Published in final edited form as:
J Nutr Biochem. 2008 September ; 19(9): 567–576. doi:10.1016/j.jnutbio.2007.10.001.

The Role of Fatty Acids in the Development and Progression of Nonalcoholic Fatty Liver Disease

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Keywords

saturated fatty acids; liver; ER stress

INTRODUCTION

Obesity is now considered a worldwide epidemic. In the US, more than 30% of the adult population is currently obese, representing a two-fold increase since 1980 [1,2]. Concomitant with this burgeoning obesity epidemic has been a significant increase in obesity-associated diseases, most notably type 2 diabetes and cardiovascular disease [1,3].

Non-alcoholic fatty liver disease (NAFLD) is a newly emerging obesity-related disorder characterized by fatty infiltration of the liver in the absence of chronic alcohol consumption [4–6]. Similar to obesity, the prevalence of NAFLD has nearly doubled since 1980 [4,6,7]. Data from the most recent National Health and Nutrition Examination Survey (NHANES 1999–2002) suggest that the current prevalence of NAFLD is approximately 8.9% of the US population, as indicated by elevated levels of serum alanine aminotransferase (ALT) [8]. Diagnosis based solely on ALT levels, however, has been shown to underestimate the prevalence of NAFLD when compared to liver biopsies and radiographic techniques (e.g. magnetic resonance spectroscopy and computed tomography) [9,10]. Using these latter techniques, it has been estimated that NAFLD may affect 25–30% of the general population and up to 80% of obese and diabetic individuals [11]. Perhaps most alarming, NAFLD is emerging as a common pediatric disease, afflicting approximately 3–9% of all children in the US and up to 50% of obese children [12].

Recent data have demonstrated that NAFLD is closely associated with visceral adiposity, dyslipidemia and insulin resistance, and has been described as the hepatic component of the metabolic syndrome [6]. NAFLD ranges from fat accumulation in the liver (steatosis), to steatosis accompanied by inflammation and necrosis with or without fibrosis (non-alcoholic steatohepatitis or NASH), to end-stage liver disease [5,7]. Individuals with NAFLD often remain asymptomatic for decades. This indolent nature of NAFLD has contributed to an under-appreciation of its potential hazards [5]. However, NAFLD is now recognized as the most common cause of chronic liver enzyme elevations and cirrhosis [5,13], and more recent data

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suggest that the disease is independently associated with the development of cardiovascular disease and overall- and obesity-related mortality [14].

In light of the increasing prevalence and health consequences of NAFLD, there is a critical need to identify the mechanisms that mediate the development and progression of the disease. Research over the last decade has greatly enhanced our understanding of the disease in this regard, although numerous questions remain unanswered. For example, what metabolic abnormalities initiate the development of NAFLD. Also, what biochemical processes mediate the transition from simple steatosis to NASH. The current review will focus primarily on data from our laboratory and elsewhere examining the potential role of fatty acid composition in the progression of the disease. A putative role for the endoplasmic reticulum (ER) in the development and progression of NAFLD will also be discussed. Finally, we will compare and contrast the role of fatty acid composition in the pathophysiology of NAFLD with that of alcoholic fatty liver disease (AFLD), a disease histologically identical to NAFLD but with some intriguing differences.

TWO-HIT HYPOTHESIS

The current working model explaining the pathogenesis of NAFLD is the “two-hit” hypothesis, first proposed by Day et al. in 1998 [15]. According to this hypothesis, steatosis represents the “first hit”, which increases the vulnerability of the liver to various “second hits” that in turn lead to the inflammation, fibrosis and cellular death characteristic of NASH. Consistent with this hypothesis, administration of variously proposed second hits (e.g. endotoxin and pro-oxidants) results in significantly greater liver damage and lethality in obese mice with fatty liver compared to lean mice with healthy livers [16–18]. Furthermore, in humans, the severity of steatosis is one of the strongest predictors of the development of NASH [19].

Several factors have been suggested to constitute the second hit(s), most notably oxidative stress, pro-inflammatory cytokines and gut-derived bacterial endotoxin [4,20–22]. A detailed discussion of each of these putative factors is beyond the scope of this paper and is available in recent reviews [4,20–22]. It is important to note here, however, that these mechanisms are not mutually exclusive; but instead, likely act in a coordinated and cooperative manner to hasten the development and progression of NASH. For example, excess adiposity is associated with increased proinflammatory cytokines and oxidative stress, as well as an exaggerated inflammatory response to endotoxin administration [23]. Once generated, cytokines can cause direct liver damage or act indirectly by increasing oxidative stress, which in turn can also directly impair liver function or act indirectly by perpetuating the inflammatory response [13]. Therefore, in environments conducive to the generation of various second hits (e.g. obesity), a perpetuating cycle of insults may cause liver injury and culminate in NASH and, over time, end-stage liver disease.

