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Mechanisms and clinical implications of hepatocyte lipoapoptosis

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is characterized by insulin resistance, elevated serum levels of free fatty acids (FFAs) and fatty infiltration of the liver. Accumulation of triglycerides in the hepatocyte results from the uptake and esterification of circulating FFAs by the liver. Contrary to current theory, hepatic steatosis appears to be a detoxification process, as FFAs are directly cytotoxic for the hepatocyte and inhibition of triglyceride formation enhances FFAs toxicity. Hepatocyte apoptosis is a key feature of NAFLD and correlates with disease severity. Since FFA-induced toxicity, or lipoapoptosis, represents a mechanism for the pathogenesis of NAFLD, this article will highlight the cellular pathways contributing to hepatocyte lipoapoptosis. To date, there is no proven effective therapy for patients with NAFLD and insights into the molecular mediators of lipoapoptosis should help promote effective therapeutic strategies for this disease.

Keywords

BH3-only proteins; CCAAT/enhancer binding homologous protein; c-Jun N-terminal kinase; death receptor; endoplasmic reticulum stress; hepatic steatosis; nonalcoholic steatohepatitis

Nonalcoholic steatohepatitis & lipoapoptosis

Nonalcoholic fatty liver disease (NAFLD) is highly prevalent in Western countries and is commonly associated with clinical features of metabolic syndrome, such as Type 2 diabetes, obesity and dyslipidemia [1]. This disease is characterized by an accumulation of fat in the liver and encompasses a wide spectrum of liver disorders, ranging from benign simple steatosis to steatohepatitis. Nonalcoholic steatohepatitis (NASH), which represents 5–10% of NAFLDs, is associated with hepatic inflammation as well as the presence of steatosis, hepatocyte damage and various degrees of fibrosis [2]. NASH represents a potentially progressive liver disease, as it can ultimately lead to cirrhosis, chronic liver disease with portal hypertension, liver failure and hepatocellular carcinoma [3,4].

A major risk factor for NASH is insulin resistance, which occurs within the context of metabolic syndrome [1,5,6]. Indeed, impaired insulin suppression of lipolysis within peripheral adipose tissue leads to increased plasma levels of free fatty acids (FFAs) [7]. As a consequence of this, increased delivery of adipose-derived FFAs to the liver directly contributes to the development

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of hepatic steatosis in patients with Type 2 diabetes [8]. Abnormal and excessive accumulation of lipids in nonadipose tissues, with limited capacity for storage of lipids, results in cellular dysfunction or cell death, a phenomenon termed lipoapoptosis [9,10]. Athough steatosis characterizes NAFLD, lipoapoptosis appears to be mediated by FFAs rather than by their esterified products (triglycerides). Indeed, numerous studies have demonstrated that nonesterified FFAs are inherently toxic to liver cells [11–17], whereas the esterification of FFAs to form neutral triglycerides appears to be a detoxification process [18,19]. Hepatic FFA levels are increased during murine experimental steatohepatitis [20,21], and NASH is characterized by both increased serum FFA levels and hepatocyte apoptosis, with the magnitude of circulating FFA correlating with disease severity [22,23]. Consistent with an increase in hepatocyte apoptosis, elevated serum caspase-cleaved cytokeratin 18 fragments distinguish simple human hepatic steatosis from NASH [24]. Therefore, FFA-induced toxicity or lipoapoptosis could represent a potential mechanism that relates apoptosis to NASH.

Hepatic lipids

Increased availability of circulating FFAs and the augmented delivery of FFAs to the liver most probably play a key role in the development of hepatic steatosis in NAFLD. Throughout this article, the term FFAs will refer to the saturated and unsaturated long-chain FFAs with aliphatic tails longer than 16 carbons atoms.

Hepatic free fatty acid

Free fatty acids entering the liver are derived from either hydrolysis of dietary triglycerides or from lipolysis of adipose tissue triglycerides in the fasting state. Excessive fat accumulation in the liver can also result from increased fat synthesis, reduced fat oxidation (β-oxidation) or reduced capacity to export fat in the form of VLDL. However, studies performed in humans and rodents have demonstrated that excessive accumulation of hepatic triglycerides is principally the result of increased delivery of adipose-derived FFAs to the liver and enhanced *de novo* lipid synthesis in the liver, and was only modestly affected by lipid disposal via β-oxidation or VLDL export [25]. In addition, dietary lipids contribute minimally to hepatic steatosis [26].

Free fatty acids enter the cells by both passive diffusion and facilitated transport involving specific fatty acid transporters such as the fatty acid transport protein (FATP) [27] and the fatty acid translocase FAT/CD36 [28]. Modifications in the expression of these fatty acid transporters directly contribute to fat-induced triacylglycerol accumulation in the cells. In particular, FATP2 and FATP5 are highly expressed in the liver and are involved in the uptake of long-chain FFAs [29]. Loss of hepatic FATP5 reduces dietary lipid deposition in murine hepatocytes by redirecting lipid fluxes toward peripheral tissues; and small hairpin RNA-targeted knockdown of FATP5 can decrease established hepatic lipid accumulation in animal models of diet-induced hepatic steatosis [30]. Furthermore, hepatic expression of FAT/CD36 is increased in mice fed a highfat diet [31,32] and enforced expression of FAT/CD36 in hepatocytes markedly increases hepatic fatty acid uptake [31]. To date, genetic polymorphisms of FATP5 or FAT/CD36 have not yet been reported, but it is possible that alterations in the expression of these proteins play an important role in susceptibility to and/or progression of NAFLD. Hepatic expression of FAT/CD36 and FATP5 are also augmented in the livers of patients with NAFLD and correlate with liver fat content [33–35]. Finally, genetic deletion of the cytosolic liver-specific fatty acid-binding protein (L-FABP) decreases incorporation of unsaturated FFAs into cellular triglycerides [36,37], and L-FABP hepatic expression is increased in early stages of human NAFLD [38].

Hepatic lipids in steatosis & nonalcoholic steatohepatitis

Circulating levels of FFAs are increased in NASH patients and correlate with disease severity [23,39,40]. Although *de novo* lipogenesis is increased in NAFLD patients [40], 60–80% of the circulating FFAs in these individuals are derived from adipocyte lipolysis and provide approximatively 60% of FFAs in the liver [26]. More precisely, de Almeida *et al*. studied the exact composition of the circulating long-chain FFAs increased in NASH patients. NASH was demonstrated to be predominantly associated with elevated levels of the saturated FFA, palmitic acid (C16:0), and the monounsaturated FFAs, oleic acid (C18:1) and palmitoleic acid (C16:1) [39]. Despite an increase in total (esterified and nonesterified) hepatic saturated and unsaturated fatty acids, no or minimal increase of hepatic saturated or unsaturated FFAs was observed in liver biopsies of NAFLD and NASH patients when compared with normal patients [41,42]. Therefore, there is evidence to suggest that excess circulating saturated and unsaturated FFAs are rapidly esterified within the hepatocytes to form diacylglycerides and triacylglycerides [41].

