REVIEW

The Role of Endoplasmic Reticulum in Lipotoxicity during Metabolic Dysfunction—Associated Steatotic Liver Disease (MASLD) Pathogenesis

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Perturbations in lipid and protein homeostasis induce endoplasmic reticulum (ER) stress in metabolic dysfunction—associated steatotic liver disease (MASLD), formerly known as nonalcoholic fatty liver disease. Lipotoxic and proteotoxic stress can activate the unfolded protein response (UPR) transducers: inositol requiring enzyme1α, PKR-like ER kinase, and activating transcription factor 6α. Collectively, these pathways induce expression of genes that encode functions to resolve the protein folding defect and ER stress by increasing the protein folding capacity of the ER and degradation of misfolded proteins. The ER is also intimately connected with lipid metabolism, including de novo ceramide synthesis, phospholipid and cholesterol synthesis, and lipid droplet formation. Following their activation, the UPR transducers also regulate lipogenic pathways in the liver. With persistent ER stress, cellular adaptation fails, resulting in hepatocyte apoptosis, a pathological marker of liver disease. In addition to the ER—nucleus signaling activated by the UPR, the ER can interact with other organelles via membrane contact sites. Modulating intracellular communication between ER and endosomes, lipid droplets, and mitochondria to restore ER homeostasis could have therapeutic efficacy in ameliorating liver disease. Recent studies have also demonstrated that cells can convey ER stress by the release of extracellular vesicles. This review discusses lipotoxic ER stress and the central role of the ER in communicating ER stress to other intracellular organelles in MASLD pathogenesis. (Am J Pathol 2023, 193: 1887–1899; https://doi.org/10.1016/j.ajpath.2023.08.007)

Metabolic dysfunction—associated steatotic liver disease, or MASLD, earlier known as nonalcoholic fatty liver disease, is the most common chronic liver disease worldwide with an overall prevalence of 32.4%.1 In the background of consistently rising obesity, MASLD affects up to 48% of the US population and is the foremost cause of liver-related mortality and morbidity.1 MASLD encompasses a clinico-pathological spectrum that includes metabolic dysfunction—associated fatty liver, a benign, nonprogressive macrovesicular accumulation of intracellular lipids and metabolic dysfunction—associated steatohepatitis (MASH), a more severe and progressive condition with evidence of cell injury, inflammation, hepatocyte degeneration, apoptosis, and fibrosis. MASH has the potential to progress to cirrhosis, an antecedent to end-stage liver disease and hepatocellular carcinoma.2 The primary insult in MASLD is hepatic lipotoxicity that occurs when the hepatocyte’s capacity to handle and export free fatty acids (FA) is exceeded either due to an excessive free FA influx or de novo lipogenesis. Several molecular mechanisms orchestrate lipotoxicity, including endoplasmic reticulum (ER) and oxidative stress, autophagy, inflammation, and lipoapoptosis.3

The ER is an intracellular organelle whose role in protein synthesis, folding, modification, and trafficking has been well studied. It plays a vital role in synthesizing glycoproteins, cholesterol, and phospholipids, while also maintaining calcium homeostasis.4,5 When ER homeostasis is perturbed, ER stress occurs, which has been implicated in various conditions including inflammation, diabetes mellitus, atherosclerosis, metabolic disorders, and cancers.6–8 Cellular stress also

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impacts other membranous organelles, including mitochondria, endosomes, and lysosomes, which have functional contacts with the ER, and in turn exert direct or indirect effects on the outcome of ER stress signaling. In this article, the authors offer succinct insights into the cellular processes that underlie ER stress, with a particular emphasis on its role in the evolution of MASLD/MASH. In addition, the global landscape of organelle crosstalk and its mediators that show promise as therapeutic targets has been reviewed.

ER Structure

The ER is an interconnected network largely made up of three main structures: the nuclear envelope, the peripheral ER consisting of smooth tubules and rough sheets, and the cortical ER that abuts the plasma membrane. The nuclear envelope is composed of two lipid bilayers, the inner and outer nuclear membrane, which has numerous pores to facilitate transport of RNAs and proteins. The outer membrane of the nuclear envelope is continuous with the ER membrane and connected to the sheets and cisternae of the peripheral ER through their shared lumen. Sheets are flat structures that have a stacked appearance due to the parallel arrangement of the layers with consistent luminal spacing. The curved regions in the membrane edges connect them to one another. Rough ER sheets possess ribosomes on the cytosolic surface, thus allowing them to partake in protein synthesis and folding. Smooth ER tubules are dynamic structures that are constantly remodeling and characterized by scant ribosome attachment and binding. Cortical ER, abutting the plasma membrane, is a combination of sheets and tubules, and plays a role in calcium signaling.

