HHS Public Access

Author manuscript

J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. Author manuscript; available in PMC 2017 December 19.

Published in final edited form as:

J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2014 ; 32(1): 83–104. doi: 10.1080/10590501.2014.881648.

Endoplasmic Reticulum Stress in Drug- and Environmental Toxicant-Induced Liver Toxicity

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Abstract

Liver injury resulting from exposure to drugs and environmental chemicals is a major health problem. Endoplasmic reticulum stress (ER stress) is considered to be an important factor in a wide range of diseases, such as cancer, neurological and cardiovascular disease, diabetes, and inflammatory diseases. The role of ER stress in drug-induced and environmental toxicant-induced liver toxicity has been underestimated in the past; emerging evidence indicates that ER stress makes a substantial contribution to the pathogenesis of drug-induced liver toxicity. In this review, we summarize current knowledge on drugs and environmental toxicants that trigger ER stress in liver and on the underlying molecular mechanisms. We also discuss experimental approaches for ER stress studies.

Keywords

Endoplasmic reticulum (ER) stress; liver toxicity; signaling pathways

INTRODUCTION

Drug-induced liver injury, one of the leading causes of drug failure and withdrawal from the market after approval, is a serious concern for public health as well as for drug development and approval [1–3]. Multiple factors and mechanisms are involved in drug-induced liver toxicity. For example, drug metabolism via liver metabolizing enzymes generates reactive electrophilic metabolites and free radicals. These toxic metabolites can covalently bind to proteins, lipids, and DNA and cause such adverse consequences as oxidative stress, glutathione depletion, lipid peroxidation, and cell death. Alteration of the immune response has often been described as an important mechanism of liver toxicity. Gunawan and Kaplowitz have summarized the mechanisms of drug-induced liver toxicity [4]. More recently, endoplasmic reticulum (ER) stress has begun to be recognized and is often

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associated with liver diseases including, but not restricted to, hepatitis, alcoholic liver disease, fatty liver disease, and drug-induced liver toxicity.

The ER is a critical organelle that plays a vital role in many cellular processes including protein synthesis, folding, assembly, trafficking, postmodulation, quality control of both secretory and membrane proteins, lipid synthesis, and regulation of intracellular calcium hemostasis. Disturbances of ER function by stimuli such as altered cellular redox status, oxidative stress, unbalanced calcium homeostasis, hypoxia, or energy deprivation lead to the accumulation of unfolded proteins in the ER, which is generally termed ER stress [5–9]. To re-establish ER function and restore homeostasis, a specific signaling pathway, the unfolded protein response (UPR), is activated as an adaptive mechanism. This adaptive mechanism is a transcriptional program to up-regulate the expression of genes involved in protein folding and quality control [9,10]. Excessive and prolonged ER stress causes apoptosis and necrotic cell death. Three key molecules that are present in the ER membrane—PERK (Protein Kinase R/PKR-like ER kinase), IRE1α (inositol-requiring enzyme 1α), and ATF6 (activation of transcription factor 6)—are ER stress sensors. When cells are under ER stress, glucose-regulated protein 78 (Grp78) dissociates from these three transmembrane ER signaling proteins, thereby leading to the activation of three major UPR branches (Figure 1). These three UPR transducers are activated by translocation of ATF6 to the Golgi and phosphorylation of PERK and IRE1α. (A) ATF6 is translocated to the Golgi and then cleaved by the Golgi resident proteases. The cleaved form of ATF6 is released to the cytosol and travels to the nucleus to regulate gene expression. (B) Phosphorylation of PERK leads to a reduction of the protein load on the ER by decreasing protein synthesis mediated by eIF2α phosphorylation. The expression of transcription factor 4 (ATF4), a member of the basic leucine-zipper (bZIP) family of transcription factors that regulate the promoters of numerous UPR genes, is induced. (C) Phosphorylation of IRE1α initiates an intron excision from the mRNA for a transcription factor, XBP1 (X-box binding protein 1), to produce a spliced form XBP1S. XBP1S binds to promoters of several genes, resulting in an increased expression of genes involved in restoring protein folding or degrading unfolded proteins [10,11]. These signaling pathways are referred to as pro-survival unfolded protein responses [9]. Excessive and prolonged ER stress stimulates death signals, which provoke apoptosis and necrotic cell death. For instance, pro-apoptotic protein C/EBP CCAAT/enhancer binding protein homologous protein (CHOP) is induced by ATF4 activation via the PERK-eIF2α pathway. ER resident caspase 12 (human form: caspase 4) is activated and becomes involved in ER-stress associated apoptosis [12].