One important aspect of the two hit hypothesis is that steatosis *per se* is not causal in the development of NASH; but rather, it sensitizes the liver to the damaging effects of second hits such that stressors innocuous to a healthy liver lead to the development of NASH in the steatotic liver. As will be discussed, however, an increasing body of literature suggests that the deposition of fat in the liver, and more specifically the *type* of fat that is deposited, may in fact directly damage the liver and precipitate the development of NASH.

LIPIDS AND NASH

Hepatocyte apoptosis is a salient feature and independent predictor of NASH [24,25]. In 1998, Unger et al. [26,27], first introduced the concept of lipoapoptosis, whereby over accumulation of lipids in non-adipose tissues leads to cell dysfunction and death. More recent data collected in various experimental models suggest that lipid-induced cell toxicity and apoptosis is specific

to or made more severe by saturated fatty acids [28–32]. These data predict that the presence of increased circulating and/or hepatic saturated fatty acids, but not polyunsaturated fatty acids, may promote the development and progression of liver damage, in part via activation of apoptosis. Recent studies by our laboratory and others have tested this prediction [33–37].

To examine the ability of individual fatty acids to induce apoptosis in liver cells, we exposed H4IIE hepatoma cells to either saturated (palmitate or stearate) or unsaturated (oleate or linoleate) fatty acids. Only palmitate and stearate increased caspase-3 activity and induced DNA fragmentation (Fig. 1A). Inclusion of the general caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone prevented palmitate and stearate-induced DNA laddering, demonstrating that saturated fatty acid-induced apoptosis was caspase-dependent. Notably, co-incubation of palmitate with oleate or linoleate reduced palmitate-mediated apoptosis (Fig. 1B). This latter finding is consistent with previous data in pancreatic β cells, Chinese hamster ovary cells (CHO), and cardiomyocytes and suggests that the ratio of saturated-to-unsaturated fatty acids in cells is an important determinant of cell viability [30,34,38,39].

To examine whether an increased ratio of saturated-to-unsaturated fatty acids could induce liver injury in vivo, we utilized dietary models of hepatic steatosis [40,41]. Male Wistar rats were fed diets enriched with starch (STD), sucrose (HSD), polyunsaturated fat (HPUFA), or saturated fat (HSAT) for 1,4 or 24 weeks (only 4 and 24 wk data are shown in figures) [33]. Liver triglycerides were increased to a similar extent in HSD, HPUFA, and HSAT compared with STD at 4 and 24 weeks; however, saturated fatty acid content of triglycerides and microsomal membranes was increased in HSD and HSAT compared with HPUFA (Fig. 2A). Liver caspase-3 activity and plasma markers of liver injury were significantly higher in HSD and HSAT compared to STD and HPUFA (Fig. 2B). In addition, HSD and HSAT were characterized by reduced proliferative capacity following partial hepatectomy and increased liver injury in response to lipopolysaccharide compared to HPUFA. Thus, an increased saturated-to-unsaturated fatty acid ratio in the steatotic liver not only induced liver injury but also reduced proliferative capacity and increased the susceptibility of the liver to endotoxin. Importantly, increased liver injury in these dietary models was observed independently of differences in cytokines and insulin action. These data are consistent with the notion that the composition of fatty acids delivered to and stored within the liver is an important determinant of liver cell integrity, and potentially an independent risk factor for progression to NASH.

INTRACELLULAR SIGNALS MEDIATING SATURATED FATTY ACID-INDUCED TOXICITY

Despite unequivocal evidence that saturated fatty acids induce apoptosis in a number of cell types [28–32,38], including liver and hepatocytes [33,34,37], the mechanisms by which they do so are unclear. Ceramide accumulation, which can occur via enhanced de novo synthesis using palmitate or increased sphingomyelin breakdown, has been linked to both insulin resistance and apoptosis [42–45]. In pancreatic β cells and bovine retinal pericytes, saturated fatty acids not only increase ceramide levels, but inhibition of ceramide production prevents saturated fatty acid-induced apoptosis [26,46]. To determine the role of ceramide in saturated fatty acid-mediated apoptosis in liver cells, we incubated H4IIE cells with palmitate in the absence or presence of the ceramide synthetase inhibitor fumonisin B_1 [34]. Palmitate significantly increased ceramide concentration in the absence of fumonisin B_1, and the presence of fumonisin B_1 prevented this increase. However, the presence of fumonisin B_1 did not reduce palmitate-mediated apoptosis. These data are consistent with previous findings in CHO cells [29], and suggest that intracellular mediators of saturated fatty acid-induced apoptosis are cell specific, and that factors other than ceramide mediate the apoptotic effect in the liver.