The distinctions in the subcellular architecture of the ER and the differences in the ratio of sheets to tubules across cell types facilitate diverse cellular functions. For instance, cells with high secretory demand such as B cells (antibody synthesis and secretion) and pancreatic acinar cells (insulin synthesis and secretion) have large amounts of stacked sheets in the rough ER, whereas cells involved in lipid synthesis such as hepatocytes and Leydig cells have more tubules in their smooth ER. This difference in the ratio of sheets to tubules has been identified because of different ER shaping proteins, most prominent being the reticulon family of proteins. In vivo studies have demonstrated that a change in ER structure with respect to tubule formation can alter changes in normal lipid metabolism leading to an increase in lipid droplets (LDs) and triglyceride content, and an up-regulation of enzymes involved in de novo lipogenesis. Primary hepatocytes from obese mice models have shown that enriching ER sheets and increasing the ER sheet to tubule ratio via ER-shaping membrane proteins such as the 63-kDa cytoskeleton-linking membrane protein (Climp-63) can decrease lipogenesis and glucose production. Thus, the spatial organization of the ER provides functional flexibility and metabolic diversity to the cell.

ER Function

Structural complexity and flexibility of subcellular components aid in meeting the complex metabolic demands and maximizing the metabolic efficiency of multicellular organisms. Numerous studies have extensively characterized the subcellular architecture in relation to metabolic homeostasis, revealing that the structural organization of cellular components is a critical factor influencing their respective functions.

A primary biosynthetic role of the ER is to ensure cotranslational folding of nascent polypeptides, whether they are secreted proteins, proteins intended for the plasma membrane and other membranous organelles, or luminal proteins within the ER, Golgi, and lysosomes. Translation of these proteins begins in the cytosol, where the ribosome–mRNA complex is formed. A topogenic signal sequence in the nascent polypeptide is identified by the signal recognition particle, or SRP. The ribosome–mRNA complex encounters the nascent polypeptide–SRP complex, and the four-component complex, composed of the ribosome, mRNA, nascent polypeptide, and SRP complex, is recruited to the ER membrane where it docks on the SRP receptor. Translation continues on the ER membrane. Depending on whether the protein is directed to be an integral membrane protein or secreted, translocation will pause embedding the nascent polypeptide in the ER membrane, or will be transported completely into the ER lumen, respectively. In the event of misfolded proteins or aggregates, proteins either remain in the ER lumen or enter ER-associated degradation. Thus, ER quality control mechanisms prevent the secretion of anomalous proteins.

Apart from protein synthesis, the second biosynthetic process integral to the ER membrane is lipid biogenesis, reviewed elsewhere in detail. In hepatocytes, the smooth ER is abundant and is a site for the synthesis of almost all lipid classes. Most lipid synthesis enzymes are transmembrane proteins located in both the smooth and rough ER membranes, with some pathways focused in subdomains of the ER membrane, such as ER–organelle membrane contact sites. Phospholipids are synthesized in the cytosol-facing lipid bilayer of the ER membrane. Ceramides formed in the ER are exported to the Golgi where they are further enzymatically modified to generate glycosphingolipids and sphingomyelin in the lumen-facing Golgi lipid bilayer. In addition to phospholipid and sphingolipid synthesis, cholesterol synthesis, triglyceride synthesis, and LD and lipoprotein formation occur in the ER membrane.

The nuclear envelope is a double membrane structure, and the outer nuclear membrane is continuous with the ER. Due to this continuity, the nuclear envelope and the ER share many proteins. Like the ER, the nuclear envelope is also a site for lipid metabolism. Mutations in protein of the nuclear envelope proteins may be pathogenic, resulting in
multisystem disease, including lipodystrophies and susceptibility to MASLD and MASH. These genetic links demonstrate that lipid metabolism at the nuclear envelope, and through their connections to chromatin, can affect lipid metabolism gene programs.21

Lastly, the ER plays a crucial rule in calcium homeostasis by employing proteins that aid in pumping Ca\(^{2+}\) from the cytosol into the lumen against the electrochemical gradient, storing Ca\(^{2+}\) by way of sequestering using luminal binding proteins and releasing Ca\(^{2+}\) back into the cytosol via channels along the electrochemical gradient. Calcium homeostasis is maintained by the smooth ER Ca\(^{2+}\) ATPase (SERCA) transporters, which pump Ca\(^{2+}\) into the ER lumen, and inositol 1,4,5-triphosphate (IP\(_3\)) receptor activation—mediated release of stored Ca\(^{2+}\) from the ER lumen into the cytosol.11 The aforementioned processes underscore that the ER is integral to both cellular and organismal lipid homeostasis.