ER stress has been reported to be associated with a wide range of human diseases [13–15]; these include, but are not restricted to, cancer, neurological, kidney, lung, and cardiovascular disease, diabetes, and inflammatory diseases. A large number of ER stress review articles focusing on cancer [16–24]; Alzheimer disease [25–29]; Parkinson [30,31], kidney [32–34], lung [35–37], cardiovascular [38–42], and inflammatory diseases [43–47]; obesity [48–50]; gastrointestinal disease [51]; and diabetes [50,52–60] have recently been published. In addition, relationships between ER stress and environmental toxicants have also been summarized [61,62]. In this article we reviewed literature on drugs and some environmental

toxicants in which the underlying mechanisms may be ER stress. We also summarize research approaches in ER stress studies.

ER STRESS AND DRUG-INDUCED LIVER TOXICITY

Acetaminophen

Acetaminophen overdose is the leading cause of drug-induced liver failure in the United State [1,63]. The liver toxicity of acetaminophen is known to be initiated by N-acetylbenzoquinoneimine, an active metabolite produced by the cytochrome P450 [64]. The toxicity of acetaminophen and its mechanisms have been extensively studied for decades because of its complexity, as it involves multiple signaling pathways and also resembles many aspects of drug-induced liver injury; however, the exact mode of cell death is not completely understood [65]. Recent studies have demonstrated a role of ER stress in acetaminophen-induced liver toxicity [65–68]. A sublethal dose of acetaminophen results in activation of ER stress markers include ATF6 and CHOP. In addition, caspase-12, an ER-resident caspase, is transiently activated [68]. It has been suggested that the mode of death for acetaminophen's liver toxicity is caspase-independent apoptosis, initiated by activation of polymerase 1 (PARP-1) [67,68], which can be attenuated by the PARP-1 inhibitor BGP-15 (O-[3-piperidino-2-hydroxy-1-propyl]-nicotinic amidoxime). Interestingly, BGP-15 did not protect against the ER-stress related early events of redox stress, such as glutathione depletion, and BGP-15 did not counteract any ER stress signaling events. Thus, the protection against acetaminophen-induced apoptosis by BGP-15 is not likely to be due to inhibition of ER stress pathways. The authors suggested that caspase-independent apoptosis is initiated by the stressed ER and nucleus [67].

A recent study assessed the roles of ER stress in acetaminophen-induced liver toxicity using CHOP knock-out mice [65]. ER stress pathways were activated when toxic doses were given orally to the wild-type mice. While all three branches of UPR were activated, PERK-elF2-CHOP cascade seemed to be the most important signaling pathway. While wild-type mice developed extensive liver necrosis, CHOP knock-out mice were partially protected from liver toxicity, showed increased survival, and hepatocyte proliferation at the sites of necrosis, indicating that CHOP plays a critical role in acetaminophen's toxicity and that up-regulation of CHOP compromises hepatocyte survival by reducing regeneration [65]. Unlike previous reports demonstrating that ER stress is an early event of apoptotic cell death and glutathione depletion [67,68], this study reported that ER stress occurred after a cascade of events such as glutathione depletion and oxidative stress [65]. The discrepancy is not clear; it could be due to different routes of administration (i.p. injection [67] vs. oral administration [65]). Further studies are warranted to explain the differences.

Anti-HIV Drugs

Liver toxicity represents one of the most common complications of immunodeficiency virus (HIV) treatments. Side effects have been reported to be associated with different classes of anti-HIV drugs, including nucleoside reverse transcriptase inhibitors, non-nucleoside analogue reverse transcriptase inhibitors, and HIV protease inhibitors [69].

