disease progression, including lipotoxic mediators such as FFA, TG, ceramide, and FC. The precise mechanisms and impact of each of these lipid types in human NAFLD remain incompletely define and will require further investigations. The uncovering of the cellular events linking lipid overloading of liver cells to hepatocellular damage may open a new era in our understanding of the pathogenic mechanisms responsible for disease progression in the context of NAFLD.

Five-year view

Over the last few years there has been an increasing knowledge on the pathogenesis of fatty liver as well as disease progression to NASH and fibrosis by studies in different experimental models and in humans with this condition. Lipid overloading and in particular certain types of lipids appear to play a central role not only as regulators of insulin sensitivity and development of fatty liver, but also in the inflammatory process, cell death, fibrogenesis, and fibrosis. Over the next 5 years, a better understanding of the role of these lipids as well as their possible interactions to regulate the spectrum of disorders seen in NAFLD may help in the development of novel diagnostic markers as well as more rational treatment strategies to halt the progression to the more severe forms of the disease.

Key Issues

- Lipid loading of the liver (steatosis) is considered the “first hit” in Nonalcoholic Fatty Liver Disease (NAFLD) development, whereas the fates of lipids accumulating may contribute to the “second hit” resulting in progression to nonalcoholic steatohepatitis (NASH) and NASH-cirrhosis.
- Hepatic triglyceride (TG) accumulation may be a protective mechanism against lipid induced toxicity or “lipotoxicity”.
- The type or “quality” as opposed to “quantity” of lipids accumulating may play a central role in risk for disease progression.
- Free fatty acids (FFA) may be important mediators of lipotoxicity through death receptors, the mitochondrial-lysosomal pathway, and endoplasmic reticulum (ER) stress.
- Toll-like receptors (TLR), specifically TLR4 may provide a link between SFA and obesity-induced hepatic inflammation.
- The sphingolipid, ceramide is an attractive candidate metabolite linking lipid overloading to the development of metabolic syndrome (MS) and NAFLD.
- Free cholesterol (FC) may become a new target for the development of novel therapeutic strategies for NASH treatment.

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Abbreviations

NAFLD, Nonalcoholic fatty liver disease
NASH, Nonalcoholic steatohepatitis
MS, Metabolic syndrome
FFA, Free fatty acid
SFA, Saturated fatty acids
MUFA, monounsaturated fatty acids
VLDL, very low-density lipoproteins
ER, Endoplasmic reticulum
UPR, unfolded protein response
JNK, C-jun N-terminal kinase
TLR, Toll-like receptors

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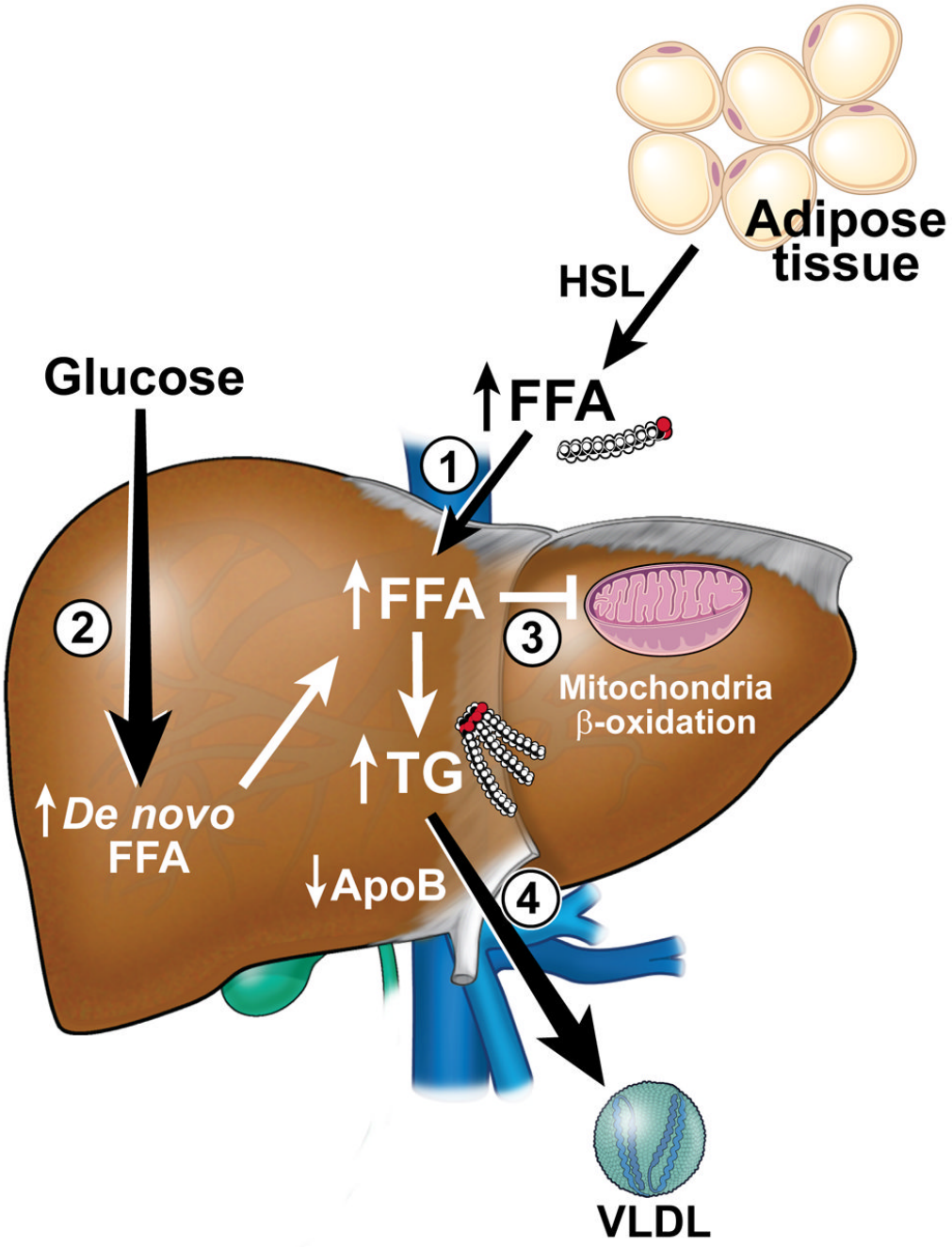