It is interesting to note that the greatest increase of total hepatic lipid content, diacylglycerides or triacylglycerides, was observed in NAFLD patients with benign simple steatosis rather than in NASH patients with a more advanced form of NAFLD [41]. Along with this observation, a new emerging concept suggests that cellular triglyceride accumulation is not toxic *per se*. Rather, accumulation of excess nonesterified saturated FFAs or their metabolic products mediate lipotoxicity [43]. Indeed, considerable data indicate that saturated FFAs are more toxic to liver cells than unsaturated FFAs [11–17]. Some recent studies have reported that this difference in toxicity between saturated and unsaturated FFAs relies on their ability to be rapidly esterified and incorporated as triglycerides [18,44,45]. Furthermore, esterified unsaturated fatty acids are the most abundant in various kinds of lipids, including phospholipids and triglycerides. Therefore, exposure of liver cells to nontoxic unsaturated FFAs (e.g., oleic acid and palmitoleic acid) results in a significant accumulation of triglycerides, whereas exposure to the toxic saturated FFA (e.g., palmitic acid) minimally increases hepatic lipid droplets [18,44,45].

Diversion of saturated FFAs into triglycerides provides hepatocytes protection against saturated FFA-induced toxicity (Figure 1). In fact, monounsaturated FFAs, such as oleic acid and palmitoleic acid, inhibit liver toxicity induced by saturated FFAs [15–18] and the protective effects of the unsaturated FFAs is suggested to be due to their ability to redirect saturated FFAs into triglyceride storage [18]. For example, the enzyme stearoyl CoA desaturase (SCD) 1, which catalyzes the desaturation of palmitic acid and stearic acid to palmitoleic acid and oleic acid, respectively, plays an important role in hepatic triglyceride accumulation; and SCD1 overexpression increases triglyceride synthesis and protects against lipoapoptosis [18]. By contrast, genetic deletion of *SCD1* decreases hepatic steatosis and aggravates hepatocellular apoptosis and liver injury during murine experimental steatohepatitis [44]. In a similar manner, antisense oligonucleotide-mediated silencing of diacylglycerol acyltransferase 2, a key enzyme in the esterification of FFAs to triglyceride, enhances liver injury and fibrosis despite reducing hepatic triglyceride content in a murine model of NASH [19].

Different patterns of L-FABP expression have been observed in human NAFLD progression, from simple steatosis to NASH [38]. L-FABP is overexpressed in the liver of patients with simple steatosis, but as the stage of NAFLD progresses to NASH, L-FABP expression dramatically decreases [38]. Similarly, feeding mice a high-fat diet, which results in extensive steatosis without hepatocellular injury or fibrosis, increases hepatic SCD1 expression, whereas feeding mice a methionine-and choline-deficient diet, which reproduces the fibrosing steatohepatitis observed in patients with severe NASH, markedly decreases SCD1 expression [44]. Hence, a potential pathogenic mechanism accounting for the development of liver injury

in NASH may involve impaired cellular capacity to incorporate toxic FFAs into neutral triglycerides.

Hepatocyte cell death in steatosis & steatohepatitis

Failure of the hepatocyte to dispose of excess FFAs by converting them into triglyceride is associated with increased risk for hepatocyte lipoapoptosis, a cardinal pathogenic feature of NASH [22]. The mechanisms involved in FFA-induced toxicity have yet to be completely defined, but recent compelling data suggest that hepatocyte lipoapoptosis mainly arises from FFA-induced lipotoxic stress of intracellular organelles, in particular the endoplasmic reticulum (ER) and mitochondria.

Endoplasmic reticulum stress

Disturbances in the ER seem to be implicated in lipoapoptosis pathways and may even be an important causative factor in mediating cell death. The ER is a critical organelle responsible, among other functions, for the synthesis, maturation, folding and transport of proteins, as well as lipid synthesis and packaging, and the regulation of cellular calcium homeostasis. Perturbation of these processes creates a condition referred to as ER stress. As a primary event, ER stress leads to the activation of an adaptive and protective signaling network termed the unfolded protein response (UPR), which serves to overcome the stress stimulus and re-establish ER homeostasis [46]. However, if the cell fails to adapt, a prolonged and persistent ER stress will trigger proapoptotic signals that will indirectly cause cell death by activating downstream molecules, mainly c-Jun N-terminal kinase (JNK) and the transcription factor CCAAT/enhancer binding homologous protein (CHOP).

Endoplasmic reticulum stress responses are induced by three distinct ER membrane-spanning signaling molecules, namely, activating transcription factor 6 (ATF6), PKR-like ER kinase (PERK) and inositol-requiring enzyme (IRE) 1α, which can be held inactive by the chaperone glucose-regulated protein (GRP) 78. As an initial event, the activation of these three kinases will induce several mechanisms to reduce the burden of unfolded proteins in the ER. However, when these adaptive mechanisms fail to re-establish ER homeostasis, excessive activation of the resident ER kinases will induce proapoptotic signals. When activated, PERK phosphorylates and inactivates eukaryotic translation initiation factor (eIF) 2α, reducing mRNA translation and decreasing protein load in the ER. Paradoxically, phosphorylation of eIF2α selectively benefits the translation of ATF-4 [47], which regulates the promoter of GRP78 [48] but also leads to the transcriptional upregulation of the transcription factor CHOP. Active IRE1α classically cleaves X-box-binding protein (XBP)-1 mRNA, and the resultant spliced protein, sXBP-1, controls the upregulation of a broad spectrum of genes involved in ER-assisted degradation, protein folding and protein quality control [49]. Furthermore, active IRE1α can recruit the adaptor molecule TNF-receptor-associated factor 2, which further recruits the apoptosis-signal-regulating kinase that activates the JNK signaling pathway [50]. While activation of PERK and IRE1α will promote both cytoprotective processes that re-establish homeostasis and proapoptotic processes, ATF6 mainly upregulates the expression of GRP78 and ER degradation enhancing-mannosidase like protein, resulting in increased ER-chaperone activity and degradation of misfolded proteins [51,52].