Efavirenz—Efavirenz is a non-nucleoside analogue reverse transcriptase inhibitor. Although generally considered safe, some evidence indicates that efavirenz disrupts lipid metabolism [70] and induces liver fibrosis [69,71]. An in vitro study demonstrated that a battery of ER stress markers was induced when primary human hepatocytes and Hep3B cells were exposed to clinically relevant concentrations of efavirenz [72,73]. The ER stress responses included induction of CHOP and GRP78, phosphorylation of eIF2a, and the production of XBP1 isoform XBP1S. Morphological changes, such as dilation of ER membranes, were also observed. Because mitochondrial dysfunction is one the mechanisms responsible for efavirenz-induced liver damage [74,75], the interplay between ER stress and mitochondrial dysfunction was investigated in Rho^o cells, liver cells with nonfunctional mitochondria [76]. Efavirenz-treated Rho^o cells showed no appreciable increase in ER stress signals, such as the induction of CHOP and GRP78, compared to wild-type cells, demonstrating that mitochondria are involved in the efavirenz-induced ER stress response.

Lopinavir, ritonavir, saquinavir, nelfinavir, atazanavir, and amprenavir

HIV protease inhibitors: It has been documented that numerous HIV pro-tease inhibitors induced ER stress in both liver cells and in the livers of animals [77–79]. In an in vitro model, five HIV protease inhibitors (lopinavir, ritonavir, saquinavir, nelfinavir, and atazanavir) were studied for their ER stress-inducing abilities at clinically relevant concentrations [78]. The induction of CHOP, ATF4, ATF3, and some ER chaperones indicated that these HIV protease inhibitors caused ER stress in HepG2 cells, the ER stress response was attributed their proteasome inhibiting functions.

The effect of three HIV protease inhibitors, amprenavir, atazanavir, and ritonavir, on ER stress was studied and compared in primary rodent hepatocytes. Lipid metabolism was also investigated in the same study because lipid disturbances have been reported to be associated with the use of HIV protease inhibitors [79]. Both atazanavir- and ritonavir-induced ER stress and apoptosis, and increased sterol regulatory element-binding proteins (SREBP) levels, while amprenavir, at the same concentrations, had no effect.

Kou and colleagues investigated lopinavir- and ritonavir-induced ER stress and also examined the synergistic effect of alcohol-induced liver lipid accumulation and ER stress response in mice, primary human hepatocytes, and primary mouse hepatocytes [77]. Both lopinavir- and ritonavir-induced ER stress response in mice, but did not show a significant increase in serum alanine aminotransferase (ALT) levels. Co-administration of these HIV protease inhibitors with alcohol caused an increase in ALT and a synergistic increase in lipid accumulation and ER stress response. In addition, co-administration of alcohol and lopinavir or ritonavir reduced intracellular calcium levels and also potentiated cell death. These data suggest that HIV protease inhibitors potentiate alcohol-induced ER stress and worsen liver functions [77]. The underlying mechanisms that contribute to the liver toxicity of HIV protease inhibitors are not, at present, well understood. The studies described previously pinpointed that at therapeutic concentrations, HIV protease inhibitors induce ER stress response as major ER stress markers had been altered (activation of SREBPs, ATF4, CHOP,

caspase-12, and splicing of XBP1), implying that ER stress maybe a new mechanism for the liver toxicities of HIV protease inhibitors.

Anti-type 2 Diabetes Drugs

Agonists of peroxisome proliferator-activated receptor γ (PPARγ) are oral drugs designed to treat type 2 (insulin-resistant) diabetes. Ciglitazone, the first PPARγ agonist to enter clinical trials, was not marketed because of liver toxicity. Troglitazone, the first PPARγ agonist approved by the US Food and Drug Administration (FDA), was withdrawn from the market after cases of severe liver failure and death were reported [80,81]. The mechanisms involved in the liver toxicity include mitochondrial dysfunction, apoptosis, disruption of calcium hemostasis, oxidative stress, and ER stress [82–89]. Studies using GN4 rat liver epithelial cells demonstrated that both troglitazone and ciglitazone activated the calcium-dependent p38 mitogen-activated protein kinase (MAPK) pathway and also induced ER stress [88,90]. ER stress induction by troglitazone and ciglitazone was indicated by the activation of the two key molecules (PERK and eIF2α) in the PERK branch [88]. A link between p38/MAPK activation and induction ER stress was also demonstrated. Treatment with KN-93, an inhibitor of calcium/calmodulin-dependent kinase II (CaMKII), which is an upstream regulator of p38, attenuated the activation of both MAPK (MKK3/6) and p38 and the phosphorylation of eIF2α. In addition, the activation of MAPK had shown to correlate with the phosphorylation of PERK and eIF2α. In contrast, the two other glitazones (rosiglitazone and pioglitazone), which have lower liver toxicities, failed to induce either MAPK or eIF2α, suggesting that induction of ER stress and the activation of MAPK signaling pathways may play critical roles in the effects of troglitazone or ciglitazone on cell growth and maturation [88].