Figure 1. Potential Mechanisms of Hepatic Steatosis in NAFLD

A net retention of fat in hepatocytes could potentially result from alterations in the (1) uptake, (2) de novo synthesis in the liver itself, (3) oxidation, and (4) export pathways of hepatic lipid homeostasis. The type and number of processes involve may potentially play an important role in whether the disease follows a benign non-progressive clinical course, or a progressive one evolving into NASH and NASH-cirrhosis. Abbreviations: FFA, free fatty acids; TG, triglycerides; ApoB, apolipoprotein B; HSL, hormone sensitive lipase.

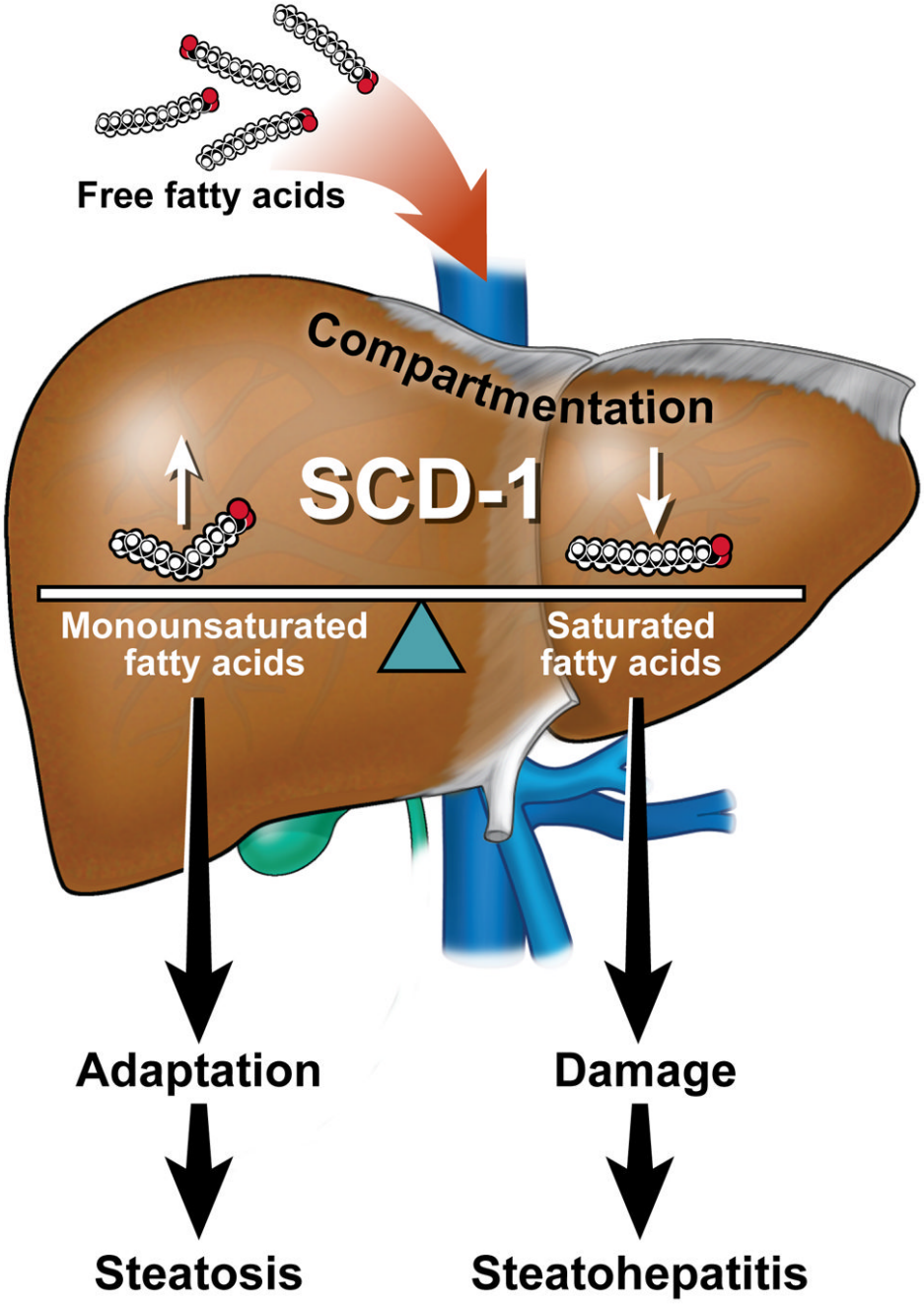

Figure 2. Hepatic lipid partitioning and liver damage in NAFLD

Hepatic steatosis characteristic of patients with NAFLD, which is commonly associated with obesity and insulin resistance results mainly from increase flow of free fatty acids from circulation. In this context, lipid compartmentation in liver cells and in particular the ratio of monounsaturated to saturated free fatty acids that is a function of SCD1 activity, plays a central role in whether