Previous studies in both genetic and dietary murine models of obesity suggest that ER stress and activation of the UPR in the liver play a determinant role in the development of obesity-induced insulin resistance and Type 2 diabetes [53]. Indeed, it was demonstrated that obesity-induced ER stress led to suppression of insulin receptor signaling through sustained JNK activation which phosphorylates and inactivates insulin receptor substrate-1 [53]. Furthermore, in dietary murine models of hepatic steatosis, ER stress-induced CHOP expression and *XBP-1* mRNA splicing are increased in rat liver and correlate with the development of liver

injury [16]. Finally, in human patients with NAFLD and NASH, a strong activation of PERK is observed, as reflected by increased phosphorylation of eIF2α [54]; and phosphorylation and activation of JNK, a downstream target of IRE1α, is also observed in liver biopsies from patients with NASH [13,54].

The mechanisms underlying ER stress and the activation of UPR signaling in obesity-induced hepatic steatosis are not fully understood. However, it appears that the composition of fatty acids in the steatotic liver, rather than hepatic steatosis itself, is an important determinant in inducing ER stress-mediated liver damage. Hence, although rats fed a diet enriched in either polyunsaturated fatty acids or saturated fatty acids accumulate hepatic triglycerides to a similar extent, only those rats fed a diet enriched with saturated fatty acids exhibited strong activation of ER stress markers and liver injury [16]. Since intracellular FFAs are trafficked to, and esterified within, the ER, inundation of liver cells with FFAs could disturb ER function and induce an ER-stress response. In accordance with this concept, increased saturated lipid content of ER directly compromises ER morphology and integrity [55]. Recent *in vitro* studies suggest that saturated FFA-induced liver cell apoptosis relies on ER stress-mediated CHOP induction and JNK activation; and the low toxicity of unsaturated FFAs on liver cells could be due to their incapacity to induce resident ER kinases, PERK- and IRE1α-dependent signaling pathways [15,17,45]. Appropriate ER-calcium levels are necessary for normal protein folding because of the calcium-dependent nature of chaperone proteins (such as GRP78 and calreticulin) [56]. Deletion of ER calcium can result in the misfolding of proteins and can trigger the UPR or ER stress. Indeed, saturated FFAs deplete ER-calcium stores in liver cells and this process partially contributes to ER stress by saturated FFAs [17]. Calcium released by the ER into the cytosol can also be taken up by mitochondria, promoting dysfunction of this organelle and thereby triggering apoptosis [57].

CHOP downstream targets

CCAAT/enhancer-binding homologous protein is an inducible leucine zipper transcription factor that plays an important role in ER stress-induced apoptosis. Although CHOP knockdown confers protection against FFA-mediated ER stress-induced apoptosis in pancreatic β-cells [58] and prevents hepatocyte apoptosis in alcoholic liver injury [59], its role in lipoapoptosis, as well as the mechanisms by which CHOP promotes lipoapoptosis, remains incompletely understood.

Considering that CHOP is a transcription factor, recent studies have revealed that CHOP regulates the transcription of several members of the Bcl-2 family. The Bcl-2 family of proteins regulate the mitochondrial pathway of apoptosis, and this protein family is comprised of both pro- and antiapoptotic members. For example, ER stress-mediated CHOP expression downregulates the antiapoptotic protein Bcl-2 [60], whereas CHOP, plus its heterodimeric partner C/EPBα, directly binds to the promoter of the proapoptotic protein Bim and upregulates Bim transcription [61]. Decrease in Bcl-xL expression and increase in Bim expression have been reported to contribute to saturated FFA-mediated apoptosis in liver cells [11,12,62]. However, whether FFA-induced modulation of these members of the Bcl-2 family depend on CHOP transcriptional activity merits further investigations.

Studies performed in human carcinoma cells demonstrated that CHOP can transcription-ally upregulate TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2 or death receptor 5 [DR5]), a member of the TNF-receptor gene superfamily [63]. Upregulation of death receptors, such as TRAIL receptors and/or Fas can promote apoptosis by the extrinsic cellular pathway [64]. TRAIL has been implicated in steatosis-associated liver injury. For example, enhanced expression of DR5 was observed in liver biopsies from patients with NASH [65]. Similarly, FFAs upregulate DR5 expression in liver cancer cell lines, and silencing DR5 expression protects against FFAs-mediated apoptosis [65]. Although healthy human

hepatocytes are resistant to TRAIL, FFA-induced steatosis sensitizes hepatocytes to TRAIL cytotoxicity [66]. Other death receptors may also be implicated in the pathogenesis of steatohepatitis. Indeed, expression of the death receptors Fas (or CD95) and TNF-α-receptor 1 is enhanced in livers of patients with NASH [22,67]. Fas is also increased in experimental models of NASH, and FFA-treated liver cells overexpress Fas [68]. Furthermore, obesity-induced steatosis increases hepatocyte sensitivity to Fas ligand-mediated apoptosis [22,69]. Whether FFA-induced ER stress regulates the expression of these DRs remains unexplored.

JNK-signaling pathway

c-Jun N-terminal kinase, which belongs to the family of mitogen-activated protein kinases, has been implicated as a central mediator of FFA-induced hepatocyte lipoapoptosis in both dietary murine models of nonalcoholic steatohepatitis [70–72] and in human NASH [13,54]; and pharmacological inhibition of JNK prevents FFA-induced hepatocyte apoptosis [11]. Sustained activation of JNK, secondary to saturated FFA-stimulated mitogen-activated protein kinase kinase kinase MLK3 [73] and/or secondary to saturated FFA-induced ER stress [15, 50], can cause cell death signals by both transcriptional and post-transcriptional mechanisms [74]. These mechanisms will be discussed in greater detail in this section.

