Review

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# Lipotoxic endoplasmic reticulum stress, β cell failure, and type 2 diabetes mellitus

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**Failure of the unfolded protein response (UPR) to maintain optimal folding of pro-insulin in the endoplasmic reticulum (ER) leads to unresolved ER stress and β cell death. This contributes not only to some rare forms of diabetes, but also to type 2 diabetes mellitus (T2DM). Many key findings, elaborated over the past decade, are based on the lipotoxicity model, entailing chronic exposure of β cells to elevated levels of fatty acids (FAs). Here, we update recent progress on how FAs initiate ER stress, particularly via disruption of protein trafficking, and how this leads to apoptosis. We also highlight differences in how β cells are impacted by the classic UPR, versus the more selective UPR that arises as part of a broader response to lipotoxicity.**

## The classic UPR

The UPR (see Glossary) comprises three major signaling arms initiated by protein kinase RNA-like endoplasmic reticulum kinase (PERK), the transcription factor activating transcription factor 6 (ATF6), and the serine/threonine-protein kinase/endoribonuclease IRE1 (Figure 1). Each of these sensor proteins straddles the ER membrane and detects alterations in the load of misfolded protein via their lumenal domains [1]. It was originally thought that the three sensors were all held in an inactive conformation by their association with the protein chaperone BiP/Grp78, and activated by release of BiP in response to an accumulation of misfolded protein. However, at least in the case of IRE1 (and possibly PERK), misfolded protein appears to interact directly with the lumenal domain [1]. IRE1 activation proceeds firstly via dimerization and then higher-order oligomerization, which promotes the alternative splicing and nuclear translocation of a transcription factor, X-box binding protein 1 (XBP-1). The IRE1 endoribonuclease also promotes the degradation of diverse mRNAs, which is beneficial when it helps restrict protein influx into the ER by targeting secretory proteins, but is detrimental when it prevents synthesis of protein chaperones during prolonged or strong stress [2]. The protein kinase function of IRE1 appears dispensable for activation, and probably serves a subtle regulatory or counter-regulatory role in stabilization and destabilization of the oligomeric complexes [1]. By contrast, PERK autophosphorylation is

## Glossary

**Akita mice:** mice that contain a C96Y mutation in one of the two mouse pro-insulin genes resulting in diabetes as a consequence of ER stress, insulin depletion, and β cell apoptosis. The mutation (among others) also contributes to rare forms of diabetes in humans.
**Autophagy:** a degradation route whereby surplus macromolecules are incorporated into lysosomes where further breakdown (such as proteolysis) occurs. Classically activated by starvation, autophagy can also be triggered by ER stress, and potentially helps remove misfolded protein, although the topology and molecular details of this role remain uncertain.
**Chaperone:** a molecule that enhances protein folding. It can be a protein itself, such as the cytosolic heat-shock proteins or BiP/Grp78, which functions in the ER lumen. Pharmacological agents, such as PBA, also act as protein chaperones.
**ER-associated degradation (ERAD):** process whereby misfolded protein is retrotranslocated out of the ER and degraded in the proteasome.
**ER stress:** a consequence of any perturbation whereby misfolded protein accumulates in the lumen of the ER. This triggers the UPR.
**Intrinsic apoptosis:** a self-programmed form of cell demise that minimizes release of toxic waste products and inflammation. It involves activation of proteolytic enzymes, executioner caspases, and fragmentation of DNA, and is triggered by irredeemable cell stress, including ER stress. Upstream signaling involves activation of BH3-only proteins, which represses members of the antiapoptotic Bcl-2 family. This in turn stimulates the pro-apoptotic Bcl-2 proteins, leading to mitochondrial disruption and caspase activation.
**Lipid raft:** a more ordered or rigid subdomain of an otherwise more fluid membrane bilayer. These are characterized by enrichment for sphingomyelin and free cholesterol, and a more saturated profile of FA side chains. Specific protein complexes segregate into lipid rafts and in the proximal secretory pathway they have a role in cargo selection.
**Protein trafficking:** refers to proteins destined for secretion from the cell or for insertion in the plasma membrane, and that are synthesized on ribosomes associated with the ER. Once folded correctly, they are packaged into small vesicles at exit sites on the ER and transported to the Golgi complex. Here, protein cargo is further sorted for delivery into secretory granules.
**Pro-insulin:** the major secretory cargo of β cells that is trafficked from the ER to Golgi and subsequently packaged into secretory vesicles (granules). Pro-insulin is processed to insulin via the action of endopeptidases in these post-Golgi compartments. Humans have one pro-insulin gene, but mice and rats have two.
**Sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA):** an ATP-consuming pump in the ER membrane responsible for transporting $Ca^{2+}$ against its own concentration gradient into the ER lumen.
**Sphingolipids:** together with phospholipids and neutral lipids, these serve as a major lipid class. Ceramide is the central sphingolipid, from which the more abundant sphingomyelin is generated.
**Unfolded protein response (UPR):** represents a signaling and transcriptional pathway triggered by accumulation of misfolded protein in the lumen of the ER. Its primary purpose is to help restore folding capacity (the adaptive UPR) but if unresolved leads to apoptosis (terminal UPR).

