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Fatty acids and the Endoplasmic Reticulum in Non-Alcoholic Fatty Liver Disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) represents a burgeoning public health concernin westernized nations. The obesity-related disorder is associated with an increased risk of cardiovascular disease, type 2 diabetes and liver failure. Although the underlying pathogenesis of NAFLD is unclear, increasing evidence suggests that excess saturated fatty acids presented to or stored within the liver may play a role in both the development and progression of the disorder. Aputative mechanism linking saturated fatty acids to NAFLD may been doplasmic reticulum (ER) stress. Specifically, excess saturated fatty acids may induce an ER stress response that, if left unabated, can activate stress signaling pathways, cause hepatocyte cell death, and eventually lead to liver dysfunction. In the current review we discuss the involvement of saturated fatty acids in the pathogenesis of NAFLD with particular emphasis on the role of ER stress.

Keywords

saturated fatty acids; ER stress; liver injury; NAFLD

The Burden of NAFLD

Nonalcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder characterized by hepatic fat accumulation (steatosis) in the absence of excessive alcohol consumption (1–2). The prevalence of NAFLD has nearly doubled since 1980, and current US estimates indicate that NAFLD may affect up to 25% of the general population and 80% of obese and diabetic individuals (3–4). Clinically, NAFLD encompasses a broad spectrum of hepatic derangements ranging from steatosis to nonalcoholic steatohepatitis (NASH), the latter characterized by hepatic fat accumulation coincident with inflammation, reduced liver function, and fibrosis (5–6). Progression to NASH occurs in approximately 10% of NAFLD patients, and 20% of these individuals, in turn, progress to cirrhosis within 10 years (7–9). Individuals with NAFLD are also at an increased risk of cardiovascular disease, type 2 diabetes and overall-and obesity-related mortality (10–12). Therefore, given the increasing prevalence and clinical consequences of NAFLD, a thorough understanding of its underlying pathology is crucial for the development of effective therapeutic strategies.

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Lipids and NAFLD

The initial stage of NAFLD involves accumulation of triglycerides in the liver. Hepatic lipids are derived from three potential sources, 1) the diet, 2) de novo lipogenesis, and 3) circulating fatty acids released from adipose tissue (13). Donnelly et al., recently found that the latter source accounts for approximately 60% of the hepatic triglyceride content in NAFLD patients (14), highlighting the importance of circulating fatty acids in the development of NAFLD.

Accumulating data suggest that, in addition to their role in the development of NAFLD, fatty acids play an important role in the progression from NAFLD to NASH. Free fatty acids are elevated in NASH patients and are positively correlated with disease severity (15–16). Experimental suppression of circulating fatty acids improves hepatic insulin sensitivity and reduces liver enzymes in healthy individuals (17). These data have led to the emerging concept that elevated fatty acids and products of fatty acid metabolism, rather than triglycerides per se, promote hepatotoxicity. Indeed, hepatic triglycerides are higher in patients with benign steatosis compared to those with NASH (18), and esterification of fatty acids into triglycerides prevents fatty acid toxicity in hepatocytes and reduces liver damage in experimental animals (19–21).

Numerous studies have indicated that saturated fatty acids are more deleterious to hepatocytes and liver function than unsaturated fatty acids (22–23,21). Wang et al., reported that a high saturated fat diet fed to experimental animals resulted in significant liver injury, greater susceptibility to endotoxin, and a reduced liver proliferative capacity. In contrast, a high *un*saturated fat diet did not induce liver damage despite similar levels of total hepatic triglyceride accumulation (24). The toxic effects of saturated fatty acids maybe due to their inability, relative to unsaturated fatty acids, to be esterified and incorporated into triglyceride. As such, genetic and pharmacologic manipulations that channel saturated fatty acids towards triglyceride accumulation attenuate liver cell dysfunction and death (19,25,21). 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 the progression to NASH.