The Unfolded Protein Response

In homeostatic conditions, several checks and balances are in place to prevent an accumulation of misfolded proteins in the ER.22 When cells accumulate unfolded and/or misfolded proteins in the ER, they undergo ER stress. In response to this, to maintain homeostasis, several compensatory mechanisms occur including translation inhibition, increase in chaperones and folding enzymes and degradation of the unfolded/misfolded proteins. Failure to recover from ER stress triggers cell death. In mammals, these signaling pathways are mediated by the three proximal UPR sensors: inositol requiring enzyme 1\(\alpha\) (IRE1\(\alpha\)), protein kinase-like ER kinase (PERK), and activating transcription factor \(\alpha\) (ATF6\(\alpha\)). The UPR sensors are inactive basally, and in this configuration, their luminal domains are bound to the chaperone 78-kDa glucose-regulated protein (GRP78)/binding immunoglobulin protein (BiP) (Figure 1). Misfolded proteins can trigger activation of the UPR sensors by binding to GRP78/BiP or direct interactions with the UPR sensors.

There are three described models of stress sensing by IRE1\(\alpha\). In the direct association model, it is postulated that the misfolded proteins trigger conformational changes, which result in stabilization of IRE1\(\alpha\) homodimers by binding to the peptide binding pocket created in the luminal domain of dimers, activating its kinase and endoribonuclease activities.19,23 In the competition model, GRP78/BiP prevents IRE1\(\alpha\) dimerization by binding the IRE1\(\alpha\) luminal domain.24 The dissociation of BiP from the IRE1\(\alpha\) luminal domain is facilitated by nucleotide exchange factors.25 In the allosteric model, binding of misfolded protein to the BiP substrate binding domain (SBD) causes dissociation of the BiP ATPase domain from the IRE1\(\alpha\) luminal domain via a conformational change.26 IRE1\(\alpha\) undergoes autoprophosphorylation, activating its RNase activity, which then splices X-box binding protein 1 (XBP1) mRNA to generate sXBP1 mRNA, which encodes a soluble active transcription factor (sXBP1). sXBP1 can transcriptionally induce genes encoding ER chaperones and ER-associated degradation proteins. Although both spliced and unspliced forms of XBP1 can activate the UPR, the sXBP1 is a more potent transcription factor.

PERK, like IRE1\(\alpha\), is a transmembrane protein, whose N-terminal domain is bound by BiP. PERK dimerization and autotransphosphorylation leads to the phosphorylation of the eukaryotic translation initiation factor 2-\(\alpha\) (eIF2\(\alpha\)).27 eIF2\(\alpha\) phosphorylation results in the global attenuation of protein translation with selective translation of activation transcription factor 4 mRNA (ATF4). ATF4 can thereafter up-regulate the expression of C/EBP homologous protein (CHOP), a proapoptotic transcription factor. In a negative feedback loop, CHOP induces the expression of GADD34, which along with protein phosphatase 1 (PP1) dephosphorylates eIF2\(\alpha\), thus allowing for translation to proceed.28

The third UPR sensor, ATF6\(\alpha\), translocates from ER to the Golgi apparatus, where it is cleaved sequentially by site-1 protease and site-2 protease to generate an N-terminal fragment (ATF6\(nf\)) from the cytosolic domain that functions as a transcription factor.29 Overall, these pathways work in concert to restore proteostasis. If restoration of proteostasis fails, sustained activation of the UPR results in apoptosis. ER stress—induced apoptosis has been implicated to occur via the transcription factor CHOP, the mitogen activated protein kinase c-Jun N-terminal kinase (JNK), the death receptor 5, Bcl-2 family proteins, calcium, redox homeostasis, and caspase activation.30

Lipotoxic ER Stress

Lipotoxicity is defined as a dysregulation of the lipid environment and/or intracellular composition that leads to accumulation or transient generation of toxic lipids, resulting in cell injury or death, described in many cell types including hepatocytes and pancreatic \(\beta\)-cells.31 Lipotoxicity can be induced by several toxic lipid species such as saturated fatty acids (SFA) like palmitate, sphingolipids (C16:0 ceramide), the phospholipid lysophosphatidylcholine (LPC), and free cholesterol. By contrast, monounsaturated free FAs, such as oleate and palmitoleate, protect from SFA-induced toxicity. Although excess palmitate can be incorporated into triacylglycerides and phospholipids, it can also serve as a substrate for ceramide synthesis and LPC formation. Ceramide C16 accumulation induced ER stress by causing a disturbance in the Ca\(^{2+}\) homeostasis, leading to cell death through PERK/ATF4 and ATF6\(\alpha\) arms of the UPR, leading to induction of CHOP expression.32–34 Ceramides can promote inflammation in MASH because palmitate induces the release of proinflammatory extracellular vesicles in an IRE1\(\alpha\)/XBP1-dependent manner via transcriptional activation of the \textit{de novo} ceramide synthesis pathway.35,36 Through motifs in the transmembrane domain, ATF6\(\alpha\) can be activated by specific
sphingolipids (Figure 2). In addition to the role of ceramides in ER stress, whether nuclear phenotypes associated with MAFLD or MASH nuclear LDs can be resolved by inhibition of ceramide synthesis remains to be elucidated.