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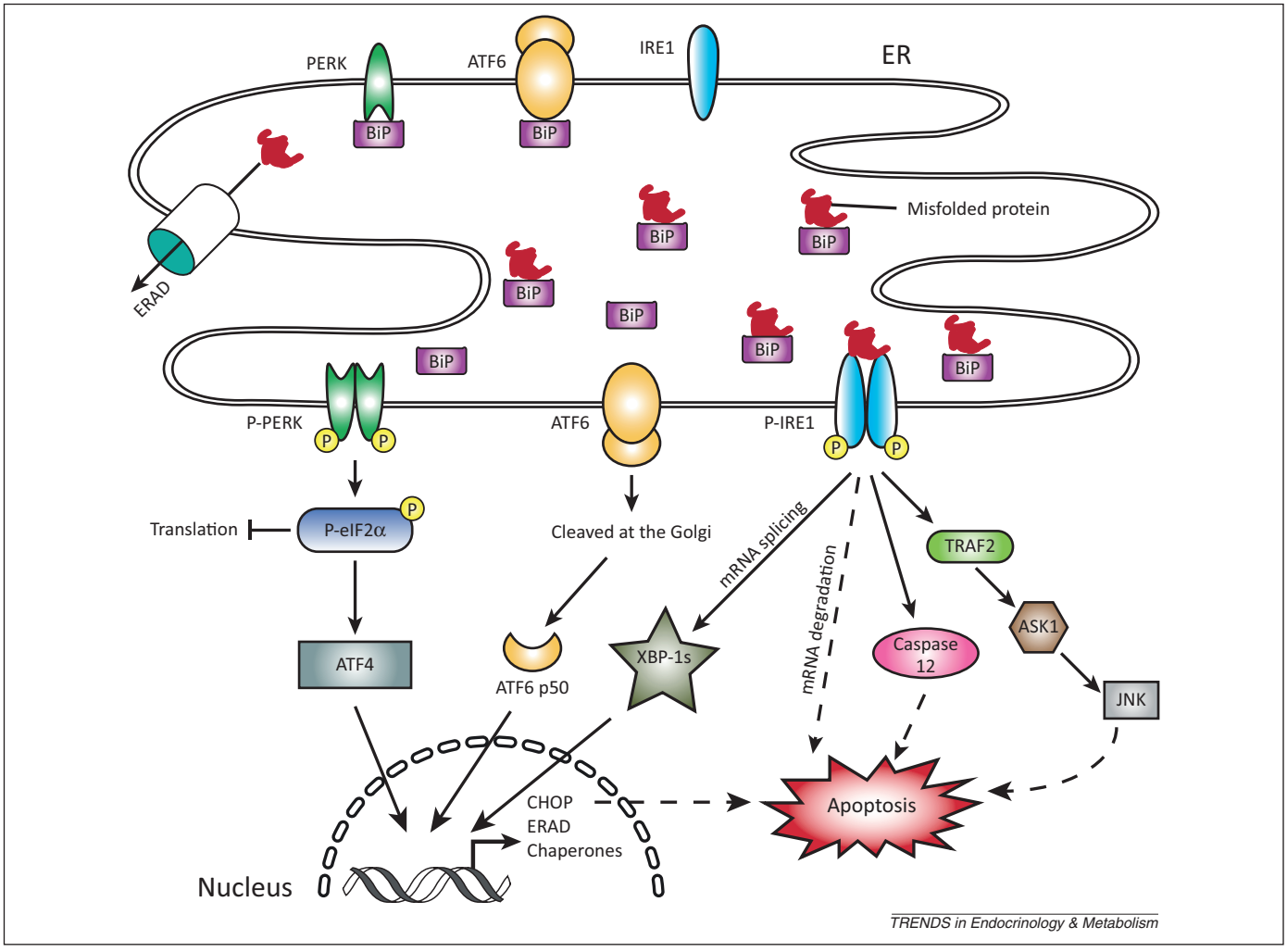


**Figure 1**. Classic pathways of endoplasmic reticulum (ER) stress and the unfolded protein response (UPR). The ER transmembrane sensors protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and IRE1 sense misfolded protein via their lumenal domains. This occurs either directly for IRE1 (and possibly PERK) and by displacement of BiP/Grp78 for ATF6 and PERK. ATF6 is a transcription factor whose activation involves release from the ER and proteolytic cleavage in the Golgi. IRE1 is both an endoribonuclease and a protein kinase. Its activation results in alternative splicing and nuclear translocation of X-box binding protein 1 (XBP-1), another transcription factor. PERK is a protein kinase, which phosphorylates the translational initiation factor eukaryotic initiation factor 2 (eIF2)-α and transiently blocks general protein translation. However, some proteins are preferentially synthesized under these conditions, including the transcription factor ATF4. The net result of this transcriptional program is the enhanced expression of proteins helping to counter ER stress (the adaptive UPR) such as protein folding chaperones. Another adaptive response is upregulation of ER-associated degradation (ERAD), whereby chronically misfolded protein is exported from the ER and broken down by the proteasome. If ER stress remains unresolved, apoptosis is triggered as part of the terminal UPR. This involves the transcription factor CCAAT/-enhancer-binding protein homologous protein (CHOP), but in some cell types, other pathways downstream of IRE1 have also been implicated. These include activation of the protease, caspase 12, degradation of mRNAs encoding protein chaperones, and a signaling cascade initiated via the adaptor protein TNF receptor-associated factor 2 (TRAF2), and the apoptosis signal-regulating kinase 1 (ASK1)/c-Jun N-terminal kinase (JNK) protein kinase pathway.

essential for downstream signaling, initially by phosphorylation of the translational initiation factor eukaryotic initiation factor 2 (eIF2)-α, which results in a transient blockade of general protein translation. However, some proteins are preferentially synthesized under these conditions, including the transcription factor ATF4 [1]. The related protein, ATF6 is activated more directly following removal of BiP/Grp78. This facilitates the vesicular transfer of full-length ATF6 to the Golgi complex, where an active p50 fragment is released by proteolysis [1].