The Endoplasmic Reticulum

The mechanisms by which saturated fatty acids contribute to liver injury are not completely understood, although accumulating data implicate disruption of endoplasmic reticulum (ER) homeostasis, or ER stress, as a proximal event. ER stress, in turn, may lead to activation of various intracellular stress pathways that can initiate or exacerbate inflammation and, in some cases, culminate in hepatocyte cell death and liver damage. The ER, one of the largest cellular organelles, is responsible for the proper assembly and posttranslational modification of proteins destined for intracellular organelles and the cell surface(26). When the demand for protein modification exceeds the capacity of the ER to fold or degrade proteins, unfolded proteins accumulate in the ER lumen, causing disruption of ER homeostasis, or ER stress. Because unfolded proteins can impair cell function, the ER has evolved a highly specialized quality control system, the unfolded protein response (UPR), that monitors the status of ER protein assembly and serves to restore ER homeostasis (27). Specifically, the UPR monitors ER protein status via three transmembrane proteins, RNA-dependent protein kinase-like ER eukaryotic initiation factor-2α kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring ER-to-nucleus signaling protein 1 (IRE1). These proteins act as proximal sensors of ER homeostasis(28–30), and when activated, initiate three distinct but inter-related signaling cascades that reduce unfolded proteins by attenuating protein translation, enhancing ER folding capacity, and facilitating protein degradation (figure 1) (31–34).

The diversity of ER stress-mediated UPR signaling likely yields outcomes that are specific to the imposed stress and the needs of the involved cell; however, signaling can be broadly categorized into three successive stages: adaptation, alarm and cell death(28,35). The initial stage, adaptation, involves transcriptional and translational modifications aimed at clearing unfolded proteins from the ER lumen. These adaptive responses likely occur under normal physiological conditions, especially in cells such as hepatocytes where high secretory rates demand elevated rates of protein folding. The second stage, alarm, involves induction of cellular stress pathways that inform the cell and surrounding tissues that homeostasis is compromised and cellular function is jeopardized. These signaling cascades, which are commonly associated with innate immunity and host defense, represent a compensatory response aimed at protecting the cell, but can themselves compromise cellular integrity if chronically activated (36). The final stage, cell death, occurs when the initial insult is sufficiently excessive or chronic that the adaptation and alarm responses are incapable of mitigating ER stress and restoring ER homeostasis (37). A detailed discussion of these categories and the proposed mechanisms that mediate the transition from adaptation to cell death is beyond the scope of this review. The reader is referred to several excellent reviews pertaining to these issues (35,38,36).

Recent evidence suggests that, in addition to promoting the progression of NAFLD, saturated fatty acids can cause ER stress and activate the UPR in numerous cell types. This activation, and in particular the signaling pathways initiated during the alarm and cell death stages, may in turn mediate the toxic effects of saturated fatty acids. In the remainder of this review, we will describe the association between excess saturated fatty acids and ER stress, particularly as it pertains to NAFLD, and discuss the potential mechanisms by which ER stress mediates the development and progression of saturated fatty acid-induced liver damage.

Saturated Fatty Acids and ER Stress

In two seminal publications, Ozcan et al., reported that dietary and genetic models of obesity display markers of ER stress in adipose tissue and liver, and administration of chemical chaperones that improve ER folding capacity abrogate systemic insulin resistance, hepatic lipid accumulation, and markers of liver injury (39–40). Subsequent experiments incorporating genetic manipulations of the UPR have supported the concept that ER stress contributes to the pathogenesis of NAFLD (41–44). Further, clinical studies have indicated that patients suffering from metabolic disorders, including NAFLD, display markers of ER stress in the liver and other tissues (45–48). Collectively, these data indicate that certain feature(s) of an obese/over nutrition environment are sufficient to disrupt ER homeostasis, and increasing evidence suggests that the accumulation of saturated fatty acids may be one feature of the obese environment that causes this disruption. The ensuing ER stress, if left unabated, may in turn mediate saturated fatty acid-induced dysfunction in metabolic tissues such as the liver. In support of this concept, high saturated fat diets, but not high unsaturated fat diets, induce hepatic ER stress and liver damage in male Wistar rats; importantly, evidence of ER stress precedes the development of liver dysfunction in these animals(49). Numerous *in vitro* studies have found that saturated fatty acid-induced liver cell dysfunction and death are accompanied by increased ER stress, and can be mitigated by co-incubation with chemical chaperones that enhance ER folding capacity (23,50,25,51–54).

These data beget the clinically relevant question of *how* saturated fatty acids induce ER stress. Several lines of evidence suggest that this induction may occur via selective, structural effects to the ER. As mentioned above, compared to *un*saturated fatty acids, saturated fatty acids are less readily converted into triglycerides, and are thus left free to travel to the ER where they may disrupt ER morphology and function. *In vitro* data suggest

that palmitoyl CoA can inhibit ER assembly and propagate ER membrane fission (55). Moffitt et al., reported that palmitate-mediated cell dysfunction and death in INS-1 cells was caused by conversion of palmitate to tripalmitin, an insoluble triglyceride formed and retained in the ER that ultimately disrupts ER architecture (56). Through a series of experiments, Schaffer and colleagues 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 ER followed by disruption of ER structure and function(19,57). Incorporation of saturated fatty acids into the ER may also disrupt ER folding capacity and chaperone function by altering ER calcium homeostasis (58–60,52). Thus, selective trafficking of saturated fatty acids to the ER membrane may be an important determinant of ER homeostasis and may ultimately mediate the toxic effects of these lipids.