Anti-depression Drugs

Sertraline is used for the treatment of depression, and also for the treatment of panic, obsessive-compulsive, and post-traumatic stress disorders. Although it is generally considered safe, acute liver failure due to sertraline use has been documented [91–99]. Sertraline was demonstrated to be toxic in primary rat hepatocytes and human HepG2 cells; mitochondrial dysfunction and apoptosis are the underlying mechanisms [100,101]. Besides mitochondrial dysfunction and apoptosis, ER stress was found to contribute to sertraline's liver toxicity (SC and LG, unpublished data). The expression of typical ER stress markers, such as PERK, IRE1α, and CHOP, was significantly increased in sertraline-treated hepatic cells. The XPB1S isoform was also observed. The ER stress response was also measured quantitatively with two reporter assays, namely, *Gaussia* luciferase (Gluc) and secreted alkaline phosphatase (SEAP). Sertraline significantly decreased the activities of both *Gaussia* luciferase and SEAP. In addition, the interplay between the apoptosis and ER stress was studied. ER stress was an early response triggering apoptosis because ER stress occurred at earlier time points and lower concentrations compared to those for apoptosis. In addition, administration of a potent ER stress inhibitor, 4-phenylbutyrate (4-PBA) attenuated sertraline-caused apoptosis. Moreover, sertraline treatment increased the expression of tumor necrosis factor and the phosphorylation of JNK, ERK1/2, and p38, which are the major players in the MAPK signaling pathway; silencing MAP4K4 (the upstream kinase of JNK)

attenuated both ER stress and apoptosis; and a JNK-specific inhibitor efficiently prevented the decreased activity of secreted Gluc, indicating that MAPK signaling pathway signaling is involved in both apoptosis and ER stress [SC and LG, unpublished data]. Taken together, these studies demonstrate that the mechanism of sertraline-induced liver toxicity is a complex with crosstalk between mitochondrial dysfunction, apoptosis, and ER stress, with the involvement the MAPK signaling pathway.

ER STRESS AND ALCOHOL-INDUCED LIVER TOXICITY

Excessive alcohol consumption leads to liver damage since alcohol is a direct hepatotoxin; it also potentiates other liver diseases, including chronic hepatitis and nonalcoholic fatty liver disease [102]. The mechanisms of alcohol-induced liver damage are complex and multifactorial. DNA adducts, protein adducts, glutathione depletion, lipid peroxidation, apoptosis, mitochondrial damage, inflammation, and cell-cycle arrest have been shown to be involved in alcohol-associated liver toxicity [102,103]. Alcohol-induced liver toxicity is metabolism-mediated; the reactive metabolite, acetaldehyde, created via alcohol dehydrogenase, aldehyde dehydrogenase, and CYP2E1, is a major contributor of the alcohol's toxicity. ER stress has been reported to participate in alcohol-caused liver toxicity in both animal and cell models [104–107].

Alcoholic liver injury has been studied using intragastric alcohol-fed mice as a model system; this model reproduces the pathology features of early alcohol liver and demonstrated the involvement of ER stress [105]. Fatty liver, increased hepatic inflammation, apoptosis, and hyperhomocysteinemia were observed in these alcohol-fed animals. The expression of ER stress–related genes, including GRP78, GRP 94, CHOP/GADD153, and caspase-12, was found to be up-regulated, indicating that ER stress response may contribute to the pathologic features of alcoholic liver disease [105]. ER stress signals GRP78, caspase 12, and sterol regulatory element binding protein-1c (SREBP-1c) were activated in alcohol fed micropigs [107].