## β cells and the UPR

ER stress arises from an accumulation of misfolded protein that can be caused by either of two broad mechanisms: (i) protein overload, whereby the rate of synthesis exceeds that of protein exit from the ER into the secretory pathway; or (ii) alterations in the ER milieu that compromise folding efficiency. In either case, the UPR is triggered in an attempt to resolve ER stress. β cells, where pro-insulin accounts for more than 50% of total protein synthesis, require a highly efficient UPR [3,4]. This is true especially under conditions of enhanced insulin demand, such as occurs during obesity in compensation for insulin resistance (Box 1). Indeed, mild activation of the UPR contributes to the acute stimulation of pro-insulin biosynthesis by glucose. Although the details are unclear, glucose promotes phosphorylation of IRE1 [5,6], and this is required for stimulating pro-insulin translation [5]. Moreover, derepression of eIF2α phosphorylation [7–9], which occurs by induction of protein phosphatase activity downstream of PERK [9], also contributes to glucose-stimulated pro-insulin biosynthesis. However, longer-term exposure to glucose (glucotoxicity; Box 2) represses pro-insulin gene expression by destabilization of its mRNA [10,11].

**Box 1. Type 2 diabetes mellitus**

Diabetes is a major global health problem, particularly for the developing world and indigenous populations. T2DM, which accounts for 80% of overall incidence, is strongly associated with both obesity and insulin resistance. However, it must be stressed that only 20% of all obese individuals ever develop the fasting hyperglycemia or impaired glucose tolerance that characterizes T2DM [95]. Thus, insulin resistance is usually countermanded by enhanced secretion of insulin from the β cells of the pancreatic islet, and T2DM only develops in those individuals where this compensatory response fails [81,95]. This suggests that a genetic defect in β cell function, or adaptation, is essential for the development of the disease. Many candidate genes have been recently proposed from genome-wide scans, but these still represent only a small fraction of the heritable risk and their investigation has not yet afforded as much mechanistic insight into the disease as has been gained from functional studies. The latter have characterized several broad features of β cell failure, including: an enhancement of basal insulin secretion; a reduction in stimulated insulin secretion particularly in the initial response to glucose; dysregulation of the pulsatility of secretion; an increase in the secreted ratio of pro-insulin to insulin; a depletion of insulin content; an enhanced release of glucagon; and a relative loss of β cell mass due to apoptosis [81]. The relative importance of these individual features, and the cellular mechanisms underlying them, are still being intensely debated and studied.

## ER stress and lipotoxicity

Although early work established that ER stress was sufficient to cause β cell death and dysfunction [12–15], relevance to the more prevalent T2DM (Box 1) was initially suggested using *in vitro* models of lipotoxicity (Box 2). Thus, ER stress was correlated [16–19] and then causally linked [18,20,21] to apoptosis in β cells chronically exposed to elevated FAs. Further studies confirmed that ER stress markers were elevated in pancreatic islets of patients with diabetes [18,22,23]. However, it needs to be emphasized that ER stress is not the sole means whereby lipotoxicity impairs β cell function (Box 3). Moreover, lipotoxic ER stress differs in some ways from the classic UPR, most obviously because of the capacity for independent modulation of downstream pathways, but potentially also from the way it is triggered. Interestingly, there are some indications that the arms of the UPR can be differentially regulated depending on whether it is initiated by protein overload versus protein misfolding [24].

**Box 2. Nutrient toxicity**

Here, we outline the different kinds of nutrient toxicity.

**Lipotoxicity**

Lipotoxicity refers to the harmful effects of lipid accumulation in peripheral tissues when the supply of nutrient FA overwhelms the storage capacity of adipose tissue. It has come to apply especially to cell death, but as originally coined in the Unger lab [52], and as used here, also covers other aspects of β cell failure (Box 3).

**Glucotoxicity**

Chronic exposure to hyperglycemia is the major cause of morbidity and mortality in T2DM through effects on the vasculature as well as kidneys, the retina, and peripheral nervous system. It also contributes to progressive β cell failure through effects on both mass and function. However, the evidence is stronger for oxidative stress rather than for ER stress as a causative mechanism [96]; moreover, the induction of ER stress in models of β cell glucotoxicity is generally less apparent than those of lipotoxicity, at least in mice and humans [21]. However, it must be emphasized that several arms of the UPR have key roles in adjusting pro-insulin biosynthesis to acute variations in glucose availability [5–9]. These are perturbed by chronic exposure to glucose, but this topic has not been investigated as fully as lipotoxicity.

**Glucolipotoxicity**

Cellular fatty acids are usually metabolized (and thus removed) by β-oxidation but in the presence of glucose are partitioned towards more complex intermediates that are potentially harmful [96]. Thus, glucose exacerbates lipotoxicity, but it remains unclear whether this entirely explains the phenomenon or whether glucolipotoxicity is something greater than the sum of its individual parts.