Of the three known JNK genes, only *JNK1* and *JNK2* are expressed in hepatocytes [75], and these two isozymes are alternatively spliced to yield α and β isoforms of both p54 and p46 proteins [76]. Although both JNK1 and JNK2 have been implicated in liver injury, these two isoforms differentially contribute to hepatocyte apoptosis depending on the injurious stimulus. Whereas JNK2 appears to induce apoptosis in a TNF-α-induced liver injury model [77], recent studies have demonstrated that enhanced JNK1-signaling plays a central role in inducing hepatocyte apoptosis in a murine model of steatohepatitis [70,71].

c-Jun N-terminal kinase-1 predominantly contributes to the phosphorylation of the transcription factor c-Jun [13,70,71,78], a critical member of the activator protein-1 (AP-1) transcription factor complex. In experimental models of steatohepatitis, high levels of phosphorylated c-Jun and enhanced AP-1 complex binding activity correlated with increased hepatocyte apoptosis [70,71]. A recent study has determined that JNK1-dependent induction of the proapoptotic member of the Bcl-2 family, p53 upregulated modulator of apoptosis (PUMA), with subsequent activation of Bax, also contributes to hepatocyte lipoapoptosis [13]. In this study, saturated FFA-mediated JNK1/c-Jun phosphorylation resulted in the formation of an active AP-1 complex that directly binds to the promoter of PUMA and upregulates its transcription in liver cells [13]. Genetic deletion of *JNK1* prevented FFA-mediated c-Jun activation and PUMA induction [13], and decreased steatohepatitis-mediated hepatocyte apoptosis in mice [70,71]. Similarly, sustained activation of JNK in patients with advanced stages of NAFLD (NASH patients) [54] correlates with a significant increase in DNA-binding activity of hepatic c-Jun containing AP-1 complex [79], a marked increase in hepatic levels of PUMA [13] and the development of hepatocyte injury [22,54].

Alternatively, JNK can also post-transcriptionally phosphorylate and regulate other members of the Bcl-2 family. For example, JNK-mediated phosphorylation of the antiapoptotic proteins Bcl-2 or Bcl-xL suppresses their antiapoptotic activities and promotes apoptosis [80]. By contrast, JNK-dependent phosphorylation of Bad, Bim or Bax enhances the proapoptotic potential of these proteins [81–83]. Whether these post-transcriptional modifications of these pro- and antiapoptotic members of the Bcl-2 family occurs during the lipotoxic insult is not known, nor the exact contribution of each JNK isoform in these processes. Finally, it has been demonstrated that JNK also transcriptionally upregulates DR5 [65] and Fas ligand [84]; two factors that may also contribute to lipoapoptosis, as mentioned previously.

BH3-only proteins & mitochondrial dysfunctions in lipoapoptosis

Proapoptotic BH3-only protein family members, which include Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa and PUMA, are critical regulators of lipoapoptosis. These proteins are the biosensors of cell death and initiate activation of the core proapoptotic machinery. Among these proteins, Bim and PUMA expression were induced in liver cells by the saturated FFA, palmitic acid [11–13]. In this context, induction of Bim was demonstrated to result from transcriptional activation by FoxO3a, which upon dephosphorylation by protein phosphatase 2A, migrates into the nucleus and binds the *Bim* promoter. Knockdown of Bim using small interfering RNA partially protects liver cells against lipoapoptosis [12]. Prior observations indicate that protein phosphatase 2A can be activated by ER stress [61], and this mechanism could account for its activation during lipoapoptosis in liver cells. As mentioned earlier, saturated FFAs also stimulate PUMA expression by a JNK1/AP-1-dependent mechanism, and genetic deletion of *PUMA* conferred resistance to murine hepatocyte against palmitic acid-mediated apoptosis [13].

Given the fact that Bim and PUMA have complementary functions, it is likely that these two proapoptotic proteins cooperate in lipoapoptosis, as demonstrated in other cell death processes [85]. FFA-mediated Bim and PUMA induction results in the activation of the multidomain proapoptotic member of the Bcl-2 family, Bax [11,13,86]. Bim and PUMA can directly bind to and activate Bax [87,88]. Furthermore, PUMA may indirectly promote Bax activation by binding to and disabling the function of prosurvival Bcl-2 proteins such as Mcl-1 and/or Bcl-xL [89,90]. Interestingly, palmitic acid-mediated hepatocyte apoptosis can also result in the loss of the antiapoptotic proteins Mcl-1 and Bcl-xL. Inhibition of Mcl-1 degradation or forced overexpression of Bcl-xL attenuates apoptosis by saturated FFAs [14,62].

Therefore, the data discussed previously demonstrate that the toxic saturated FFAs activate the mitochondrial cell death pathway [11]. Indeed, activation and oligomerization of Bax in the outer mitochondrial membrane results in mitochondrial dysfunction, downstream activation of the effector caspases 3, 6 and 7, and ultimately, cell death by apoptosis [91]. FFA-induced Bax activation is regulated by Bim and PUMA [11–13], but lysosomal permeabilization with release of protease, such as cathepsin B, may also contribute to FFA-induced mitochondrial dysfunctions [92]. Bax expression may also be increased during murine experimental steatohepatitis [72], and increased hepatocyte apoptosis, as well as structural and functional mitochondrial abnormalities, are well documented in liver tissue from NASH patients [22, 93–95].

Other lipid-signaling molecules

Lipoapoptosis is not only restricted to FFAs. When present in excess, other lipids, such as ceramide and cholesterol, can also trigger cellular apoptotic processes. Although the mechanisms by which these lipids act as signaling molecules are not completely clear, some evidence suggests that they could also contribute to hepatocyte lipoapoptosis.

Ceramide

The role of ceramide in insulin resistance and obesity has been well documented [96,97]. Furthermore, ceramide signaling and, most importantly, *de novo* synthesis of ceramide, was suggested to play a significant role in lipoapoptosis processes in nonhepatic cells [96,98,99]. Ceramide is the central core lipid in the metabolism of sphingolipids. Ceramide accumulation in the cell can occur from either hydrolysis of sphingomyelin by sphingomyelinases, or by *de novo* synthesis via serine palmitoyltransferase (SPT) and ceramide synthase. *De novo* ceramide synthesis occurs in the ER, where a fatty acid moiety, usually palmitoyl CoA, is combined to the amino group of sphingosine. SPT is the rate-limiting enzyme of *de novo* biosynthesis of

ceramide and its activity depends on the availability of the long chain FFAs. Therefore, ceramide concentration increases in liver cells after treatment with saturated FFAs [15]. Palmitic acid and stearic acid-induced apoptosis was demonstrated to be associated with increased *de novo* ceramide synthesis in murine hematopoietic cell lines [100]; and saturated FFA-induced apoptosis was attenuated with pharmacological inhibitors of *de novo* ceramide synthesis. Furthermore, increased adipose ceramide levels with associated augmentation of SPT activity are observed in mice fed a high-fat diet [101], and accumulation of ceramide in subcutaneous adipose tissue seems to reflect the development of human fatty liver [102]. A lipidomic analysis revealed a positive correlation between accumulation of triglycerides and ceramide levels in the liver of genetically obese mice when compared with control mice [201]. However, saturated fat diet-induced obesity in mice led to liver injury via extensive ER-stress activation and hepatocyte apoptosis, independently of ceramide generation [16]. Saturated FFA-induced apoptosis in liver cells was also ceramide independent [15,62], as well as the induction of Bim expression by saturated FFAs [12]. The role of ceramide in the pathogenesis of human fatty liver is unclear. Stimulation of the death receptor Fas activates sphingomyelinases, leading to rapid accumulation of ceramide, and this process seems to play an important role in the regulation of Fas-induced apoptosis [103]. As Fas expression is enhanced in livers of patients with NASH [22], it cannot be excluded that ceramide may play a role in lipoapoptosis through the Fas-induced apoptotic signaling cascade. Nevertheless, a recent study has demonstrated that the hepatic ceramide content was unchanged in patients with NAFLD when compared with normal patients [104].