In addition to ER lipid composition, membrane stiffness also affects ER function. Sterol content is a determinant of membrane fluidity and is normally maintained at low amounts in the ER membrane. Abnormally increased sterol and SFA concentrations stretch the membrane, increasing its stiffness, triggering oligomerization of IRE1α and PERK (Figure 2) and thus activating UPR. Exploring how UPR transducers detect lipid accumulation in ER membranes might elicit a physical basis of chronic lipotoxic ER stress and reveal potentially druggable targets.

LPC is a phospholipid that is an important mediator of lipotoxicity in MASH. LPC is a primary lipid species of cell membrane bilayers, LD envelope monolayers, and very low density lipoprotein (VLDL). This toxic lipid is synthesized either intracellularly by the action of phospholipase A2 (PLA2) from phosphatidylcholine (PC) or extracellularly by the action of plasma lecithin-cholesterol acyltransferase. Thus, inhibition of PLA2 has shown to decrease intracellular LPC and palmitate-induced apoptosis. PLA2 activation also depletes membrane PC resulting in loss of hepatocyte membrane integrity, lipotoxic extracellular vesicle (EV) release, inflammation, and apoptosis. Additionally, LPC induces ER stress via eIF2α phosphorylation, increased CHOP expression, and JNK activation leading to the induction of the BH3-only protein PUMA (p53 upregulated modulator of apoptosis). Increased PUMA results in Bax and caspase 3/7 activation and thus apoptosis.

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**Figure 1** The three unfolded protein response transducers. Inositol-Requirement Enzyme 1α (IRE1α), PKR-like ER kinase (PERK), and activating transcription factor 6α (ATF6α) are the three endoplasmic reticulum (ER) stress sensors that trigger a transcriptional program termed the unfolded protein response (UPR). GRP78/BiP is an ER chaperone that is associated with all three transducers and inhibits them under normal physiological conditions. When ER stress or misfolded proteins accumulate, BiP dissociates and allows the initiation of downstream signaling: IRE1α pathway: ER stress induces IRE1α homodimerization and autophosphorylation, which triggers its RNase activity to splicing XBP1. As a transcription factor, X-box binding proteins 1 (XBP1s) activates genes related to the UPR, ERAD, and chaperones. PERK pathway: The activated PERK phosphorylates the alpha subunit of eIF2α, which attenuates protein translation to reduce the burden of misfolded proteins. Phosphorylated eIF2α up-regulates ATF4, which increases proapoptotic CHOP and UPR genes. ATF6α pathway: ATF6α is cleaved by site-1 and site-2 proteases (S1P and S2P) in the Golgi apparatus to produce ATF6N. ATF6N further initiates the transcription of its target UPR genes in the nucleus. All these pathways collectively aim to improve the protein-folding capacity and decrease the protein-folding burden by shutting down translation and degrading the ER-bound mRNAs. When this adaptive response fails, upregulated UPR signaling induces apoptosis. This figure was generated using BioRender.com (Toronto, ON, Canada).
Bidirectional Association between Lipotoxic and Proteotoxic Stress

The ER functions in protein and lipid homeostasis, and disruption in either process triggers ER stress. This could be due to unfolded/misfolded protein accumulation detected by the luminal domain or lipid-induced activation of UPR sensors, as discussed above. Additionally, SFA accumulation-induced ER stress changes the integrity and structure of the ER, leading to a downstream disturbance in ER proteostasis. The bidirectionality between these two stressors has also been evidenced by SFA-induced lipotoxic stress leading to the degradation of ER proteins, which disrupts normal ER structure and function in a Saccharomyces cerevisiae ER stress model, where the gene OPI3 is deleted, inhibiting PC synthesis. To demonstrate that proteotoxicity can occur downstream of lipotoxic stress, 4-phenylbutyric acid, on binding to ER misfolded proteins, stabilized and rescued lipotoxic stress-induced UPR. Studies have shown the possible existence of a threshold, only beyond which one stressor could trigger the occurrence of the other. In Caenorhabditis elegans, ablation of mdr-15, a regulator of lipid biosynthesis genes, activated both IRE1α and PERK independent of proteotoxic stress, possibly due to the lower threshold of lipotoxic stress. To understand and explore the bidirectional role of lipotoxic and proteotoxic stress in hepatocytes in the background of MASLD, more studies are necessary.