It has been suggested that the accumulation of intrahepatic fatty acids can promote redox imbalance and the formation of reactive oxygen intermediates. A study performed in CHO cells demonstrated that palmitate-induced apoptosis required the generation of reactive

intermediates [29]. In addition, other studies have found that reactive intermediates play a primary role in the activation stage of apoptosis [47–50]. Preliminary data (unpublished observations) from our laboratory suggest that both α-tocopherol (200 μM) and taurine (1%) reduce, but do not prevent, saturated fatty acid-induced apoptosis. Thus, other as yet unidentified intracellular signals, in addition to reactive intermediates, contribute to saturated fatty acid-induced apoptosis in liver cells.

The mitogen-activated protein kinase family of proteins is critical for the cellular response to a variety of stresses [51,52]. In particular, c-Jun NH_2 terminal kinase (JNK) has emerged as a central metabolic regulator in obesity-related insulin resistance, appears to be a direct target of ceramide, and is activated by lipids [37,43,53,54]. Furthermore, a recent study demonstrated that saturated fatty acid-induced apoptosis in both primary mouse hepatocytes and HepG2 cells was mediated in part by activation of JNK [37]. Data from our laboratory supports an important role for JNK in saturated fatty acid-induced apoptosis in the liver [36]. In this study, we examined insulin-mediated protection against saturated fatty acid-induced apoptosis in the rat hepatoma cell line, H4IIE and in primary rat hepatocytes [36]. Cells were provided a control media (no fatty acids) or the same media containing 250 μmol/L of albumin-bound oleate or palmitate for 16 h. Insulin concentrations were 0, 1, 10 or 100 nM. Palmitate, but not oleate, activated caspase-3 and induced DNA fragmentation in the absence of insulin. Insulin reduced palmitate-mediated activation of caspase-3 and DNA fragmentation in a dose-dependent manner. PI3-kinase inhibitors abolished these effects of insulin. Palmitate, but not oleate, increased JNK activity in the absence of insulin. Insulin or SP600125, a chemical inhibitor of JNK, blocked palmitate-mediated activation of JNK and reduced apoptosis. These data not only support a role for JNK in palmitate-mediated apoptosis, but also suggest that insulin is an important determinant of saturated fatty acid-induced apoptosis in liver. Thus, these findings may have implications for fatty acid-mediated liver cell injury in insulin deficient and/or resistant states.

THE ENDOPLASMIC RETICULUM IS A TARGET FOR SATURATED FATTY ACIDS

The endoplasmic reticulum (ER) is one of the largest cellular organelles, its membranes representing as much as one half of the total membranes in a cell [55]. The ER lumen comprises over 10% of the cell volume and is characterized by a unique environment that includes the highest concentration of calcium within the cell and an oxidative environment to support disulfide bond formation [55,56]. An essential function of the ER is the proper assembly of proteins that are ultimately destined for intracellular organelles and the cell surface. The status of protein assembly and folding is monitored and relayed to the cytosol and nucleus by the unfolded protein response (UPR) [56–59]. A variety of stressors, including loss of the luminal oxidizing environment, imbalance in calcium homeostasis, and aberrant N-glycosylation disrupt ER homeostasis and lead to the accumulation of unfolded proteins and protein aggregates in the ER lumen, both of which can be detrimental to cell survival. Disruption of ER homeostasis, collectively termed ER stress, activates the UPR. In mammals, ER stress is sensed and the UPR activated by three ER transmembrane proteins, PERK (RNA-dependent protein kinase-like ER eukaryotic initiation factor-2α kinase), ATF6 (activating transcription factor 6), and IRE1 (inositol-requiring ER-to-nucleus signaling protein 1) (Fig. 3A). PERK activation leads to phosphorylation of the α-subunit of the translation initiation factor eIF2 and subsequent attenuation of translation initiation, and increases the expression and selective translation of activation transcription factor 4 (ATF4). Increased expression of GADD34, a member of the growth arrest and DNA damage family of proteins, is involved in dephosphorylation of eIF2α and, therefore, reversal of translational attenuation. Upon UPR activation, ATF6 is transported to the Golgi where it is cleaved and subsequently migrates to the nucleus, as a 50 kDa fragment, and activates transcription of UPR target genes. Activation of IRE1 promotes the splicing of X-box-binding protein-1 (XBP1) mRNA and subsequent