The role of CHOP in hepatocellular apoptosis and liver injury has been studied using CHOP knock-out mice [106]. Although no significant differences in pathological parameters and occurrence of ER stress were observed between wild-type and knock-out mice, apoptosis was present in wild-type mice but absent in CHOP knock-out mice. These results implied that ER stress may be responsible for apoptosis through CHOP and that CHOP up-regulation occurred later than apoptosis [106].

A recent study assessed the effect of alcohol on the integrated stress response (ISR) signaling pathway and defined the role of CYP2E1 in this response, using primary human hepatocytes and CYP2E1 over-expressing HepG2 cells [104]. ISR triggered by diverse stresses is a defense mechanism, which leads to suppression of global protein synthesis. ISR is one branch of ER stress response and ATF4 is a key component whose translation is turned on by the phosphorylation of eIF2a [6] (Figure 1). In alcohol-treated primary human hepatocytes and HepG2 cells over-expressing CYP2E1, ATF4, the master coordinator of ISR, was up-regulated. In CYP2E1 over-expressing cells, a set of ISR target genes, including

HMOX-1, GCLC, AsnS, IGFBP-1, GADD34, CHOP, ATF3, and CHAC1, was induced. The antioxidant glutathione and CYP2E1 inhibition protected against both oxidative stress and ISR induced by alcohol. These results demonstrate that both CYP2E1 and ATF4-mediated ISR are detrimental cellular effects in alcohol-induced liver disease [104].

ER STRESS AND ENVIRONMENTAL TOXICANT-ASSOCIATED LIVER TOXICITY

Acrolein

Acrolein, a common environmental pollutant and a major component of cigarette smoke, is a highly reactive α,β-unsaturated aldehyde and is cytotoxic to many cells, including hepatocytes. The toxicities are considered to be due to oxidative stress via loss of glutathione and the formation of Michael-type addition adducts with proteins and DNA [108–111]. In hepatic cells, including both primary human hepatocytes and HepG2 hepatoma cells, acrolein caused apoptosis and necrosis, and triggered ER stress at pathophysiological concentrations [112]. Numerous typical ER stress markers, including eIF2α, ATF-3 and -4, and CHOP, were induced, presenting strong evidence of ER stress; however, the protective/adaptive components of ER stress, such as chaperone proteins GRP78 and GRP94 (assisting in protein refolding), were not changed. It appears that exposure to acrolein selectively impairs the ER protective mechanisms, thus leading cell death. The mechanisms behind the failure of the ER adaptive response in this case remain to be determined [112].

Arsenic

Humans are exposed to arsenic and inorganic arsenic compounds via different routes, such as drinking water, contaminated foods or soil, and dermal exposure. Such inorganic arsenic exposure is associated with adverse health effects, including skin lesions and increased risks of cancer of the lung, skin, liver, kidney, and bladder [113,114]. The mechanisms underlying arsenic-associated toxicity, such as DNA damage, formation of DNA and protein adducts, and production of reactive oxygen species (ROS), have been recognized [115–117]. Inorganic arsenicals are transformed by the liver into monomethylated and dimethylated arsenicals through methylation reactions and excreted into urine as pentavalent methylated forms. Although inorganic arsenic had been extensity studied, it still remains unclear which forms of inorganic arsenic are more toxic to particular organs; conclusions appear to also depend on the experimental approaches.

A study aimed to characterize and compare the different forms of inorganic arsenic-associated liver toxicity demonstrated that ER stress was induced by trivalent dimethylarsinous acid (DMAIII) but not the forms of trivalent ar-senite (iAsIII) and monomethylarsonous acid (MMAIII) [118]. The phosphorylation of PERK was induced in rat liver RLC-16 cells treated with DMAIII, but no significant PERK activation was detected when cells were treated with iAsIII or MMAIII. In the same study, it was reported that ER was a site of generation of ROS after DMAIII exposure, indicating that DMAIII targeted the ER for its toxicity. ER stress induction and ROS generation in the ER occurred

simultaneously, as both were attenuated by the addition of cycloheximide, a protein biosynthesis inhibitor [118].