ER Stress Sensors and Lipid Homeostasis

Independent of their roles in ER stress, each UPR sensor plays a role in regulating lipid metabolism. Although these roles are independent of canonical ER stress signaling, proteotoxic or lipotoxic activation of each sensor is necessary for most of their functions in lipid homeostasis.
IRE1α Pathway

Structurally, IRE1α is a transmembrane protein that possesses serine/threonine kinase and endoribonuclease (RNase) activity on the cytosolic domain. At homeostasis, it remains inactive because it is bound by the ER chaperone BiP. Yet once activated, functionally, IRE1α represses lipid accumulation and maintains lipoprotein secretion, thereby regulating lipid homeostasis. This has been studied in hepatocyte-specific deletion of IRE1α, which increased hepatic steatosis and expression of transcriptional genes and enzymes involved in lipid metabolism. Studies have also demonstrated that IRE1α deletion impairs VLDL assembly and secretion by reducing protein disulfide isomerase, an enzyme that aids in delivery of lipids to the smooth ER lumen, thus highlighting the role of IRE1α in attenuating hepatic steatosis via repressed lipolysis and export of VLDL.

Manipulating transcription downstream of IRE1α by XBP1 deletion in mice resulted in IRE1α hyperactivation, which induced regulated IRE1-dependent decay of mRNA (RIDD), reduced plasma triglycerides, cholesterol and de novo hepatic lipogenesis by gene down-regulation (DGAT2, SCD, and ACAA1). When nitric oxide synthase-mediated nitrosylation of IRE1α occurs, there is a resultant reduction in XBP1-mediated splicing causing impaired glucose homeostasis and decreased PPARα and deacetylase sirtuin 1-mediated FA oxidation and lipolysis.

Recent data suggest that IRE1α activation leads to release of proinflammatory extracellular vesicles from hepatocytes. This ties into the understanding that IRE1α activation promotes hepatic inflammation. Factors that determine the fate of IRE1α are Bax inhibitor 1 (BI-1), a negative regulator of IRE1α activation, and the degree of RIDD. BI-1 deletion accelerates MASH by hyperactivation of IRE1α and subsequent XBP1 activation. Concomitantly, IRE1α hyperactivation results in greater RIDD with activation of inflammasome, inflammation, and liver injury.

ATF6α Pathway

There are several unique aspects to the intersection of lipid metabolism and ATF6α. The activation of ATF6α shares some of the machinery employed by SREBP, including translocation to the Golgi and cleavage by site-1 protease and site-2 protease. ATF6α can also interact with PPARα to regulate FA oxidation. In keeping with these observations, ATF6 knockout mice showed hepatic steatosis secondary to increased SREBP-1c expression and blockage of FA β-oxidation. Overexpression of active ATF6α up-regulates the transcription of genes involved in PC biosynthesis, independent of XBP1. Uniquely, ATF6α, is the only UPR sensor that can be activated directly by two sphingolipid species, dihydrophosphoginose and dihydroceramide, activating a downstream transcriptional program. This activation is mediated by the transmembrane domain of ATF6α; mutations in the transmembrane domain abolish sphingolipid-induced activation while retaining proteotoxicity-induced activation of ATF6α via its luminal domain, and vice-versa. Proteotoxic activation of ATF6α led to predominant transcriptional up-regulation of chaperones, whereas sphingolipid-induced activation of ATF6α induced lipid metabolism genes, including COX1, LRP1, and PPARα, reflecting nonspecific up-regulation of multiple pathways that affect lipid homeostasis. The specificity of activation of ATF6α by dihydro-species of sphingolipids and activation of a distinct transcriptional signature suggest that there may be an ATF6α-mediated lipo-stasis pathway similar to the canonical proteostasis mediated by all of the UPR sensors.

EVs as a Language of ER Stress

EVs are being studied intensely with growing interest in the development of minimally invasive biomarkers for MASLD diagnosis and prognostication. EVs are a heterogeneous group of membranous structures, which are released by multiple cell types and have a diverse repertoire of bioactive compounds and metabolites, including RNA, surface proteins, and cytosolic constituents. EVs can be derived from endosomal compartments where intraluminal vesicles of multivesicular bodies are released upon multivesicular body fusion with the plasma membrane. The resultant EVs are termed exosomes. Alternatively, EVs released by direct blebbing from the plasma membrane are termed microvesicles. The need for disease-specific EV biomarkers is justified given the evidence from recent studies that demonstrate an increase in circulating EVs in patients with MASH, alcoholic liver disease, and cirrhosis. In an earlier study, the authors demonstrated that plasma levels of
hepatocyte-derived small and large EVs correlate with steatosis in MASLD patients and steatosis and inflammation in MASH patients with a decrease in levels following MASLD resolution secondary to bariatric surgery.53