Saturated Fatty Acids and the UPR-Mediated Alarm Response

As described above, the initial stage of the UPR, adaptation, is likely a common physiological response to increased folding demands in the ER that does not necessarily result in cell damage. If the steps taken during the adaptation stage do not restore ER homeostasis, the UPR has the capacity to activate stress signaling pathways (figure 2). C-Jun NH_2 terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family of proteins, has emerged as a critical mediator of cellular stress responses and has been implicated in the pathogenesis of various metabolic disorders, including NAFLD (61–62,39). JNK activation is present in human NAFLD and correlates with disease severity, whereas JNK inhibition protects experimental animals from NASH (63,46–47,64–65). The mechanisms by which JNK may potentiate liver damage include regulation of inflammatory genes, disruption of hepatic insulin signaling and induction of hepatocyte apoptosis (66,61,22).

A link between ER stress and JNK was first provided by Srivastava et al., who demonstrated that JNK was activated by thapsigargin, a chemical that disrupts ER homeostasis via inhibition of the endoplasmic reticulum-associated calcium ATPase (67). Urano et al., found that ER stress-mediated JNK activation is specific to the proximal UPR sensor IRE1α, which, upon its phosphorylation following ER stress, activates JNK by complexing with the adaptor protein TRAF2 (68). Collectively, these data support a sequence of events whereby elevated fatty acids, in particular saturated fatty acids, induce a protracted ER stress response in hepatocytes that leads to JNK activation and subsequent inflammation, cell injury and death. Indeed, saturated fatty acids, but not unsaturated fatty acids, activate JNK in hepatocytes, and inhibition of JNK signaling prevents saturated fatty acid-induced hepatocyte injury (22,50,69–70,53).

NFκB is a critical transcriptional regulator of inflammation and may also play a role in the development of NASH. Under normal physiological conditions, NFκB remains inactive through binding to IκB, and signal-induced phosphorylation and degradation of IκB allow for the activation and nuclear translocation of NFκB. Hepatic levels of NFκB are increased in NASH patients and correlate with disease severity (71–72). These data are consistent with most (73–74), but not all studies (75) that have reported deleterious effects of NFκB on liver function. Saturated fatty acids activate NFκB in various cell types, including liver cells, and this activation may play a role in saturated fatty acid-induced liver cell inflammation/toxicity (76–77). Disruption of ER homeostasis also activates NFκB (78–80), and all three proximal sensors of the UPR-ATF6, IRE1a, and PERK-appear capable of mediating this activation (figure 2) (81–82). The mechanisms of ATF6 activation of NFκB are unclear, whereas IRE1α-mediated activation occurs, much like JNK, by an IRE1α-TRAF2 complex. PERK-mediated activation occurs via an eiF2α-mediated reduction in general translation, which leads to an increase in the NFκB-IκB ratio and therefore frees NFκB to translocate to

the nucleus (83–84). In support of a role for NFκB in ER stress-induced cell dysfunction, inhibition of NFκB prevents thapsigargin-mediated apoptosis in INS1E cells (85).

NFκB-mediated liver damage occurs largely by transcriptional up-regulation of proinflammatory cytokines, such as IL-6 and TNFα, which have been implicated in hepatocyte apoptosis and liver fibrosis (73,86–88). TNFα, in particular, may play an important role in the development and progression of NAFLD. Patients suffering from NAFLD display increased circulating and hepatic levels of TNFα (89–92) and certain TNFα polymorphisms are associated with disease prevalence and severity (93–94). Inhibition of TNFα protects animals from dietary-and genetically-induced NASH (95–96), and early clinical studies utilizing TNFα inhibitors have yielded promising results in patients with NAFLD (97–98). Although TNFα is increased by fatty acids in hepatocytes and has been linked to ER stress (99), future studies are needed to determine whether TNFα mediates saturated fatty acid-induced ER stress and liver injury.