**Box 3. Lipotoxicity as a model of T2DM: pros and cons**

Circulating FAs are elevated in obesity, but this in itself is insufficient to justify the focus on lipotoxicity for the simple reason that only 20% of obese humans ever develop the β cell failure that defines and underlies T2DM. Therefore, we need to invoke subtle further elevations in FAs in diabetic individuals, or consider that disease susceptibility might be determined by genetic variations in the ability of β cells to metabolize FAs appropriately. There is evidence for both possibilities [97,98].

Experimentally, chronic exposure to FAs recapitulates many of the key features of β cell failure associated with T2DM, including: apoptosis; elevated basal secretion; diminished GSIS; reduced insulin content and gene expression; and defective pro-insulin processing [81]. It is also sufficient to reproduce other features of islets from T2DM subjects including (of most relevance here) ER stress, autophagy, and dysregulation of protein ubiquitination (see main text). This list is compelling and, thus, β cell lipotoxicity is the most commonly used *in vitro* model for screening mechanisms with potential relevance to T2DM. However, it is not infallible, especially in some protocols where cells are exposed to high concentrations of FAs. Free FA availability is determined by the FA:bovine serum albumin (BSA) molar ratio but in a nonlinear manner: doubling this ratio from 2.5:1 to 5:1 raises the free FA concentration by more than fivefold [96]. Studies involving ratios at the higher end of this scale are difficult to interpret, and protocols using no BSA at all should be avoided.

High-fat feeding is most commonly used as an *in vivo* model of lipotoxicity. Although useful in studying defects in insulin synthesis and secretion, unfortunately high-fat diets generally evoke little ER stress (or apoptosis) in β cells [63,99]. This may be due to metabolic adaptations in the β cell or to the difficulty in selectively elevating saturated FAs *in vivo*. Despite these drawbacks, there are numerous examples where the effects of genetic manipulation of ER stress on β cell function are exacerbated by, or only become manifest upon, high-fat feeding [57,63,71,100].

## Triggers of lipotoxic ER stress

### *Protein misfolding*

Although not implicated in T2DM, there are several rare human mutations that cause misfolding of pro-insulin, resulting in ER stress and β cell failure. The Akita mouse provides a useful model of this [25]. By contrast, perturbations in the ER milieu (alterations in pH, redox, or $Ca^{2+}$) that compromise the overall folding capacity, might have a more common role in β cell failure (Figure 2A). Certainly, many ER stressors, including nitric oxide, cytokines, and glucotoxicity, appear to act by diminishing lumenal $Ca^{2+}$ content [14,26,27]. A similar mechanism has been invoked to explain lipotoxic ER stress [21,27] but the evidence is not yet definitive. Given that measuring ER $Ca^{2+}$ depletion is technically challenging, variable effects of individual FAs have been reported in terms of extent, timing, and specificity [16,21,28]. In a recent study, both palmitate (albeit tested only at high concentrations) and the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pump inhibitor,