Cholesterol

It is well established that free cholesterol is highly cytotoxic. Analyses of the hepatic lipid composition of subjects with fatty liver disease have demonstrated a progressive increase in free cholesterol in patients with NAFLD and NASH, as compared with controls [41], and despite this increase in free cholesterol, liver cholesterol ester levels are unchanged between the three groups. A recent study has demonstrated that rats fed a high cholesterol diet, with increased free cholesterol loading in hepatocytes, developed a microvesicular steatosis and were sensitized to TNF-α- and Fas-induced hepatocellular death and inflammation [105]. In these rats, mitochondrial free-cholesterol loading accounted for the hepatocellular sensitivity to TNF-α, owing to mitochondrial glutathione depletion. The type rather than the amount of fat was an important determinant in the hepatocellular susceptibility to Fas and TNF-α, as dietary-induced triglyceride accumulation in the liver was insufficient to sensitize rats to DR-mediated toxicity [105]. Dietary supplementation with cholesterol was demonstrated to increase total ceramide levels in plasma in the adipose tissues of rats, which could account for the cytotoxic effect of cholesterol [106]. Free-cholesterol accumulation in atherosclerotic macrophages results in depletion of ER-calcium stores with activation of the UPR and ER stress-induced apoptosis [107,108]. However, free cholesterol loading of the ER in rodent hepatocytes was insufficient to induce an ER-stress response [105]. Therefore, the role of free cholesterol in the pathogenesis of human fatty liver disease is unclear, and the mechanisms by which free cholesterol induce hepatotoxicity requires further investigation.

Therapeutic approaches

To date, there is no proven effective therapy for patients with NASH. Therapy has focused on improvements of associated conditions of the disease, such as obesity, diabetes mellitus and hyperlipidemia. Weight loss in overweight patients with liver disease has demonstrated a sustained improvement in liver function and hepatomegaly [109] and therefore remains the primary nonpharmacological treatment option. Unlike weight loss, pharmacological therapies have not been as consistently positive. Several drugs that decrease insulin resistance and increase hepatic insulin sensitivity are of interest. For example, metformin improved fatty liver

disease, reversed hepatomegaly, steatosis and serum alanine aminotransferase abnormalities in obese mice [110]. In human trials, metformin improved insulin sensitivity and decreased serum alanine aminotransferase concentrations in NASH patients but these beneficial effects were transient [111].

Thus, new treatment strategies focus on the molecular causes involved in the development and progression of NASH. Hepatocyte apoptosis is a critical feature of NASH [22], and development of antiapoptotic therapies may be useful in this syndrome. As specific regulation of this apoptosis could be exploited in order to develop new drug therapies, several potential targets of the apoptotic process will be reviewed in this section (Figure 2).

Caspase inhibitors

As mentioned previously, Feldstein *et al*. have demonstrated that caspase 3 activation and hepatocyte apoptosis are prominent pathologic features of human NAFLD and correlate with disease severity [22]. Saturated FFA-induced apoptosis in liver cells depends on mitochondrial dysfunction, cytochrome *c* release and caspase 3/7 activation. Inhibition of caspase activity using pan-caspase inhibitors, Z-VAD-fmk or IDN-6556, markedly reduced cell death in liver cells treated with palmitic acid or stearic acid [11]. Similar effects were observed *in vivo* in experimental murine models of steatohepatitis. Indeed, prolonged treatment with the pan-caspase inhibitor, VX-166, decreased caspase-3 activation and reduced the number of tunnel-positive cells in mice fed a murine model of NASH [112]. Despite amelioration in hepatic steatosis and an improvement in liver fibrosis, the inhibitor was not sufficient to completely abrogate liver cell death and did not decrease serum alanine aminotransferase concentrations in this mouse model of NASH [112]. Nonetheless, a novel caspase inhibitor, GS9450, was developed by Gilead Sciences, Inc. (CA, USA) and is now being evaluated in a Phase IIA clinical trial in NASH patients.

Cathepsin B inhibitors

Some evidence suggests that structural and functional mitochondrial abnormalities, which are observed in liver cells from NASH patients [94,95], are involved in the progression of NAFLD [113]. Saturated FFAs induce mitochondrial dysfunction in both primary and transformed hepatocytes [11,62,92]. Recent data suggest that impaired mitochondrial functions in this context results from FFA-induced Bax translocation to lysosomes, with subsequent lysosomal permeabilization and cathepsin B release [62,92]. Thus, both genetic and pharmacological inhibition of cathepsin B in liver cells reduced saturated FFA-induced mitochondrial membrane permeabilization and cytochrome c release [62,92]; genetic deletion of cathepsin B attenuated steatohepatitis-mediated mitochondrial dysfunction, hepatocyte apoptosis and liver damage in mice [92,114]. Lysosomal permeabilization and release of cathepsin B into the cytosol was also observed in human liver tissues from patients with NAFLD, and correlated with the degree of inflammatory activity [114]. Therefore, inhibition of cathepsin B enzyme activity could represent a potential strategy to prevent mitochondrial dysfunctions associated with NASH.

Dietary supplementation with unsaturated free fatty acids

Saturated FFAs are more toxic to liver cells than unsaturated FFAs [11–17], and unsaturated FFAs can rescue liver cells from saturated FFA-induced caspase activation and apoptosis [15,17,18,44,45]. The protective effects of unsaturated FFAs include the inhibition of biomarkers of ER stress, namely CHOP induction and JNK activation, the prevention of ER stress-dependent upregulation of the proapoptotic BH3-only proteins, PUMA and Bim, and the reduction in Bax activation and subsequent mitochondrial dysfunction [45]. Thus, supplementation of n-3 polyunsaturated fatty acid (PUFA) *in vivo* to high-fat diet-fed rats ameliorated fatty liver and the degree of liver injury [115].