transcription of molecular chaperones (e.g. GRP78) and genes involved in ER-associated degradation [(e.g., ER mannosidase (EDEM)] [56,60–66]. Thus, activation of the UPR serves to attenuate global protein synthesis and enhance the capacity for protein folding and degradation. Failure of the UPR to re-establish ER homeostasis can lead to programmed cell death [56,67].

Several studies have linked ER dysfunction and the UPR to impairments in glucose homeostasis and diabetes. For example, PERK −/− mice develop diabetes due to a rapid and progressive decline in endocrine and exocrine pancreatic function [68]. Conversely, mice with a homozygous mutation of serine 51 on eIF2α die within 18 h of birth as a result of hypoglycemia and impaired induction of genes involved in hepatic gluconeogenesis [69]. Programmed cell death in response to ER stress is mediated, in part, through transcriptional activation of CCAAT/enhancer binding homologous protein (CHOP) [70,71]. Targeted disruption of the CHOP gene in Akita mice, a mouse line that spontaneously develops hyperglycemia with reduced β-cell mass, delayed the onset of diabetes [72]. Thus, it has been proposed that chronic disruption of ER homeostasis may contribute to the attrition of β-cell function and to impaired regulation of glucose homeostasis in diabetes [73–75].

An elegant study also identified the UPR as a molecular link between obesity and deterioration of insulin action in liver and adipose tissue [76]. However, this study did not examine how obesity led to disruption of ER homeostasis. The ER membrane is characterized by a low concentration of cholesterol and a high concentration of polyunsaturated fatty acids, a lipid environment consistent with a "disordered" membrane [77]. Recent evidence has demonstrated that cholesterol loading activates the UPR and induces apoptosis in macrophages, suggesting that the UPR senses changes to the membrane cholesterol environment [78]. To determine whether the UPR senses changes in the fatty acid environment, we exposed H4IIE hepatoma cells to either saturated (palmitate or stearate) or unsaturated (oleate or linoleate) fatty acids [34–36]. Incubation with palmitate or stearate resulted in a significant increase in the expression of biochemical markers of the UPR (GRP78, ATF4, GADD34, CHOP) and XBP1 splicing at concentrations ranging from 100 to 500 μM [34,35]. Saturated fatty acid-activation of the UPR preceded apoptosis [34,35]. Neither oleate nor linoleate altered any markers of UPR activation, and co-incubation of palmitate with oleate or linoleate reduced palmitate-induced UPR activation [34]. To determine whether saturated fatty-acids compromise ER homeostasis in vivo, we measured several markers of ER stress in the aforementioned study in which male Wistar rats were fed diets enriched with starch (STD), sucrose (HSD), polyunsaturated fat (HPUFA), or saturated fat (HSAT) for 1,4 or 24 weeks (Fig. 2) [33]. Livers and hepatocytes from HSD and HSAT rats, but not STD or HPUFA, were characterized by the presence of spliced XBP-1 mRNA and increased GRP78 and CHOP protein [33]. These results suggest that the UPR may sense and respond to the fatty acid environment and also indicate that the ratio of saturated to unsaturated fatty acids may be an important determinant of hepatic ER homeostasis. Future studies are necessary to determine whether ER stress and activation of the UPR are causally linked to saturated fatty acid-induced apoptosis and liver injury.

It is presently unclear how saturated fatty acids induce ER stress. Saturated fatty acids disrupt ER homeostasis and induce apoptosis in liver cells via mechanisms that do not appear to involve ceramide accumulation [34]. Several studies suggest that saturated fatty acids-induce cytotoxicity and/or disrupt ER homeostasis via selective, structural effects to the ER. For example, in vitro data suggest that palmitoyl CoA can inhibit ER assembly and propagate ER membrane fission [79]. In pancreatic β-cells, Busch et al [80] demonstrated that saturation per se provoked cytotoxicity. In INS1 cells, palmitate was converted in the ER to solid tripalmitin, thus induction of ER stress and apoptosis was attributed to physicochemical properties of these "saturated" triglycerides [81]. In a highly innovative series of experiments, Borradaile et al. [82] demonstrated that palmitate-induced ER stress in CHO cells and H9c2 cardiomyocytes

was associated with the rapid incorporation of palmitate into lipid components of the rough ER followed by disruption of ER structure and function. Thus, it is possible that the trafficking of saturated fatty acids to the ER membrane may be an important determinant of ER homeostasis [38,82]. Further work is necessary to determine whether selective lipid trafficking to the ER is a component of saturated fatty acid-induced ER stress in hepatocytes.