APPROACHES FOR DETECTING AND MONITORING ER STRESS

The study of ER stress is a growing research area and various experimental approaches have been applied. The most commonly used methods include immunohistochemistry and Western blots for protein detection, qPCR measurement of ER stress markers, PCR-based assays to detect the alternate splicing of XBP1 mRNA, and reporter assays for measuring the activity of ER stress-responding transcriptional factors such as XBP1 and ATF6 [119,120]. Recently, we developed two reporter assays in HepG2 cells, with secreted alkaline phosphatase (SEAP) and secreted *Gaussia* luciferase (Gluc) as reporters, and demonstrating the usefulness of these two in vitro assays [SC and LG, unpublished]. Genetically modified animal models include transgenic models and knock-out models of essential ER stress genes [65,121]. Descriptions of different methods follow.

1. Protein detection of ER stress markers: induction of various protein expression such as Grp78, PDI, CHOP, ATF4, ATF6, spliced XBP1, IRE1, phosphorylated-eIF2α, and phosphorylated-PERK have been reported in response to ER stress and these markers are very often to be chosen for ER stress identification. The detection of increased expression of these markers also can help to elucidate mechanisms of ER stress, that is, which branch (es) of UPR is altered (Figure 1). Both standard Western blots and immunohistochemistry have been applied.

2. Measurement of ER stress marker gene expression: a set of ER stress-related genes, including, but not restricted to, Atf4, Atf6, Bip (Grp78), Erp72, Chop, Perk, Ire1α, Gadd34, and Herp, can measured using PCR; real-time PCR is often applied nowadays because of its sensitivity and reliability compared to conventional PCR. ER stress pathway-focused PCR arrays can be used for screening and mechanism exploration (http://www. sabiosciences.com/rt_pcr_product/HTML/PAMM-089A.html). Gene expression analysis with microarray studies or next-generation sequencing can also be used for mechanism discovery.

3. Semiquantitative PCR and real-time quantitative PCR to measure the splicing of XBP1 mRNA: as depicted in Figure 1, in response to ER stress, Grp78 is released from the complex of Grp78/IRE1. IRE, an ER-located transmembrane RNase, is then activated by auto-phosphorylation of its ribonuclease domain. Activated IRE1 causes an unconventional splicing of the mRNA encoding XBP1, a transcription factor, excising a 26 nucleotide to form a spliced XBP1, referred to as XBP1S. XBP1S is more stable than XBP1, increasing its ability to act as a transcription factor that binds to the ER stress response element (ERSE) sequence of a variety of UPR target genes and leads to the transcription of many chaperone proteins [122–125]. The ratio of XBP1 to XBP1S correlates with the expression levels of other ER stress markers; thus, XBP1 mRNA splicing is often measured in ER stress studies [120,126,127]. The most commonly used method

to measure XBP1 mRNA splicing is semiquantitative RT-PCR that can be visualized by gel electrophoresis. The detailed procedures, PCR conditions, and the primers used to detect both unspliced and spliced isoforms are given in Samali and colleagues [120].

Quantitative real-time RT-PCR has been suggested for measuring XBP1S mRNA instead of conventional RT-PCR [127]. The primers for real-time are designed to span the 26 base pair intron that is removed from the unspliced form of XBP1 mRNA; in this way, only spliced XBP1 (XBP1S) is amplified and measured. Using two well-known ER stress inducers, thapsigargin and tunicamycin, the correlation of the expression of CHOP and Bip to XBP1S was shown to be 0.962 and 0.884, respectively [127].