Furthermore, depletion of long-chain n-3 PUFAs has been observed in the hepatic tissue of patients with NAFLD [116] and likely promotes the pathogenesis of the disease [117]. Dietary supplementation with n-3 PUFAs reduced hepatic triglyceride content and serum alanine aminotransferase levels, and improved insulin resistance in patients with NAFLD [118,119]. Nevertheless, clinical trials have yet to confirm the therapeutic benefit of a dietary supplementation with n-3 PUFAs in patients with NAFLD.

JNK inhibitors

Chronic or excessive JNK activation results in systemic insulin resistance, promoting diabetes and metabolic syndrome. JNK, which is activated exclusively in liver biopsies from NASH patients [13,54], contributes to the pathogenesis of NAFLD [11,13,70,71]. In cellular models of saturated FFA-induced toxicity, sustained JNK activation mediates PUMA and Bim-dependent Bax activation and apoptosis in liver cancer cells and isolated murine and human hepatocytes [13], both of which are prevented when using JNK inhibitors [11,13]. While the JNK1 isoform mediates the proapoptotic processes in experimental steatohepatitis, the JNK2 isoform appears to have an antiapoptotic effect on lipoapoptosis [71]. Indeed, genetic deletion of *JNK2* exacerbates hepatocellular injuries induced by a high-fat diet in mice; the cytoprotective function of JNK2 is mediated by its ability to enhance Bim degradation and cellular elimination [71]. Thus, while indiscriminate JNK inhibition could interfere with the beneficial antiapoptotic effect of JNK2 isoform, selective inhibition of the JNK1 isoform could represent a potentially effective treatment for NASH.

Chemical chaperones

Perturbations in the ER may be associated with lipotoxicity, and ER stress was detected in both rodent and human steatohepatitis [16,54]. Saturated FFAs activate the PERK-and IRE1α-dependent arm of ER stress, which results in CHOP induction and JNK activation in various liver cancer cells [15,17,45]. Interestingly, saturated FFAs seem to regulate only selective components of the UPR. Indeed, saturated FFAs did not promote the beneficial ER responses involved in protein folding, such as upregulation of molecular chaperones (GRP75 and calreticulin), as well as enhancing-mannosidase like protein [15]. Similarly, the level of the major ER chaperone, GRP78, is not, or is only modestly, altered after saturated FFAs treatment [Akazawa y *et al*., Unpublished Data,15]. Given that ATF6 activation controls the expression of GRP78 [51,52], ATF6 may remain silent during lipoapoptosis processes and no study has ever reported the involvement of ATF6 activation during saturated FFA-mediated apoptosis.

Since the failure to activate the protective responses of the UPR may contribute to lipotoxicity, re-establishment of ER homeo-stasis may represent a novel strategy to limit lipoapoptosis. A recent study has reported that oral administration of chemical chaperones, (e.g., 4-phenyl butyric acid) or bile acid derivatives (e.g., tauroursodeoxycholic acid) to genetically obese mice decreased biochemical markers of ER stress in the liver (e.g., PERK, IRE1α and JNK activation), improved insulin sensitivity, reduced hepatic steatosis and normalized the liver functional enzyme, alanine aminotransferase [120]. Thus, molecular agents that modulate ER and increase folding capacity may have therapeutic potential in reducing hepatic impairments associated with obesity and NAFLD. However, it should be noted that ursodeoxycholic acid failed to improve NAFLD in a human trial [121].

Conclusion

Nonalcoholic fatty liver disease, the most common liver disorder in the developed world, is associated with insulin resistance, a component of metabolic syndrome, which leads to the accumulation of fat, mainly triglycerides, in the liver. In NAFLD, the liver fails to cope with an excess of lipids. Although hepatic steatosis is not toxic, the high serum concentration of

FFAs observed in advanced stages of NAFLD (NASH) induce hepatocyte apoptosis, and lipoapoptosis may represent a key mechanism resulting in the progression of simple steatosis to steatohepatitis. The lipotoxic effects of FFAs are multiple and complex, and involve impairment of the proper function of liver cell organelles, such as the ER. FFA-mediated ER stress probably plays a pivotal role in the activation of the numerous intracellular processes leading to hepatocyte apoptosis. These include JNK- and CHOP-dependent upregulation of proapoptotic BH3-only proteins and death receptors, Bax activation, mitochondrial permeabilization and subsequent activation of effector caspases. At present, there are no proven effective therapies for NASH. Thus, a better understanding of the molecular mechanisms involved in lipoapoptosis should help identify novel therapeutic approaches for this disease process.

Future perspective

The phenomenon of lipotoxicity is among a multitude of events associated with NASH. Although an important amount of information has been accumulated in the past 10 years concerning the molecular factors involved in lipoapoptosis, the mechanisms by which saturated FFAs induce an ER-stress response remain obscure. A plausible explanation is the impairment of ER morphology and function, as the result of an overload of saturated lipid content of the ER [55]. However, the nontoxic FFA, oleate, which failed to induce ER stress in liver cells [15,17,45], has also been reported to cause ER distension in pancreatic cells [122]. Therefore, future studies will need to focus on uncovering the specific upstream mechanisms induced by saturated FFAs which lead to the activation of ER stress.

Furthermore, saturated FFAs seem to regulate only selective components of the UPR. Indeed, saturated FFAs did not induce, or only modestly induced, the protective ER responses, such as upregulation of ER chaperones [Akazawa y *et al*., Unpublished Data, 15] in order to increase protein folding and re-establish ER homeostasis. This observation suggests that FFAs may not induce a full ER-stress response, as selective ER responses remain silent during the lipoapoptosis processes. A further examination of the ER stress-mediated mechanisms during lipoapoptosis is necessary.

Executive summary

Nonalcoholic steatohepatitis & lipoapoptosis

- Nonalcoholic steatohepatitis (NASH), an inflammatory stage of nonalcoholic fatty liver disease (NAFLD), is characterized by high serum concentration of circulating free fatty acids (FFAs) and hepatocyte apoptosis.

Hepatic lipids

- Circulating FFAs, mainly derived from the lipolysis of adipose tissue triglycerides, are transported into hepatocytes by the fatty acid transporter protein 5 and FAT/CD36. Within the hepatocyte, these FFAs can be esterified to form neutral triglycerides resulting in hepatic steatosis, as observed in NAFLD patients.
- Esterification of FFAs appears to be a detoxification process, as nonesterified FFAs are inherently toxic to hepatocytes and induce apoptosis.
- Saturated and unsaturated FFAs differ with regard to their potential for lipoapoptosis; saturated long-chain FFAs are significantly more toxic than unsaturated FFAs and unsaturated FFAs can rescue liver cells from saturated FFA-induced apoptosis.