Mechanistic studies demonstrated that lipotoxic hepatocytes release EVs that are proinflammatory by virtue of signaling molecules such as damage-associated molecular patterns and chemokines contained within the EVs. The formation of EVs from palmitate-stimulated hepatocytes was mediated by IRE1α.34 Examination of the mechanism of biogenesis of EVs in MASH demonstrated that lipotoxic EVs, enriched in S1P, are released following the activation of IRE1α leading to XBP1-mediated transcriptional up-regulation of de novo ceramide synthesis.36 At a pathophysiological level, proinflammatory S1P-containing EVs from hepatocytes mediate liver inflammation in MASH by recruiting monocyte-derived macrophages into the liver.35,36

Others have demonstrated the release of misfolded proteins and chaperones in EVs.64,65 Thus, ER stress could be communicated to the extracellular milieu by the release of EVs.

EVs from other cell types can also communicate and elicit stress or salutary responses in hepatocytes by regulating ER function. EVs isolated from the supernatant of LPS-stimulated macrophages can induce expression of inflammatory genes and ER stress in hepatocytes through the PERK pathway. Additionally, treated hepatocytes exhibit altered lipid metabolism, where de novo ceramide synthesis enzyme serine palmitoyltransferase expression decreases, whereas cholesterol synthesis gene expression increases. However, the composition of these EVs that activate the UPR when co-cultured with hepatocytes remains unknown.66 Additionally, adipocyte-derived EVs have been shown to induce hepatic steatosis due to their abundance in resistin. Resistin, by inhibiting phosphorylation of S5'-adenosine monophosphate-activated protein kinase α (pAMPKα) on Thr172, generates ER stress and ultimately contributes to the development of MASLD/MASH.67 Melatonin treatment led to an increase in the transcription factor brain and muscle Arnt-like protein-1, which can reduce cellular resistin levels and content in EVs and has thus been explored as a therapeutic modality as discussed in later sections. Mesenchymal stem cells have therapeutic potential; one postulated mechanism is via mitigation of ER stress in an ischemia reperfusion injury model.68

EVs are a heterogenous population released from different sites and under various cellular states, both of which influence their physical and biological characteristics. This and the inherent complexities of biological fluids increase the difficulty of isolating EVs and identifying candidate prognostic and diagnostic biomarkers. Furthermore, there are no standardized techniques for isolating EVs; studies rely on a general consensus, but many variations in isolation methods exist.59 These variations contribute to inconsistencies in the literature and pose a challenge to biomedical applications. Nonetheless, it is important to identify the different populations of EVs to better understand whether the EV number, cargo, or physical characteristics are altered in disease. Characterization of EVs is limited to population-based assays, which hinders the identification of relevant subpopulations. Although single particle analysis of EV size and number is possible using nanoparticle tracking analysis, quantitative analysis of EV cargo on a single particle analysis remains an area for growth. In summary, EVs are a heterogenous population that contributes to health and disease with diagnostic, prognostic, and therapeutic potential. However, there are technological hindrances to identifying and isolating relevant populations for their use. A greater understanding of their origin and characteristics may help in overcoming these difficulties.

**ER—Mitochondria Contact Sites**

ER membranes make contact with the mitochondria (Figure 3); these contact sites are termed mitochondria-associated membranes, or MAMs. MAMs are dynamic protein bridges that tether the mitochondria to the ER and integrate cellular processes that require an interfacing platform for efficient execution. These processes include Ca2+ homeostasis, lipid metabolism, autophagy, NOD-like receptor protein 3 (NLRP3) inflammasome activation, and apoptosis, all of which are relevant to the pathogenesis of fatty liver.69 Proteomics studies have identified greater than 1000 proteins in isolated MAM fractions, many of which play structural roles. The ER proteins MOSPD2, ORP5, ORP8, VAPB, and PDCD8 are associated with MAMs. MOSPD2 and VAPB interact with the mitochondrial protein PTPP51. Additionally, inositol 1,4,5-triphosphate receptors (IP3Rs) in the ER membrane form a MAM by binding VDAC1 and GRP75 harbored in the outer mitochondrial membrane. These interactions facilitate lipid exchange and Ca2+ transport, respectively.70,71 The UPR sensors IRE1α and PERK are both present at MAMs where they are linked to canonical, noncanonical, and regulatory properties. Deletion of tethering proteins can activate the UPR, for example, mitofusin 2 (mfn2) represses PERK activation, such that deletion of mfn2 leads to activation of PERK, IRE1α, and ATF6α.72 ER stress is communicated to mitochondria via MAMs, and this communication helps ameliorate ER stress. Studies have shown that ER stress causes mitochondria to relocate toward the perinuclear ER. These mitochondria are different as they have an increased transmembrane potential and calcium uptake resulting in higher ATP production, reductive power, and oxygen consumption. This increase in ATP is needed to buttress the prosurvival ER stress transcriptional factors that lead to an increase in chaperones. An increase in ER—mitochondrial MAMs is reported in fatty liver and correlates with the severity of hepatic steatosis and inflammation.73 Conversely, disruption of ER—mitochondrial MAMs is
noted to be an early event in high-fat diet—fed mice. In other models, NLRP3 activation, which is a prominent feature of fatty liver, occurs at MAMs. Future functional studies are necessary to determine how lipotoxic ER stress associated MAMs regulate cellular fate and inflammation.