CREBh is a recently identified, liver-specific, endoplasmic reticulum (ER)-localized transcription factor (100). Genetically altered mice lacking CREBh have scarcely detectable levels of the acute phase reactant, C-reactive protein, in the basal state and following stimulation with the ER stress inducer tunicamycin, suggesting that CREBh is required for initiation of the acute phase response and systemic inflammation subsequent to ER stress (101–102). More recent data have demonstrated that fatty acids up-regulate CREBh expression *in vitro* and *in vivo*, but interestingly, both saturated and unsaturated fatty acids appear capable of mediating this up-regulation (103–104). Thus, CREBh may represent a link between excess fatty acids and inflammation, although more studies are needed to address how different fatty acids regulate CREBh, as well as the effects of CREBh activation on liver function.

Oxidative stress has been linked to the development and progression of NAFLD and to saturated fat-induced cell damage (105–109). Recent evidence suggests that the ER may be a potent source of reactive oxygen species (ROS) production. Each disulfide bond formed during oxidative protein folding in the ER produces a single ROS, and it has been estimated that this process accounts for ~25% of all ROS generated in a cell (110–113). Malhotra et al., using cells that were engineered for transcriptional induction of wild-type human FVIII, demonstrated that FVIII misfolds in the ER lumen, activates the UPR, causes oxidative stress and induces apoptosis. All of these responses to FVIII misfolding were reduced by antioxidant treatment (114).

UPR-mediated ROS generation is at least partly due to activation of CCAAT/enhancer-binding protein homologous protein (Chop), also known as growth arrest-and DNA damage-inducible gene 153 (GADD153) (115). *Chop* expression is regulated by the ATF6 and PERK arms of the UPR, the latter via activation of the transcription factor ATF4 (116–117). As described in the following section, Chop is best known as an important mediator of ER stress-induced cell death, but Song et al., recently found that Chop deletion reduces oxidative damage in mouse models of diabetes, suggesting that Chop activation may enhance oxidative stress.

To manage the endogenous creation of ROS, the UPR is linked to the regulation of antioxidant defense systems via numerous pathways (figure 2). Most notably, the antioxidant transcription factor Nrf2 is a substrate of the proximal UPR sensor PERK, and PERK-mediated activation of Nrf2 maintains redox homeostasis and prevents cell death following ER stress (118–119). Interestingly, Nrf2 is highly expressed in the liver, and its deletion results in rapid onset and progression of steatohepatitis in mice provided a methionine-choline deficient diet (120). The PERK pathway may also provide protection

from ROS via the downstream effector, eiF2α. Mice lacking the ability to phosphorylate eiF2α are characterized by a severe diabetic phenotype that can be attenuated by a high antioxidant diet, suggesting a role for eiF2α phosphorylation in preventing oxidative stress (121). The UPR protein XBP1 may also protect cells from oxidative damage. Liu et al., reported that mouse embryonic fibroblasts deficient in XBP1 were more prone to cell death and less able to activate antioxidant defenses following exposure to hydrogen peroxide (122). Future studies are necessary to determine if ROS play a role in ER stress and liver injury secondary to saturated fatty acids, although substantial evidence indicates that direct links exist among ER stress, ROS, and NAFLD.

Several points regarding our overview of the alarm stage of the UPR warrant consideration. First, the signaling molecules/pathways discussed above do not represent an exhaustive list of those that may mediate liver injury secondary to saturated fatty acids and/or ER stress. Second, the signaling molecules/pathways discussed above are not mutually exclusive, but may interact with one another to facilitate and exacerbate liver injury, and it is probable that some of these deleterious effects are independent of ER stress. Third, the alarm and cell death stages of the UPR are not entirely dichotomous, and it is likely that several of the mediators of the alarm stage also play an important role in hepatocyte cell death secondary to saturated fatty acids and ER stress. An important issue for future studies is to identify the conditions under which the UPR transitions from a survival response to a cell death response. Finally, the relation between ER stress and many of the molecules/pathways described above appears to bebi-directional, which raises the intriguing possibility that saturated fatty acids initiate a self-perpetuating cycle whereby ER stress activates intracellular stress pathways, which further exacerbate ER stress, with NASH developing as collateral damage.