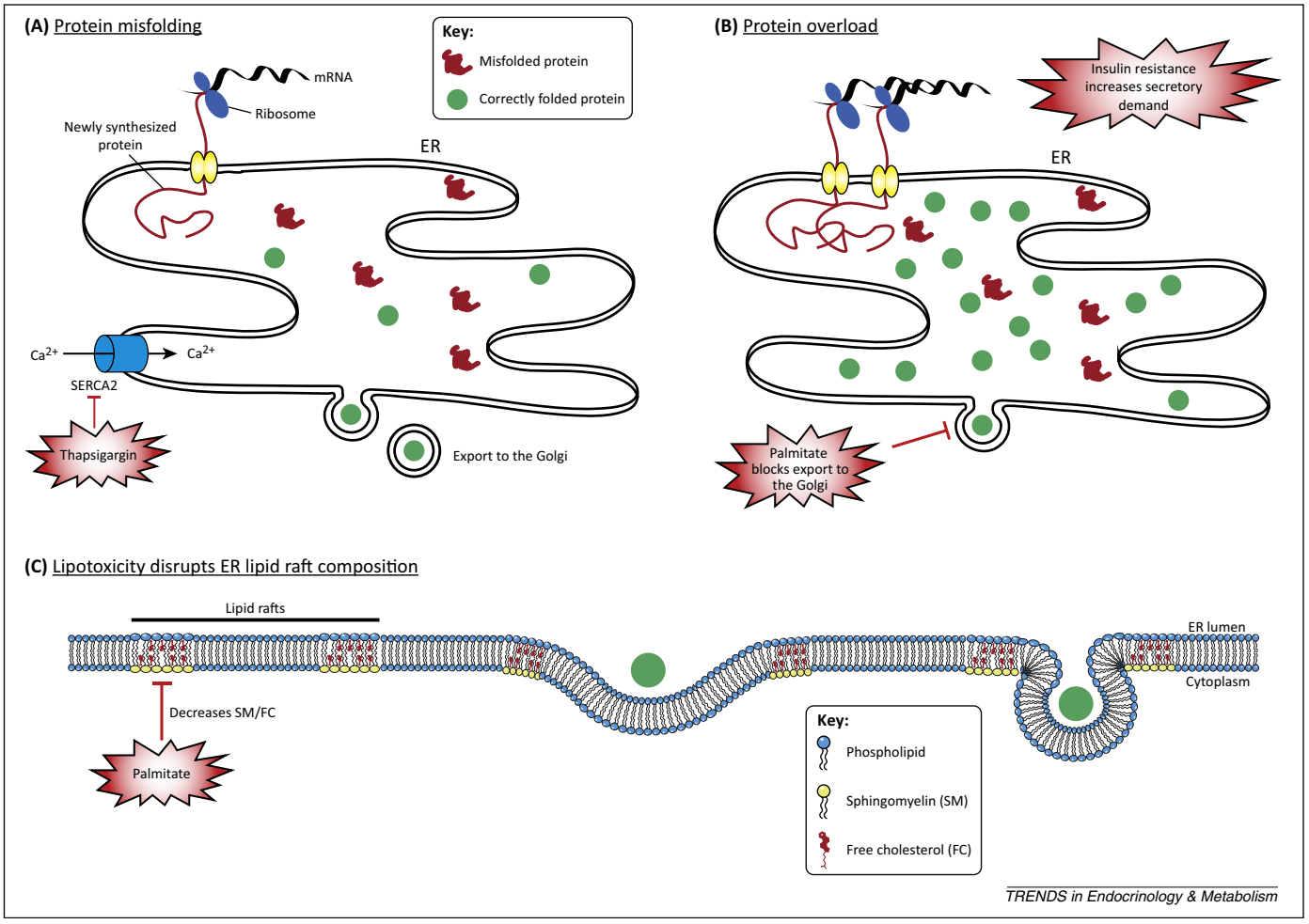


**Figure 2**. Protein misfolding, protein overload, and lipotoxicity. Alterations in folding efficiency can arise from various disturbances in the endoplasmic reticulum (ER). One of the best characterized of these is depletion of $Ca^{2+}$ via blockade of the $Ca^{2+}$ pump using agents such as thapsigargin. This can cause ER stress without any changes in amount of protein transiting through the ER **(A)**. Alternatively, misfolded protein could accumulate due to enhanced protein synthesis, without any change in folding efficiency. For pro-insulin, this would probably occur in compensation for insulin resistance in other tissues, but this is unlikely to be sufficient to cause ER stress in the absence of other factors **(B)**. Protein overload could also arise if exit of secretory cargo from the ER is impaired. This appears to be the case for lipotoxicity. More specifically, chronic exposure to palmitate reduces the level of ER-localized sphingomyelin (SM) and cholesterol, and thereby disrupts the lipid rafts that largely comprise these lipids **(C)**. Although the molecular mechanisms remain to be determined, this would be expected to inhibit protein trafficking. It is also unclear whether ER lipid rafts impact selectively on transmembrane or soluble cargo (or subsets thereof). Abbreviation: SERCA2, sarco/endoplasmic reticulum $Ca^{2+}$-ATPase 2.

thapsigargin, were more effective at lowering ER $Ca^{2+}$ than were cytokines or glucotoxicity [27]. Interestingly, overexpression of mutant secretory proteins also depleted $Ca^{2+}$ from the ER, suggesting that this phenomenon occurs as a consequence, as well as a trigger, of ER stress [27]. Nevertheless, diminished ER $Ca^{2+}$ is necessary for the induction of ER stress in response to cytokines, glucotoxicity, and nitric oxide donors, because the effects of these agents on apoptosis are partially rescued by overexpression of SERCA2b [14,26,27]. However, no such causation has been demonstrated for lipotoxic ER stress. Finally, whereas both glucotoxicity [27] and cytokines [26,27] inhibit SERCA2b expression in β cells, there is no evidence that this occurs with lipotoxicity [21,27].

By contrast, reduced expression of SERCA2b is observed in islets of patients with T2DM [27,29], and accompanies impaired $Ca^{2+}$ handling and β cell dysfunction in db/db mice [27,29–31]. The latter model is more defined by glucotoxicity rather than by lipotoxicity [32], so perhaps loss of SERCA2b and, by extension, protein misfolding, contribute to the progression rather than initiation of T2DM. In conclusion, arguably the role, and certainly the mechanism, of ER $Ca^{2+}$ depletion in β cells is better established for other ER stressors than for lipotoxicity. Moreover, ER $Ca^{2+}$ is only an indirect measure of the actual state of protein folding. However, this has been analyzed directly in a study making use of an ER-localized reporter, encompassing the vesicular stomatitis virus glycoprotein tagged with GFP (VSVG-GFP). Using an antibody specific for the native (correctly folded) conformation of VSVG-GFP, palmitate was not shown induce protein misfolding, whereas thapsigargin did [33]. Hopefully, techniques for assaying the folding state of endogenous protein cargo under lipotoxic or diabetic conditions will become available to help better address these issues.

### *Protein overload*

Recent studies using both lipotoxic and genetic models of β cell failure and ER stress attest to the importance of protein overload. Most obviously, it might be expected that ER stress would arise from a compensatory increase in proinsulin biosynthesis within β cells in an attempt to counter insulin resistance (Figure 2B). Although it might contribute in the background, it is important to realize that this

mechanism is not sufficient to promote terminal ER stress in β cells, because T2DM only develops in a minority of insulin-resistant patients (Box 1). Moreover, *in vitro* or *in vivo* models of (nonmutant) pro-insulin overexpression display only modest ER stress [34–36]. This suggests that coping with enhanced pro-insulin biosynthesis is itself part of the normal compensatory response for insulin resistance and, conversely, that an inability to do so might contribute to diabetes susceptibility [37].