- Impaired cellular capacity to incorporate toxic FFAs into neutral triglycerides may account for the development of liver injury in NASH.

Hepatocyte cell death in steatosis & steatohepatitis

- Hepatocyte lipoapoptosis could mainly arise from the disturbance of endoplasmic reticulum (ER) function and the induction of an ER-stress response mediated by the phosphorylation and activation of two ER-resident kinases, inositol-requiring enzyme) (IRE) 1α and PKR-like endoplasmic reticulum kinase (PERK).
- A prolonged and persistent ER stress triggers proapoptotic signals mediated by PERK-dependent activation of the transcription factor CCAAT/enhancer-binding homologous protein (CHOP) and IRE1α-dependent induction of c-Jun N-terminal kinase (JNK) signaling pathways. JNK and CHOP upregulate the expression of the proapoptotic BH3-only proteins, p53 upregulated modulator of apoptosis (PUMA) and Bim, respectively.
- PUMA, in concert with Bim, activates the proapoptotic executioner protein, Bax, resulting in mitochondrial dysfunction and activation of effector caspases. Both contribute to FFA-induced apoptosis.
- In NASH patients, upregulation of death receptors, such as TNF-related apoptosis-inducing ligand receptors and/or Fas, can also sensitize fatty hepatocytes to the extrinsic cellular pathway of apoptosis.

Other lipid-signaling molecules

- Lipids others than FFAs, such as ceramide and free cholesterol, could also contribute to hepatocyte lipoapoptosis. Although both ceramide and free cholesterol are proapoptotic molecules, the mechanisms by which these lipids trigger apoptosis are not completely clear and the contribution of these two lipids in human nonalcoholic steatohepatitis merits further investigation.

Therapeutic approaches

- To date, there are no proven effective therapies for NASH and the development of an antiapoptotic therapy may be useful in this disease.
- Caspases inhibitors, cathepsin B inhibitors, dietary supplementation with polyunsaturated FFAs, JNK inhibitors and chemical chaperones could represent efficient novel therapeutic approaches in the treatment of lipoapoptosis associated with NASH.

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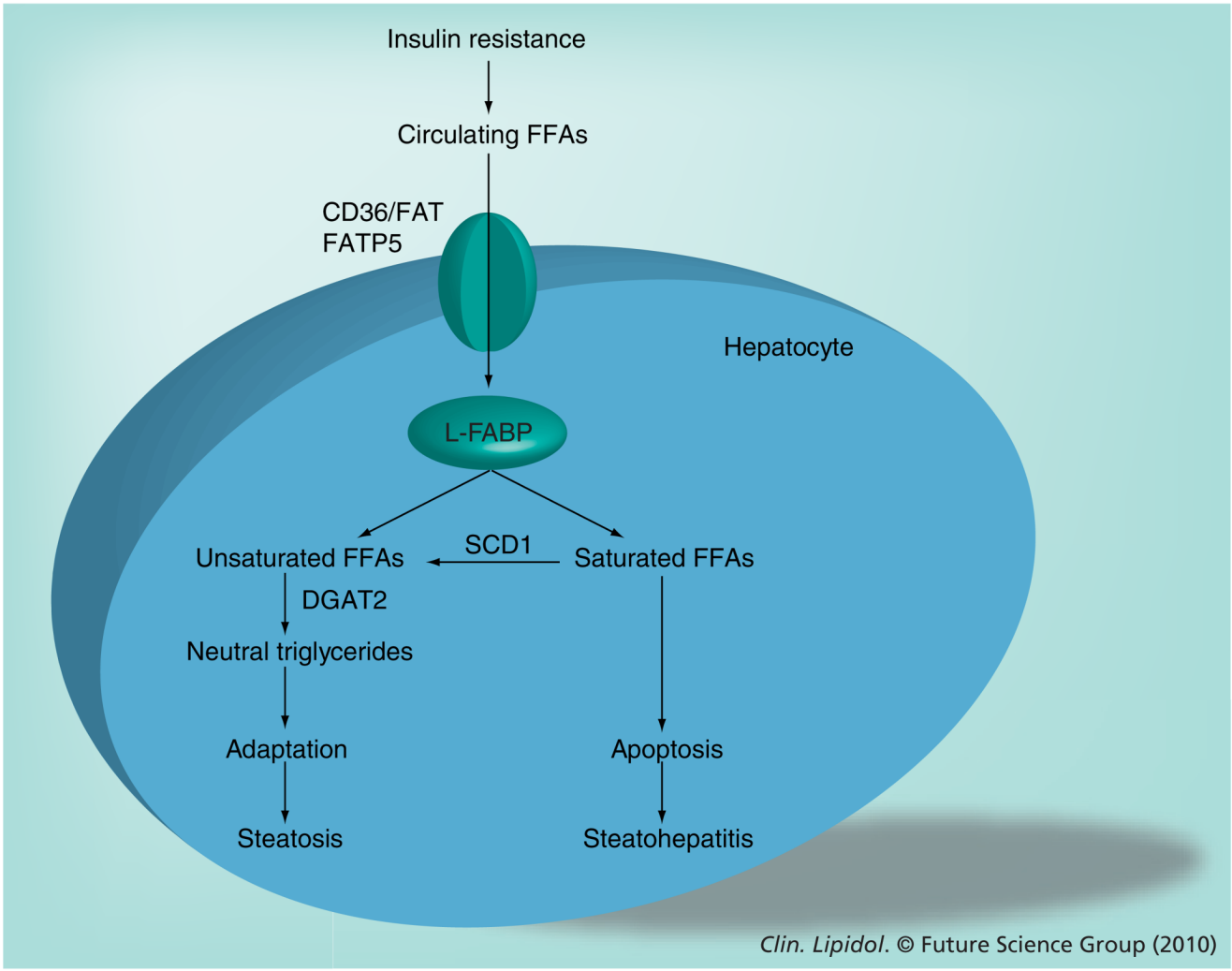

Figure 1. Diversion of saturated free fatty acids to neutral triglyceride formation as a protective mechanism against lipoapoptosis in the liver

Insulin resistance, a hallmark of NAFLD, leads to an increase in serum concentration of circulating FFAs. These circulating FFAs are transported into hepatocytes by specific fatty acid transporters (CD36/FAT and FATP5) and binding proteins (L-FABP). Within the hepatocytes, these FFAs can be esterified to neutral triglycerides, resulting in hepatic steatosis. Esterification of FFAs acts as a buffering mechanism, allowing cells to maintain viability in the face of excess nonesterified FFA exposure. Saturated and unsaturated FFAs differ with regard to their potential for lipoapoptosis. Unsaturated FFAs are less toxic than saturated FFAs and are rapidly esterified by the enzyme DGAT2 and incorporated as triglycerides. By contrast, conversion of the saturated FFAs into unsaturated FFAs by the enzyme SCD1 seems to be necessary in the diversion of saturated FFAs to triglyceride formation. Failure to partition nonesterified FFAs as triglycerides, by inhibiting SCD1 or DGAT2 activities, results in hepatocellular apoptosis and liver damage, which further leads to the development of steatohepatitis.