Saturated Fatty Acids and the UPR-Mediated Cell Death Response

If ER stress is particularly severe or protracted, and the UPR cannot re-establish homeostasis, downstream signaling molecules can initiate apoptotic cell death. ER stress-related apoptosis can occurvia the intrinsic pathway, whereby mitrochondrial outer membrane permeabilization (MOMP)leads to cytochrome c release from the mitochondria and subsequent activation of initiator and effector caspases (123–124). The importance of this pathway in the pathogenesis of NAFLD is supported by data demonstrating that elevated caspase activity is a prominent feature of NAFLD and correlates with disease severity (125–126). Furthermore, inhibition of caspase activity via the general caspase inhibitor, FMK (Z-Val-Ala-Asp-fluoromethylketone), reduces apoptosis and fibrosis in an animal model of NASH and prevents saturated fatty acid-induced apoptosis in hepatocytes (22–23,127). Thus, hepatocyte apoptosis secondary to prolonged ER stress may be an important mechanism by which excess saturated fatty acids play a role in liver injury and the development of NASH.

As mentioned in the previous section, the transition of the UPR from a cell survival pathway to anapoptotic pathway is, at least in part, mediated by the same effectors that are activated in the alarm stage. For example, JNK has been identified as an important mediator of ER stress-related apoptosis, and pharmacologic or genetic inhibition of JNK attenuates saturated fatty acid-induced ER stress and cell death in liver cells (22,50). Chop, an ER resident transcription factor that lies downstream of the transmembrane proteinsATF6 and PERK, is perhaps the most well characterized mediator of ER stress-induced cell death. Silencing Chop reduces hepatocyte apoptosis in alcohol-induced liver disease, and attenuates indices of liver injury in some, but not all models of liver disease (128–130,54). Furthermore, Chop appears to mediate liver cell death induced by higher (i.e. 400–800 μM), but not lower (i.e. 100–300 μM) saturated fatty acid concentrations (131–132,54), suggesting that

Chopactivation reflects the presence of ER stress and UPR activation, and may play a role in, but is not required for, saturated fatty acid-induced death in liver cells.

The ER lumen is a major site of calcium storage, and calcium homeostasis is critical in maintaining both ER folding capacity and cell viability (133–135). As such, disruption of ER calcium homeostasis, for example via inhibition of the sarco/endoplasmic reticulum ATPase (SERCA) uptake pump, reduces the folding capacity of the ER, induces ER stress, and is an important mediator of ER-associated apoptosis (136–137). Recent data also indicate that disruption in ER calcium stores may be an important mechanism by which saturated fatty acids induce cell death in H4IIE liver cells and primary rat hepatocytes (52), although future studies are needed to determine whether disruption of intracellular calcium homeostasis secondary to excess saturated fatty acids plays a direct role in the development and progression of NAFLD.

The aforementioned pathways appear to all converge on the Bcl-2 family of proteins, which in turn propagates the apoptotic signal and acts at the mitochondria to induce MOMP and cytochrome c release (138–140,141). The Bcl-2 protein family consists of approximately 20 members that can be broadly divided into three categories: multi-domain anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL); multi-domain pro-apoptotic proteins (e.g. Bax and Bak); BH-3 domain pro-apoptotic proteins (e.g. Bim, Bid, Bad, Puma). Collectively, these proteins monitor incoming stress signals and help determine cell fate by altering the balance between pro-and anti-apoptotic family members. Several lines of evidence suggest tht Bcl-2 proteins may play a direct role in saturated fatty acid-induced hepatocyte cell death and the progression of NAFLD. First, the expression of Bcl-2 proteins is altered towards a pro-apoptotic profile in patients with NAFLD and NASH (142,69). Second, saturated fatty acid-mediated hepatocyte cell death is accompanied by increases in pro-apoptotic Bcl-2 family members (e.g. Bax) and decreases in anti-apoptotic members, such as Bcl-2 and BclxL (22,143,69,53). Third, alterations in Bcl-2 proteins occur downstream of Chop and JNK, and inhibition of the BH-3 domain proteins Bim or Puma prevents saturated fatty acid-mediated hepatocyte cell death (22,69,53).

Although best known for their actions on mitochondria, recent data suggest that Bcl-2 proteins may localize at the ER and directly modulate ER function and the UPR (144–148). For example, Hetz et al., reported that Bax and Bak function at the ER membrane to regulate IRE1α activation, and Bax and Bak double knockout mice demonstrate deficient IREα signaling (146). ER localization of Bcl-2 proteins may also affect ER function and promote apoptosis by modulating ER calcium stores (149–151,140). Collectively, these data strongly suggest that Bcl-2 proteins may mediate saturated fatty acid-induced apoptosis by acting at both mitochondria and the ER.