Protein overload might alternatively result from impaired exit of protein from the ER, as opposed to enhanced biosynthesis (Figure 2B). Indeed, several groups have now made use of β cells transfected with the temperature-sensitive VSVG-GFP reporter. This accumulates in the ER of cells maintained at a nonpermissive temperature, but is rapidly trafficked to the Golgi after switching to 32°C. In this manner, lipotoxicity has been conclusively shown to disrupt ER-to-Golgi protein trafficking [33,35,38,39]. This defect was specific for saturated FAs [33], and occurred upstream of, and rather than secondary to, ER stress because thapsigargin only modestly slowed trafficking despite a stronger induction of ER stress than by saturated FAs [33]. Retention of GFP-tagged pro-insulin in the ER, suggestive of impaired export into the secretory pathway, has also been observed during glucolipotoxicity [40]. However, a drawback of all these studies is that they rely on fluorescent reporters, and trafficking of endogenous cargo has not yet been addressed. However, an independent line of evidence suggests that the budding of ER-derived vesicles, which contain endogenous secretory cargo, is also inhibited in β cells chronically exposed to saturated FA, consistent with an impairment of protein trafficking [41].

There is also evidence implicating defective protein trafficking as an early feature of β cell failure in animal models [42,43]. Surprisingly, these include Akita mice that might have been expected to reflect a pure model of protein misfolding. However, the mutant pro-insulin acts in a *trans*-dominant manner to block export of secretory cargo from the ER [25,44]. The resultant protein overload promotes ER stress, and leads to an inhibition of protein synthesis, loss of insulin content, and eventually apoptosis [25,44]. Interestingly, in models where the pro-insulin mutant protein is only modestly expressed relative to wild type protein, the mice are not overtly diabetic [34]. However, they do display impaired glucose tolerance associated with some depletion of pro-insulin and activation of ER stress markers. In addition, individual β cells showing the greatest loss of insulin content also displayed an accumulation of (native) pro-insulin in the ER and enhanced ER stress [34]. Importantly, a similar pattern was observed in pre-diabetic db/db mouse islets [42]. Morphologically, this is also associated with a distended ER and depletion of insulin granules, features observed in many murine models of diabetes [25] as well as human disease [23]. These studies suggest that impaired ER-to-Golgi protein trafficking is an early general defect that leads to ER stress due to protein overload, resulting in impaired pro-insulin maturation and loss of insulin content before apoptosis. However, the exact sequence of these events, and the mechanisms that link them, remain to be elucidated. Finally, it should be noted that strong ER stress itself compromises protein trafficking [33,35,45]; thus, protein overload might also amplify a response triggered initially by protein misfolding.

### *The toxic metabolite*

Another key issue, highly relevant for therapeutic intervention, is the identity of the metabolite or mechanism by which saturated FAs compromise β cell protein folding or trafficking. Studies with non-β cell models suggest several possibilities. Firstly, SERCA activity might be disrupted by increases in either cholesterol or the ratio of phosphatidylcholine:phosphatidylethanolamine (PC:PE) in the ER membrane [46,47]. Another possibility would be a general enhancement in ER phospholipid saturation that might impair ER structure and/or the budding of secretory vesicles [48,49]. Finally, there is evidence that PERK and IRE1 can be activated by ER lipid saturation completely independently of the interaction of their lumenal domains with misfolded proteins [50]. By contrast, recent work argues against these mechanisms having a role in palmitate-treated β cells, where neither PC:PE nor phospholipid saturation of the ER membrane was altered by genetic manipulations of lipid metabolism that relieve defective protein trafficking and ER stress [38,41]. Instead a role for sphingolipids, initially ceramide [38], was highlighted, consistent with observations linking this metabolite with lipotoxic apoptosis in β cells [51,52] and ER stress in yeast [53]. Surprisingly, however, an increase in ER ceramide appeared not to be the toxic mechanism, but rather a selective and ER-localized decrease in both sphingomyelin and free cholesterol, leading to dysregulation of ER lipid rafts [41]. The latter are implicated in the loading of cargo into secretory vesicles [54]; therefore, their disruption in lipotoxic β cells could provide a novel explanation for the accompanying protein overload and ER stress (Figure 2C). Given that other lipid species, including high-density lipoprotein (HDL) and sphingosine-1 phosphate, have also been shown recently to rescue protein trafficking and β cell function [35,39], it will be of interest to determine whether these also impact on ER lipid rafts. Finally, there is a least one other mechanism whereby lipotoxicity could disrupt protein trafficking, given that FAs are known to downregulate the expression of key enzymes involved in pro-hormone processing [55,56]. Although this occurs in post-Golgi compartments, it is possible that reduced processing of pro-insulin might feed back to disrupt ER-to-Golgi transport and, thus, also contribute to protein overload.