**Lipid Droplets**

LD are dynamic, ER-derived, regulatory organelles of lipid homeostasis that are encircled by a phospholipid monolayer peppered with an array of proteins, and a hydrophobic core composed of neutral lipids such as triglycerides and sterol esters. This arrangement allows for the storage of lipids at the core and their use for signaling pathways, membrane biosynthesis, and energy while preventing lipotoxic damage. Defects in either their formation or breakdown have been shown to contribute to disease pathogenesis in obesity, fatty liver, diabetes, and atherosclerosis, among others. Although in-depth mechanistic understanding of LD biogenesis remains elusive, it has been established that many of the constituents of LDs are formed in the ER membrane from which LD budding occurs (Figure 3).

Several protein constituents of LDs have been identified, including seipin, sorting nexin 14 (SNX14), and Rab18. Given this intimate structural and functional relationship, it is not surprising that LDs are affected by ER stress. ER stress leads to LD formation; indeed, when IRE1α function is intact, LD formation is attenuated under conditions of ER stress. Conversely, failure of LD formation can lead to ER stress, and free FA esterification into triglyceride and LD formation mitigate lipotoxic ER stress.

In addition to the cytoplasm, LDs have been observed in the nucleus of various cell types including hepatocytes. Nuclear LDs arise from Apo-B free ER luminal LDs, and LDs increase in pathologic conditions including high fat diet.
feeding. Liver-specific deletion of the nuclear envelope proteins TorsinA and lamina-associated polypeptide 1 (LAP1) lead to impaired VLDL secretion, increased nuclear LDs, and steatosis.\(^8^5\) Thus, these data suggest an interdependence in VLDL biogenesis and nuclear LD formation, such that increased flux in this pathway or accumulation of VLDL precursors, both increase nuclear LDs. The physiological function of nuclear LDs and their role in MASH remain unknown. Several LD proteins determine MASH progression.\(^8^3\) Mechanosensing by LDs plays a role in hepatocyte function.\(^8^4\) It remains to be determined whether nuclear LDs contribute to cellular injury in MASH or represent a spillover phenomenon. Studies in yeast have demonstrated a role for LDs in the clearance of misfolded protein inclusion bodies by supplying a sterol-based metabolite.\(^8^5\) In mammalian cells, LDs are involved in protemporal degradation, as demonstrated for ApoB100 and 3-hydroxy-3-methylglutaryl CoA reductase.\(^8^6^,8^7\) LD and LD proteins play a key role in fatty liver, reviewed in detail elsewhere.\(^8^3\) Many of the susceptibility loci for fatty liver also occur in LD proteins. Collectively, the findings from these studies provide evidence that LDs play a crucial role in mediating the interplay between lipotoxicity and proteotoxicity. As a result, they are likely to impact the outcome of ER stress in the context of fatty liver.

**ER—Endosome Contact Sites**

The presence of ER—endosome contact sites contributes to various aspects of endosomal function, including fission, maturation, and the formation of late endosomes. These contact sites become more abundant as late endosomes are established.\(^8^8\) Perturbations of proteins that form endosomal ER contact sites are associated with diseases, such as mutations in Niemann-Pick type C protein 1 (NPC1), which mediates cholesterol transport. Endosomal heterogeneity is reflected in the several types of contacts formed and the functions they serve. For example, annexin A1 and its ligand S100A11 form ER—endosome membrane contact sites upon epidermal growth factor receptor (EGFR) stimulation, facilitating the interaction with ER-localized phosphatase PTP1B, which dephosphorylates EGFR on the endosomal membrane.\(^8^9\) Annexin A1 treatment improves injury and inflammation in fatty liver without impacting steatosis.\(^9^0\) It is unknown whether annexin A1’s role as ER—endosome contact site plays a role in MASLD progression in addition to its role in inflammatory signaling. S100A11 is upregulated in MASH and may also perturb endosomal function.\(^9^1\) Cholesterol transfers from the ER to the endosomes via formation of contacts between endosomal proteins such as ORP1L, STAR D3, and STAR D3NL with the ER protein, VAP-A.\(^8^8\) The ER membrane contact sites with LDs and endosomes contribute to lipid transfer between the tethered organelles; how these membrane contact sites participate in lipotoxic ER stress is an opportunity for future studies.