Conclusion

NAFLD is a progressive disorder that can lead to impaired liver function and ultimately liver failure. An accumulation of saturated fatty acids in the liver may play an important role in the pathogenesis of NAFLD, and a growing body of literature suggests that ER stress may mediate the toxic effects of saturated fatty acids. Chronic ER stress induces numerous intracellular pathways that, if left unabated, can lead to systemic inflammation, hepatic fibrosis and hepatocyte cell death. Future studies are necessary to determine the precise mechanisms by which saturated fatty acids cause ER stress, and the proximal pathways that mediate ER stress-induced liver damage and hepatocyte cell death. Such studies could identify novel therapeutic strategies for the prevention and treatment of NAFLD.

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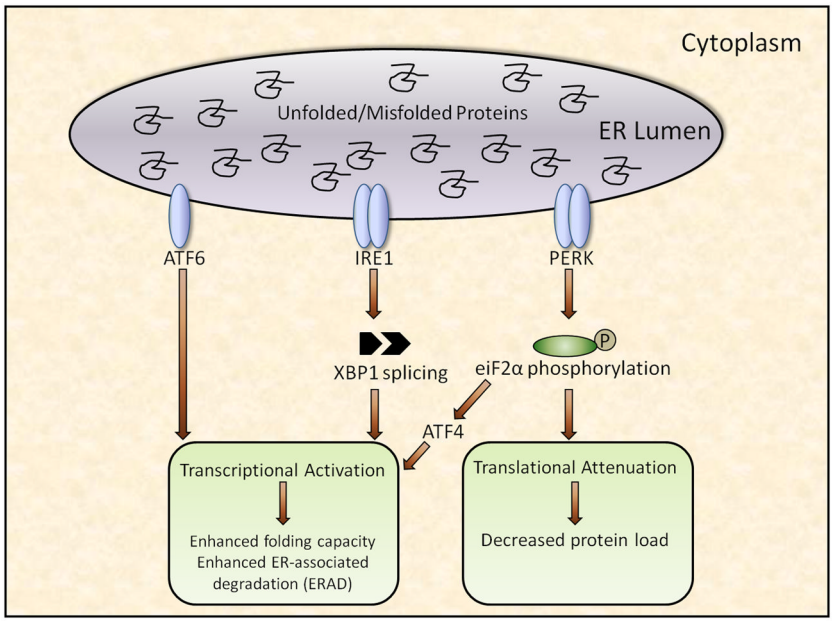

Figure 1.

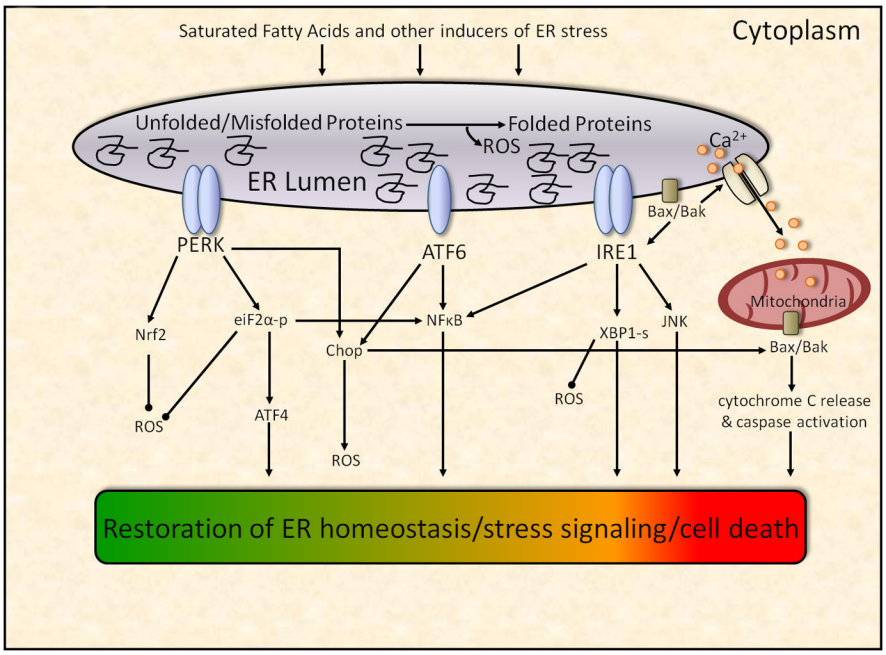

Figure 2.