## Mechanisms linking ER stress to β cell apoptosis

### *CCAAT/-enhancer-binding protein homologous protein*

Apoptosis has generally been the major focus of studies addressing β cell failure downstream of ER stress, but there is only limited agreement about the distal effector mechanisms (Figure 3). The best studied of these is CCAAT/-enhancer-binding protein homologous protein (CHOP), which is required for full apoptosis in β cells due to lipotoxicity [20,21,57] as well as other inducers of ER stress [13]. One postulated mechanism is the inhibition by CHOP of antioxidant genes [57]. CHOP also induces

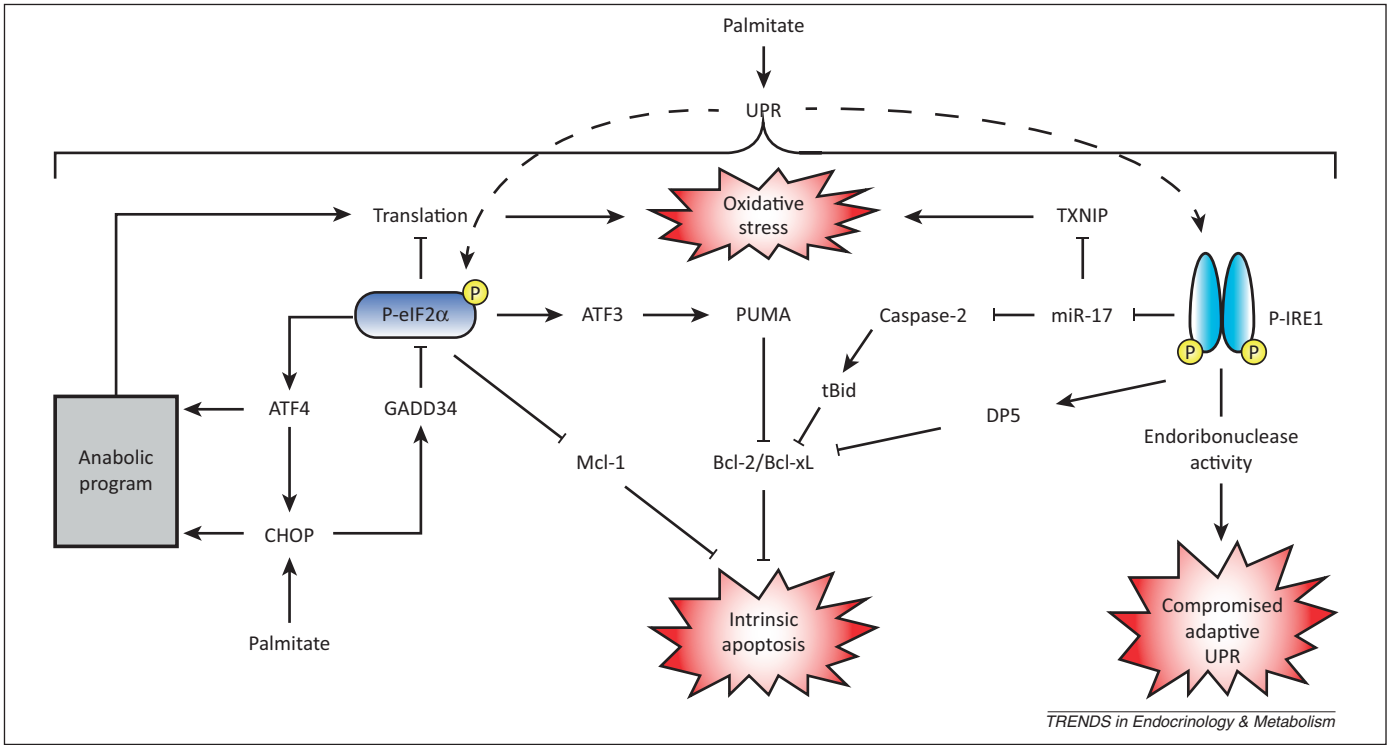


**Figure 3**. Effector pathways connecting endoplasmic reticulum (ER) stress to β cell apoptosis. There are several broad mechanisms by which lipotoxic β cell death might be triggered downstream of ER stress. The translational recovery model postulates the existence of a transcriptional program designed to help cells recover from the early blockade of protein synthesis due to eukaryotic initiation factor 2 (eIF2)-α phosphorylation. This is mediated not only directly by DNA damage-inducible protein (GADD34), a component of the phosphatase complex that targets eIF2α, but also by broader upregulation of genes involved in amino acid transport and handling. An inappropriate recovery of protein synthesis would both further exacerbate ER stress, and promote oxidative stress as a consequence of the generation of reactive oxygen species during oxidative protein folding. An alternative model highlights IRE1 as a key switch between the adaptive and terminal unfolded protein response (UPR): it helps resolve mild ER stress via activation of X-box binding protein 1 (XBP-1) splicing, but turns executioner under harsher conditions via its capacity to degrade mRNAs encoding key components of the ER protein homeostatic machinery. Finally, more direct links to the intrinsic apoptotic pathway have been proposed, via regulation of the BH3-only proteins death protein 5 (DP5) and p53 upregulated modulator of apoptosis (PUMA), as well the Bcl-2 family members, such as Mcl-1. Only some of these mechanisms have been directly implicated in lipotoxic ER stress, which is likely to represent a special case, if only because it can regulate the central player CCAAT/-enhancer-binding protein homologous protein (CHOP), through transcriptional and post-transcriptional mechanisms that are independent of the classic UPR. Abbreviations: ATF, activating transcription factor; TXNIP, thioredoxin interacting protein.

growth arrest and DNA damage-inducible protein (GADD34), which by promoting dephosphorylation of eIF2α, helps counter the effects of PERK activation and derepresses translation [57,58]. The net consequence of this program would be an oxidative stress that might promote β cell death not only directly, but also indirectly by further compromising protein misfolding and exacerbating ER stress [57,58]. These studies are based on elegant *in vivo* models, involving selective modulation of eIF2α phosphorylation, but might represent a more restricted setting than occurs with lipotoxicity. The strongest support for physiological relevance arose from crossing CHOP null mice onto the db/db background, resulting in clear protection against β cell oxidative stress [57]. However, in a complex whole-body phenotype, this might also be explained by improvements in hyperglycemia, or by lipid sparing in β cells secondary to enhanced adiposity [57,59].