A DILEMMA: SATURATED FATTY ACIDS ARE PROTECTIVE IN ALCOHOL-INDUCED FATTY LIVER DISEASE

Alcoholic fatty liver disease (AFLD) affects nearly 50% of alcohol abusers and is a major cause of illness and death among these individuals [83]. AFLD shares numerous similarities with NAFLD. The natural history of both diseases is characterized by an initial over accumulation of fat in the liver, which progresses in some individuals to steatohepatitis and cirrhosis. Obesity and insulin resistance, the two principal risk factors for NAFLD, appear to also increase the incidence of all stages of AFLD in heavy drinkers [84,85]. Histologically, the two diseases are indistinguishable, and pathologically, the two diseases appear to share at least two mechanistic pathways, oxidative stress and pro-inflammatory cytokines [86,87].

Recent evidence also suggests that AFLD is associated with ER stress. Using a murine model of intragastric ethanol feeding, Ji et al., found that the development of steatosis following 6 weeks of ethanol ingestion was accompanied by increases in several ER stress-related proteins, including GRP78, GRP94, CHOP and caspase 12 [88,89]. The induction of ER stress was mediated, in part, by hyperhomocysteinemia, and was independent of TNF-α. In a subsequent study by the same group, CHOP null mice were protected against ethanol-induced apoptosis despite the development of fatty liver, suggesting a causal role for this transcription factor in alcohol-related cell death [90].

Despite the numerous similarities between AFLD and NAFLD, some notable differences exist. One of the more intriguing relates to the role of fatty acid composition in the development of liver injury. In liver and hepatocytes not exposed to alcohol, saturated fatty acids appear to promote apoptosis and liver injury [33,34,37,91]. In contrast, the opposite appears to be true in AFLD; that is, saturated fatty acids reduce/prevent and unsaturated fats promote alcohol-related liver injury [91–94]. The protective effect of saturated fatty acids was initially observed in an intragastric rat feeding model of alcoholic liver disease, in which ethanol in combination with a liquid diet containing corn oil produced severe liver pathology, whereas equicaloric liquid diets containing either beef tallow or lard produced no or minimal to moderate pathology, respectively [91]. In fact, this study suggested that linoleic acid may be an essential factor in the development of AFLD. Notably, the protective effects of saturated fatty acids in this model of ALFD appear to be associated with a reduction of steatosis via a combination of reduced fatty acid synthesis and increased fatty acid oxidation and lipid export [94].

The mechanism(s) by which saturated fats protect against alcohol-induced liver injury are unclear. A large body of literature supports a role for oxidative stress in AFLD. Cytochrome P450 2E1, which assists in alcohol metabolism during excessive or chronic alcohol consumption, can contribute to oxidative stress via formation of oxygen radicals and lipid peroxidation [95]. Therefore, it is of note that saturated fats reduced alcohol-induced lipid peroxidation and upregulation of CYP2E1 [96]. However, upregulation of CYP2E1 by dietary saturated fat has not been a universal finding [94]. Saturated fats have also been shown to reduce proinflammatory mediators, including TNF-α, cyclooxygenase-2 and NFκB [92,93]. It has also been suggested that dietary saturated fat alleviates ALFD, in part, via upregulation of adiponectin expression and production in adipose tissue [97]. These changes in adiponectin, in turn, may contribute to the enhancement of fatty acid oxidation and thus reduced steatosis.

Collectively, the existing data provide compelling evidence that saturated fatty acids protect against the development and progression of AFLD. The mechanisms by which they elicit these protective effects are unclear, although reductions in the magnitude of steatosis, oxidative stress and inflammatory pathway activation appear to be involved. Since long chain saturated fatty acids promote oxidative stress and activate inflammatory pathways in cells and tissues not exposed to alcohol [29,37,46,98–100], it seems likely that the presence of alcohol alters metabolism of specific fatty acids within tissues. Subsequent studies that directly compare the effect of saturated fatty acids in models of alcoholic- and nonalcoholic fatty liver disease are needed to address these discrepancies.