**Therapeutic Opportunities**

ER stress and the UPR cascade are implicated in multiple liver pathologies, owing to which, therapeutic targets that modulate these pathways have gained importance.\(^9^2\) Both naturally occurring compounds and pharmaceutical agents have been studied in the background of ER stress in MASLD. A selection of the naturally occurring compounds or supplements are discussed here. Vitamin C ameliorates murine hepatic steatosis by way of down-regulation of ATF6\(\alpha\), eIF2\(\alpha\), HSPA5, and XBP1. Taurine suppresses ER and oxidative stress via caspase 3 activation and inducing apoptosis in diet-induced MASLD models. Curcumin has been shown to reduce ER stress markers [BiP, PERK, IRE1, TRAF2, tumor necrosis factor (TNF), IL1B, MAPK14, MAP3K5, and CEGBP] in diabetic rat liver. Quercetin resolves ER stress, oxidative stress, and hepatotoxicity by reducing IRE1\(\alpha\) and MAPK8 levels in rat livers subjected to feed. Berberine, a naturally occurring plant alkaloid found in *Coptis chinensis*, has been studied in oriental medicine for its glucose-lowering and LDL-lowering effects. In *in vitro* studies have shown the berberine-reversed ER stress—activated lipogenesis via the ATF6/SREBP-1c pathway. Additionally, it reduces protein aggregation, and FA-induced lipid accumulation and tunicamycin-induced triglyceride and collagen deposition, altogether reducing hepatic inflammation, fibrosis, and lipid peroxides.\(^9^3\) Obeticholic acid, an agonist of farnesoid X receptor, in advanced clinical trials for MASH reduces ER stress, likely via regulating lipid metabolism.\(^9^4\)

Several pharmaceutical agents, including empagliflozin, liraglutide, metformin, pioglitazone, and rapamycin, are associated with a reduction in ER stress, likely indirectly given their mechanisms of action are not direct components of the ER stress response.\(^9^2\) Among the multiple small molecule compounds that have been discovered, few have been studied in the context of MASLD and warrant future studies in this direction. 4\(\mu\)C binds to the IRE1\(\alpha\) endoribonuclease domain and thereby inhibits the RIDD activity and XBP-1 splicing.\(^9^5\) In studies that explored the interplay of stellate cells, neoplastic hepatocytes, and ER stress in mice with fibrotic hepatocellular carcinoma, 4\(\mu\)C reduced tumor burden and collagen deposition by blocking IRE1\(\alpha\)-induced stellate cell activation.\(^9^6\) Additionally, 4\(\mu\)C has also shown to reduce carbon tetrachloride (CCL4)-induced liver injury and fibrosis.\(^9^7\) A modulator of the PERK pathway, salubrinal prevents eIF2\(\alpha\) dephosphorylation and improves HepG2 cell viability in response to tunicamycin-induced ER stress.\(^9^8\)

Ursodeoxycholic acid (UDCA), a chemical chaperone that promotes protein folding and its appropriate assembly is US Food and Drug Administration—approved to treat primary biliary cholangitis.\(^9^9\) Additionally, taurolursodeoxycholic acid (TUDCA) reduces apoptosis and mitochondrial depolarization, and enhances insulin sensitivity. A compound with similar mechanism of action, 4-PBA is also
approved for treatment of urea-cycle disorders. 4-PBA lowers the eIF2α phosphorylation and inhibits ER stress—mediated apoptosis and hepatic inflammation, evidenced by a reduction in plasma TNFα and MPO levels.22 Outside of its protein folding ability, 4-PBA also increases the secretion of mutant alpha 1 antitrypsin (AAT) protein, allowing for its use in reduction of lung and liver injury in AAT deficiency.100

These studies suggest that reducing the overall burden of lipotoxic species leads to an improvement in ER stress—induced deleterious signaling outcomes. Additionally, therapies that improve ER proteostasis and optimize ER lipo-stasis may serve to mitigate lipotoxicity. For example, selective transmembrane-domain—mediated activation of ATF6α may serve to increase FA oxidation. Fine-tuning IRE1α activation to avoid deleterious consequences of hyperactive IRE1α may benefit steatosis. Inhibition of de novo ceramide synthesis, which is activated downstream of IRE1α in MASH, is efficacious in mouse models, yet remains untested in humans. Exploiting the PERK pathway by increasing ATF4 activation while inhibiting CHOP activation may also be of benefit in lipotoxic diseases.

Summary and Conclusions

Obesity and insulin resistance have increased the rates of MASLD at an epidemic scale, both in the United States and worldwide. The interconnectedness of the ER to other membranous organelles allows it to function as a stress-sensing platform. Lipotoxic stress in fatty liver is sufficient to activate the UPR sensors with downstream effects on stress.

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Disclosure Statement

None declared.

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