DGAT: Diacylglycerol acyltransferase; FATP: Fatty acid transport protein; FFA: Free fatty acid; L-FABP: Liver-specific fatty acid-binding protein; NAFLD: Nonalcoholic fatty liver disease; SCD: Stearoyl CoA desaturase.

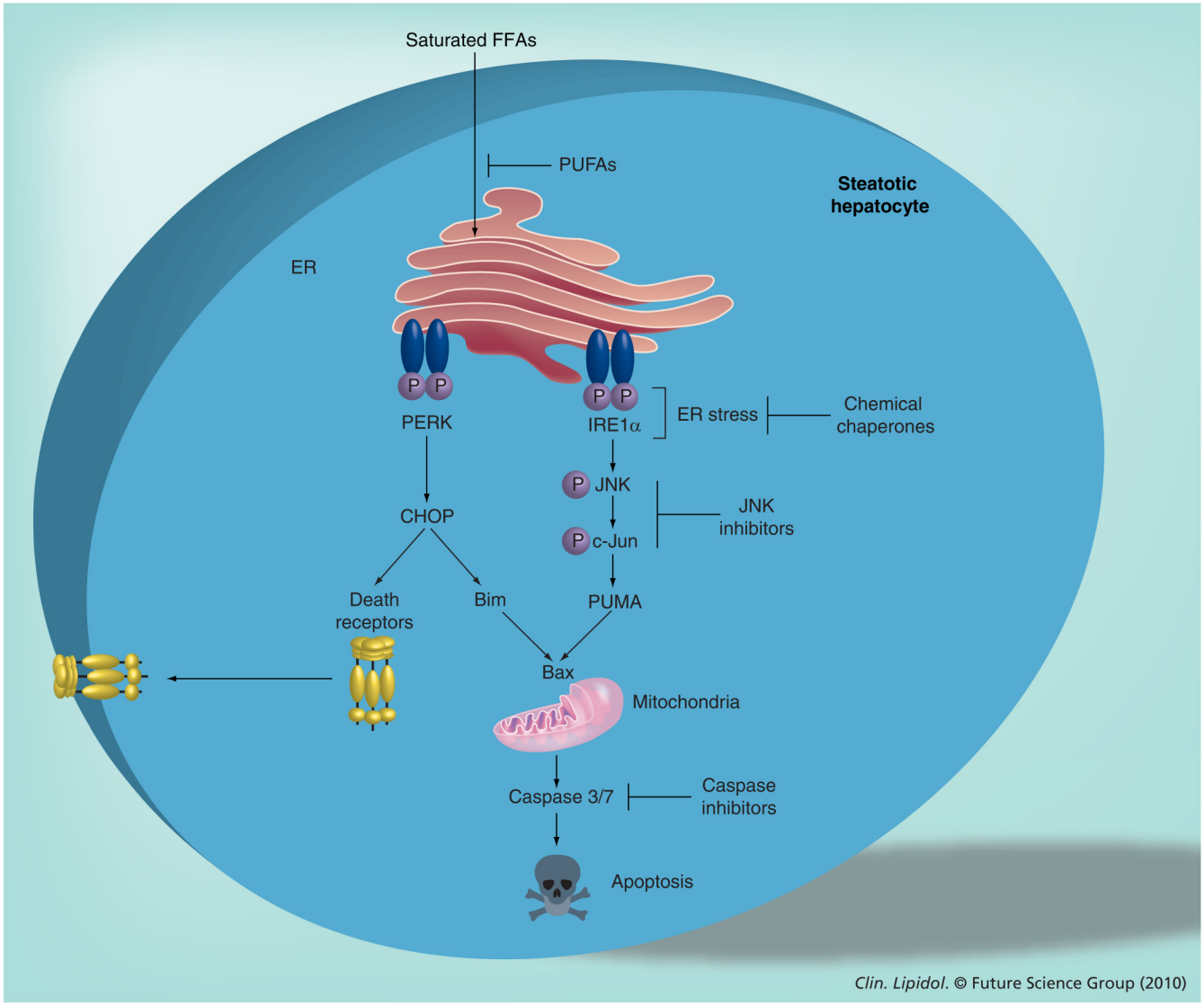

Figure 2. Molecular mechanisms involved in saturated free fatty acid-induced hepatocyte apoptosis

Saturated FFAs accumulate in the ER, which leads to disturbance of ER function and induction of an ERstress response, mediated by the phosphorylation and activation of two ER-resident kinases, IRE1α and PERK. Phosphorylated IRE1α activates an apoptotic pathway involving JNK activation. Active JNK phosphorylates the transcription factor c-Jun, which leads to the transcriptional upregulation of the proapoptotic BH3-only protein, PUMA. Activation of PERK results in the induction of the transcription factor CHOP, which upregulates the expression of the proapoptotic BH3-only protein, Bim. Bim, in concert with PUMA, activates the proapoptotic executioner protein, Bax, resulting in mitochondrial dysfunction and activation of effector caspases and apoptosis. CHOP can further upregulate the expression of death receptors, such as death receptor 5, which sensitize fatty hepatocytes to circulating death ligands (e.g., TNF-related apoptosis-inducing ligand).

CHOP: CCAAT/enhancer-binding homologous protein; ER: Endoplasmic reticulum; IRE: Inositol-requiring enzyme; FFA: Free fatty acid; JNK: c-Jun N-terminal kinase; P: Phosphate; PERK: PKR-like endoplasmic reticulum kinase; PUFA: Polyunsaturated fatty acid; PUMA: p53 upregulated modulator of apoptosis.