SUMMARY AND PERSPECTIVE

NAFLD has emerged as a serious and widespread obesity-related disorder. The full spectrum of NAFLD ranges from hepatic fat accumulation in the absence of major histological aberrations to fat accumulation accompanied by fibrosis and necrosis. The two-hit hypothesis postulates that hepatic fat accumulation per se is not injurious, but rather, secondary insults (e.g. ROS, inflammatory cytokines) imposed upon the fatty liver are necessary for progression to steatohepatitis. However, a growing body of literature strongly suggests that hepatic fatty acid composition may impact the degree of liver injury and therefore disease progression. We propose that an increased ratio of saturated-to-unsaturated fatty acids delivered to or stored within the liver may contribute to progression from simple steatosis to NASH. Therefore, within the context of the two-hit hypothesis, saturated fatty acids may represent an intrinsic second hit that hastens the development of NASH.

It is important to emphasize that cellular and murine models of NAFLD are far removed from the free living conditions in which people typically develop the disease. Thus, it is important to determine if the cytotoxic effects of saturated fatty acids observed in animal and cell culture models are relevant to the development of the disease in humans. In this context, it has recently been found in NAFLD patients that a considerable portion of hepatic triglycerides are derived from the diet [101]. Given that saturated fatty acids and simple sugars constitute a significant portion of the American diet [102–105], and that at least some patients with NASH consume more saturated fat and carbohydrate and less unsaturated fats than healthy weight-matched controls [106–108], it is reasonable to speculate that the amount of saturated fat in the liver of NAFLD patients that progress to NASH may be increased. Indeed, the presence of increased saturated fatty acids in serum cholesterol esters has been observed in individuals with type 2 diabetes [109]. In future studies, it will be important to examine the relationship between circulating and intrahepatic fatty acid composition and liver damage in patients with NAFLD.

Acknowledgements

The authors would like to acknowledge the Pagliassotti Lab and support from the NIH, DK072017

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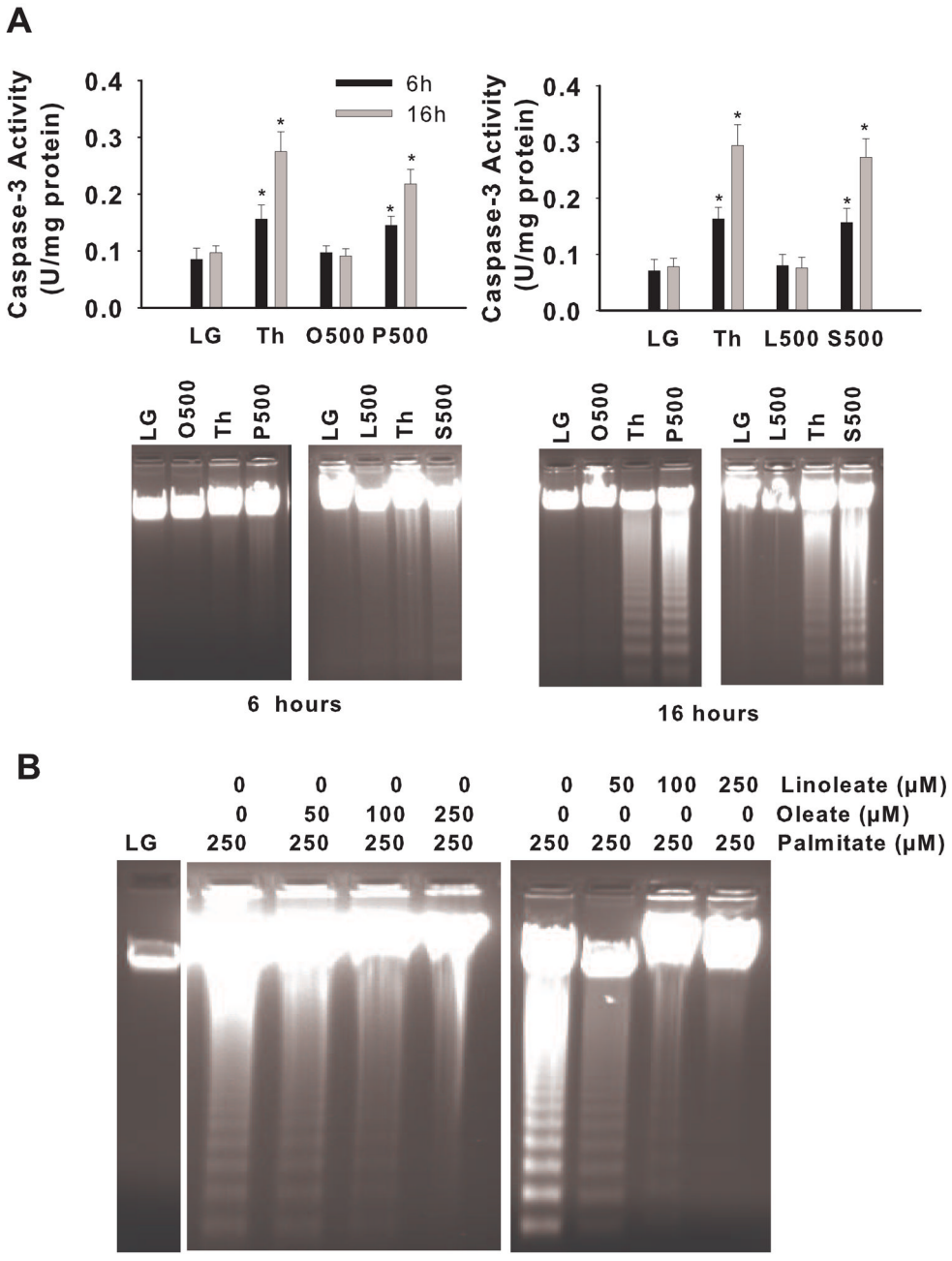