4. Reporter assays: transcriptional regulators ATF6, ATF4, and XBP1S are activated when ER is stressed (Figure 1). These transcription factors bind to ER stress-response elements (ERSE) or unfolded protein response elements (UPRE) on the promoters of their regulated downstream genes [128,129]. Based on the transcriptional activation of these regulators, reporter assays have been developed to detect ATF6 or XBP1S activation. A luciferase-based assay has been generally used, with the luciferase gene being under the control of promoters containing the ERSE or UPRE of the ATF6 or XBP1S gene or other ER stress-related genes [128,130]. Because blocking of or decreasing protein secretion is a distinct feature of ER stress, seeing a reduction of protein secretion indicates an ER stress response. Based on this feature, reporter assays for monitoring protein secretion have also been developed and used to study ER stress in various cells [119,131–135]. One of these, secreted alkaline phosphatase (SEAP) reporter, has been used in vitro for monitoring different biological processes [131–133]. Recently, the secreted *Gaussia* luciferase (Gluc) has gained popularity due to its high sensitivity [119,134,135]. Both reporter assays are capable of monitoring ER stress by measuring a decrease in Gluc or SEAP being excreted into the medium because ER stress blocks or decreases processing in the secretory pathway and trafficking [119]. Two stable cell lines were established recently in our laboratory, HepG2-Fluc-SEAP and HepG2-Fluc-Gluc to study ER stress associated liver toxicity (SC an LG, unpublished). Lentivirus vectors carrying the expression cassettes for Gluc or SEAP were used to generate stable cell lines (Figure 2); firefly luciferase (Fluc) was used as an internal control for normalizing the cell number. Both cell lines showed significantly decreased activity of SEAP or Gluc when treated with the well-known ER stress inducers brefeldin A or thapsigargin. The usefulness of these in vitro systems was demonstrated in a study on ER stress induced by sertraline, an antidepressant drug [101]. It is anticipated that such assays can be used in high-throughput settings to screen drugs for liver toxicity mediated by ER stress.

5. A fluorescence-based detection method has been applied to detect ER stress in liver cells [136]. Thioflavin T, a small molecule with fluorescence properties, exhibits enhanced fluorescence when it binds to protein aggregates. Thioflavin T

fluorescence correlates directly with ER stress response and, thus, can be used to measure and quantify ER stress.

6. Genetically manipulated models: both cell and animal models with modified ER stress-related genes have been used in ER stress studies [65,121,137–144]. For animal models, both ER stress related gene knock-in and knock-out animals have been shown to be useful to study ER stress. For example, transgenic mice expressing the ER stress indicator XBP1S have been used to monitor physiological and pathological ER stress in vivo [121]. In this model, the stress indicator was constructed by fusing XBP1 and green fluorescent protein (GFP). When ER stress occurred, the spliced indicator mRNA is translated into an XBP1-GFP fusion protein and green fluorescence can be detected. Strong fluorescence in the kidney and pancreas was detected when a typical ER stress inducer, tunicamycin, was injected i.p. [121].

   Transgenic animal models are not only used in monitoring ER stress but also to study mechanisms. For example, using IRE knock-out mice, it was shown that the inflammation occurred earlier than in IRE wild-type mice when exposed to dextran sodium sulfate to induce inflammatory bowel disease, indicating that perturbations in ER function participate in the development of colitis caused by environmental agents [144]. In a more recent study, CHOP knock-out mice were used in a mechanistic study of acetaminophen-induced liver toxicity [65]. Following administration of a toxic dose to wild-type mice, ER stress was activated; all three branches of UPR were activated, with PERK-elF2-CHOP being the most important pathway. While wild-type mice developed extensive necrosis, CHOP knock-out mice exhibited hepatocyte proliferation at the sites of necrosis and protected against acetaminophen-induced liver damage, indicating that CHOP plays a critical role in acetaminophen toxicity, and that up-regulation of CHOP compromises hepatocyte survival by reducing regeneration [65].

It should be stated that no single individual assay will be the most appropriate for all ER stress evaluations and mechanistic studies; thus, a set of assays should be considered and chosen to be used sequentially or in parallel, based on the requirements of a particular study. As an initial step to evaluate ER stress-mediated liver toxicity in our laboratory, we perform reporter assays using the established cell lines HepG2-Fluc-Gluc and/or HepG2-Fluc-SEAP and real-time PCR on a group of ER stress-related genes because of their sensitivity and ease of use.