adaptation occurs resulting in a benign clinical course, or there is ongoing hepatocellular apoptosis, and liver damage resulting in steatohepatitis and fibrosis development.. Abbreviations: SCD1, stearoyl-CoA desaturase-1.

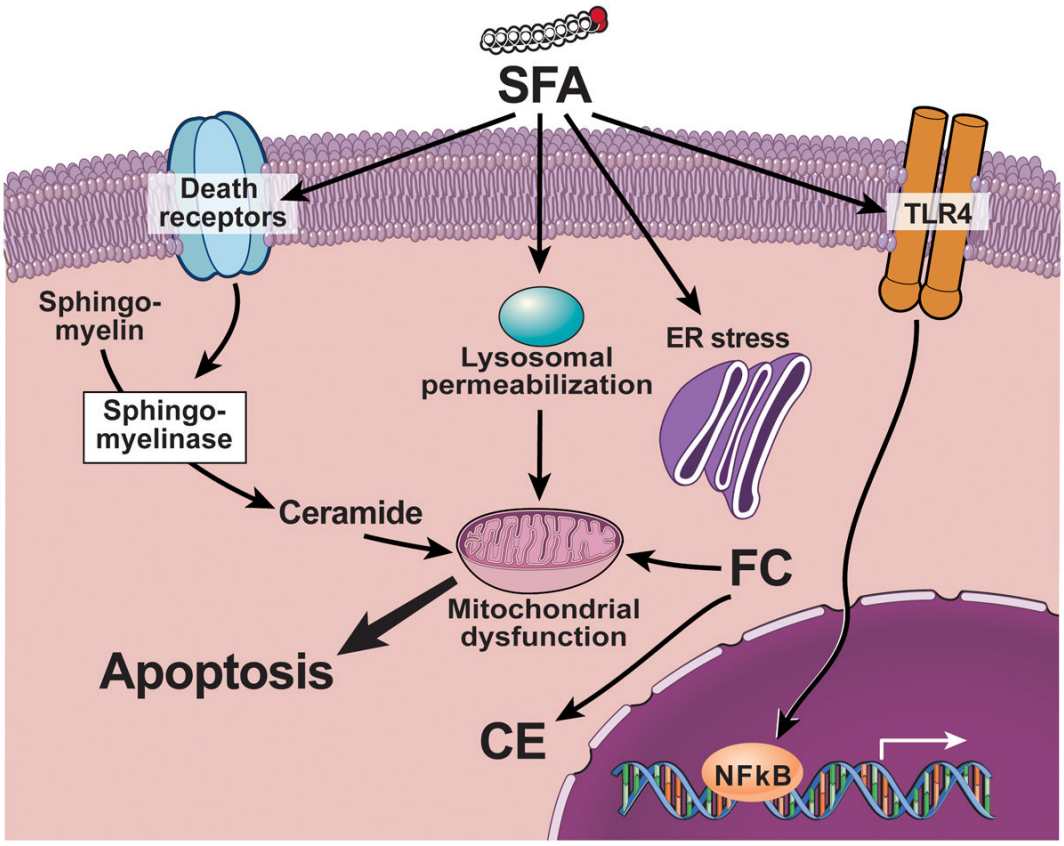

Figure 3. Lipotoxicity pathways in NAFLD

FFA may activate several signaling pathways of apoptosis including up-regulation and increased number of death receptors such as Fas and TRAIL receptor 5 (DR5), at the level of the plasma membrane, lysosomal permeabilization, and ER stress both coupled to mitochondrial dysfunction resulting in activation of the mitochondrial pathway of apoptosis. These toxic fatty acids may also activate TLR4 signaling resulting in up-regulation of several pro-inflammatory cytokines. Finally, other lipid types such as free cholesterol (FC) and ceramide may induce mitochondrial dysfunction and activate the mitochondrial pathway of apoptosis. Abbreviations: FFA, free fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids, FC, free cholesterol, CE, cholesteryl-ester; ER, endoplasmic reticulum.