Figure 1.

Caspase activity and DNA fragmentation in H4IIE liver cells. A) Caspase-3 activity and DNA fragmentation were measure in liver cells following 6 or 16 hours of exposure to a control media (LG) or a control media supplemented with thapsigargin (Th, positive control), oleate at 500 μM (O500), palmitate at 500 μM (P500), linoleate at 500 μM (L500), or stearate at 500 μM (S500). B) DNA fragmentation was measured in liver cells following 16 hours of exposure to control media (LG) or control media supplemented with the noted concentrations of fatty acids. *, significantly different from LG and O500 or LG and L500 ($p<0.05$) [34].

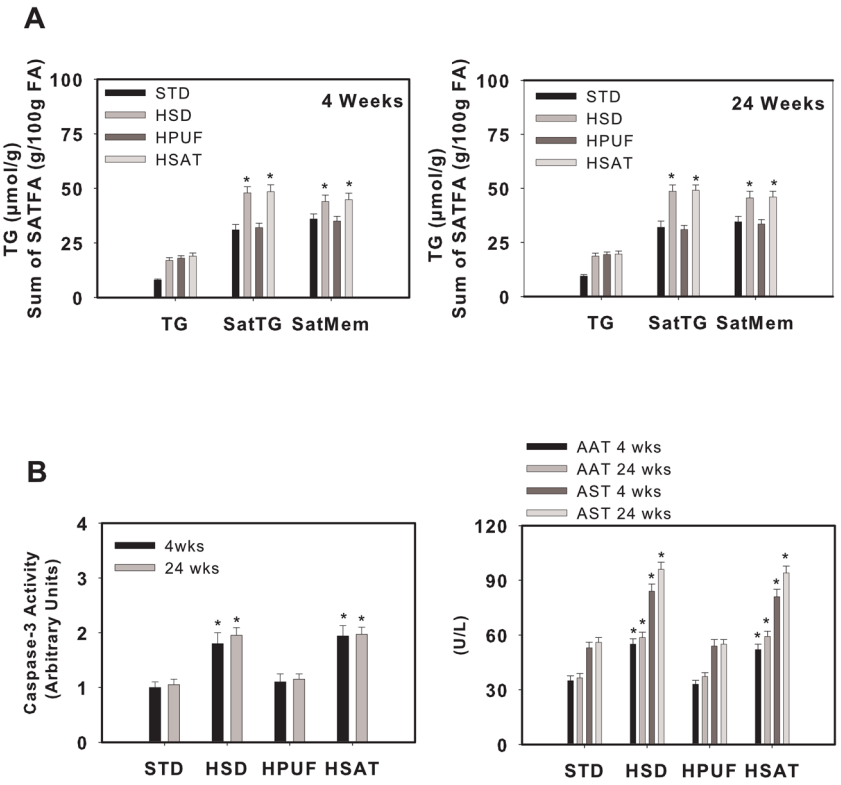

Figure 2.