CONCLUSIONS

Drug-induced liver injury is one of the leading causes of acute liver failure. Besides drugs, herbal dietary supplements and environmental pollutants are also reported to be associated with liver damage [145–149]. The causes of liver damage vary, including factors such as genetic makeup, personal lifestyle, environmental factors, and pre-existing diseases. Understanding of molecular mechanisms of liver damage is fundamental for preventing and curing liver injury caused by drugs and chemicals. Endoplasmic reticulum stress plays an

important role in a range of diseases. The role of ER stress in drug-induced liver toxicity is beginning to be recognized, although not yet extensively studied and therefore the precise mechanisms remain elusive. Better knowledge of ER stress can help identify risk factors and decrease the incidence of drug-induced liver toxicity. It is anticipated that as more studies aimed at studying ER stress associated liver toxicity and the interplay between different organelles and pathways appear, the role of ER stress in liver toxicity will become widely recognized.

Acknowledgments

SC was supported by appointments to the Postgraduate Research Program at the National Center for Toxicological Research administered by the Oak Ridge Institute for Science Education through an interagency agreement between the US Department of Energy and the US FDA.

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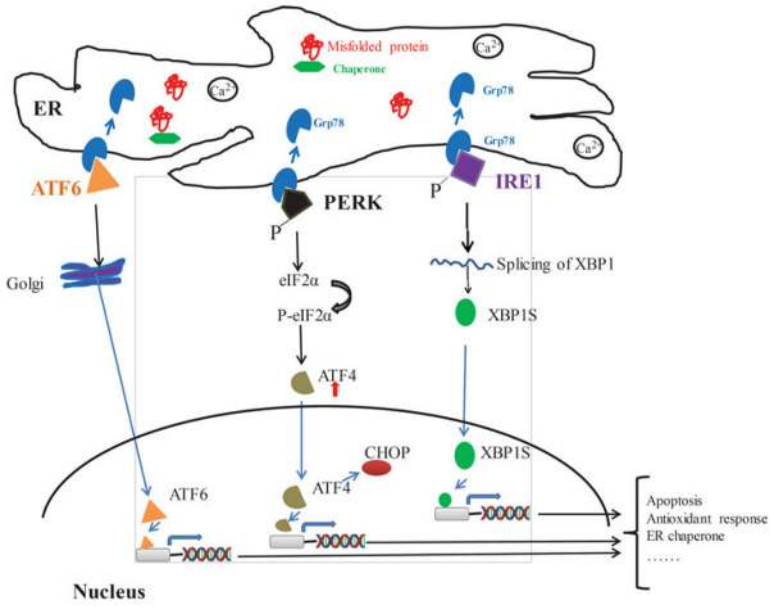

Figure 1.

The unfolded protein response: Three UPR transducers are activated upon stimulation of ER. (A) ATF6 is translocated to the Golgi and then cleaved by resident Golgi proteases. The cleaved form of ATF6 is released to the cytosol and then travels to the nucleus to regulate gene expression; (B) Phosphorylation of PERK leads a reduction in the protein load on the ER by decreasing protein synthesis through eIF2α phosphorylation. The expression of transcription factor 4 (ATF4) is induced; ATF4 is a member of the bZIP family of transcription factors that regulate the promoters of numerous UPR genes; (C) Phosphorylation of IRE1α initiates an intron excision from the mRNA for the transcription factor, XBP1 (X-box binding protein 1), to produce a spliced form XBP1S. XBP1S binds to promoters of several genes, resulting in increased expression of genes involved in restoring protein folding or degrading unfolded proteins.

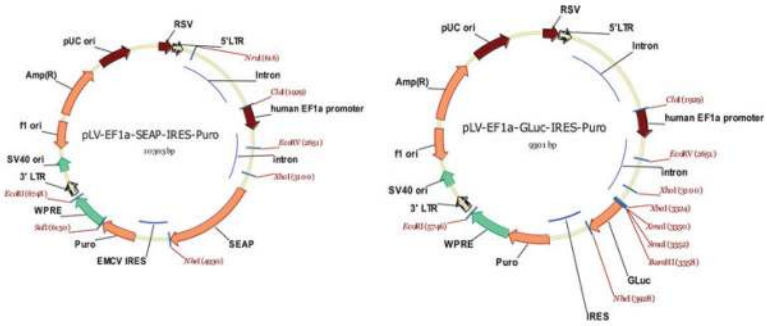

Figure 2.

Construction of plasmids used to establish Gluc or SEAP reporter gene assays for monitoring ER stress in HepG2 cells.