Liver triglycerides, saturated fatty acid composition, caspase-3 activity and liver enzymes in dietary models of hepatic steatosis. Rats were fed a high starch (STD), high sucrose (HSD), high polyunsaturated fat (HPUF) or high saturated fat (HSAT) diet for 4 or 24 weeks. A) Liver triglyceride (TG) concentration and the sum of saturated fatty acids in triglycerides (SatTG) and microsomal membranes (SatMem). B) Liver caspase-3 activity and plasma concentrations of alanine aminotransferase (AAT) and aspartate aminotransferase (AST). *, significantly different from STD and HPUF ($p<0.05$) [33].

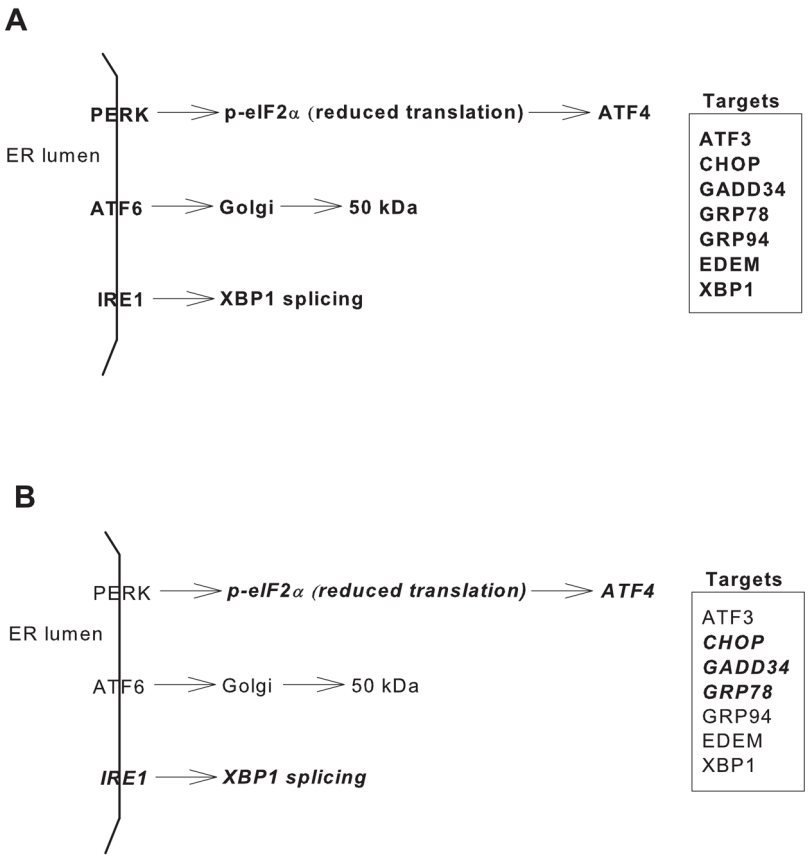

Figure 3.
A) Schematic diagram depicting major components of the unfolded protein response as described in text. B) Schematic diagram depicting (bold and italics) the components of the UPR that are known to be activated in response to long chain saturated fatty acids.

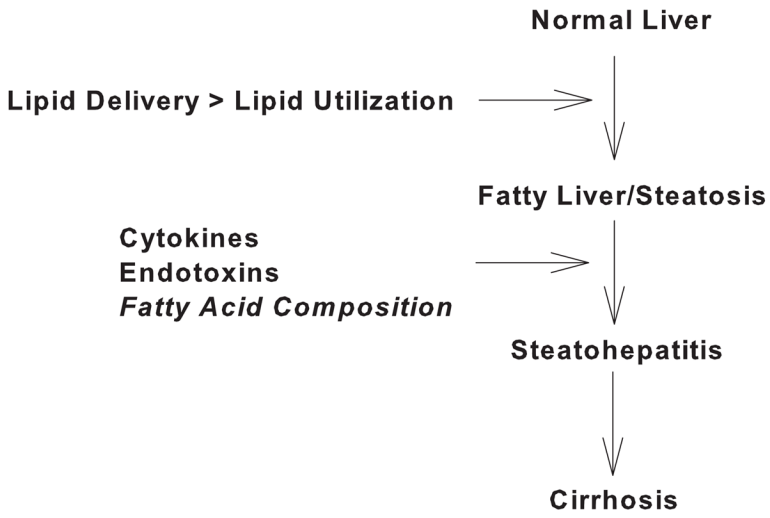

Figure 4.
Schematic diagram depicting disease progression in NAFLD and hypothesized second hits.