### *ATF4*

More recent work, although supporting the translational recovery model for oxidative cell death, argues for a more important role for ATF4 versus CHOP [60,61]. The two transcription factors induce a partially overlapping set of genes, constituting an anabolic program of amino acid handling and protein synthesis (Figure 3), which in addition to GADD34, further promotes translational recovery [60]. Expression of several of these genes is also increased in islets of Akita mice, corresponding to an enhancement of protein synthesis versus control islets [61]. However, other observations argue against a cytotoxic role for ATF4: one of its direct target genes, eukaryotic translation initiation factor 4E-binding protein 1 (eIF4EBP1), promotes β cell survival [62], and its upregulation has been postulated to contribute to improved β cell function both downstream of the master gene regulator pancreatic and duodenal homeobox 1 (Pdx1) [63], and following treatment with the insulin secretagog glucagon-like peptide-1 (GLP-1) [64].

Perhaps one explanation for these apparently conflicting findings would be that the translational recovery hypothesis, delineated mainly using strong pharmacological stressors *in vitro*, or mice with defective pro-insulin folding or eIF2α/PERK signaling, represents a more simplified situation than occurs with T2DM or even lipotoxic models. At one level, this appears obvious because the direct transcriptional induction of CHOP by palmitate differs from that due to pharmacological stressors [65]. Moreover, recent evidence points to another role for palmitate in regulating the deubiquitylation and stabilization of CHOP protein [66]. Thus, lipotoxicity can impact on CHOP expression independently of upstream activation of

the UPR (Figure 3). However, there are more specific examples of a discrepancy between translational recovery and lipotoxicity. Most notably, whereas pharmacological inhibition of eIF2α dephosphorylation (thus repressing translational recovery) protects against β cell apoptosis due to chemical ER stressors [61], the same approach worsens ER stress and cell death due to lipotoxicity [67]. Moreover, chronic exposure to FAs inhibits pro-insulin synthesis both *in vivo* [68] and *in vitro* [69], as opposed to stimulating it, as might be expected from the translational recovery model.

### *Intrinsic apoptotic pathway*

CHOP/ATF4 are not the sole effectors of lipotoxic ER stress in β cells and more direct links to the apoptotic pathway have been proposed (Figure 3). In one scenario, early translational repression via PERK/eIF2α phosphorylation leads to loss of the rapidly turned-over protein, myeloid cell leukemia sequence 1 (Mcl-1), an antiapoptotic member of the BH3 family [70]. Conversely, pro-apoptotic members, p53 upregulated modulator of apoptosis (PUMA) and DP5/Hrk, are also upregulated by palmitate, downstream of PERK but independently of CHOP/ATF4 [71]. Instead, ATF3 is a key player, synergizing with IRE1/JNK signaling in the case of DP5/Hrk, and serving upstream of TRB3 and forkhead box O3 (FoxO3a) for PUMA. A partial requirement for each of these various proteins in lipotoxic ER stress was established by modulation of their expression *in vitro* [70,71], and further supportive evidence was provided *in vivo* using a whole-body knockout mouse for DP5/Hrk [71].

### *mRNA degradation*

Although it had been known that IRE1 activation directly regulates degradation of many ER-associated mRNAs, an elegant recent study [72] invokes a novel mechanism involving depletion of the miRNA miR-17 that in turn controls stability of its own mRNA targets (Figure 3). One such is thioredoxin interacting protein (TXNIP), which was previously implicated in mediating oxidative stress due to glucotoxicity [73]. TXNIP is induced by strong ER stress [72,74] and its deletion rescues glucose intolerance and β cell apoptosis in the Akita mouse [72]. TXNIP potentially acts downstream of ER stress to induce not only oxidative stress, but also inflammation [72,74], both of which have been linked to β cell failure in T2DM. Yet, TXNIP is neither induced in islets by chronic exposure to FAs, nor necessary for lipoapoptosis [73]. Thus, although TXNIP is an important downstream mediator of pharmacological or genetic ER stress, its relevance to lipotoxicity, and arguably T2DM, remains to be established. However, miR-17 targets other mRNAs in addition to TXNIP, such as caspase-2, which might link ER stress to the intrinsic apoptotic pathway via activation of the pro-apoptotic BH3 protein Bid [75]. Clearly, more work is needed to establish a more complete picture of how ER stress mediates β cell apoptosis in the setting of T2DM.

## ER stress and defective insulin release

### *Regulation of insulin content*

As discussed above, individual β cells displaying a build up of pro-insulin in the ER also show evidence of ER stress and loss of insulin content even before cell death [34,42]. Impaired ER-to-Golgi trafficking itself probably contributes directly to this insulin depletion by compromising the maturation of pro-insulin in post-Golgi compartments. However, there is also extensive evidence for the role of the UPR, most notably because mRNA for pro-insulin, and related secretory or processing proteins, is targeted by the endoribonuclease activity of IRE1 following strong ER stress [2,10,11]. ER stress can also repress pro-insulin gene expression in β cells via indirect effects of ATF6 [76,77]. A similar role for XBP-1 splicing has been proposed, mediated in part by regulation of Pdx1 [78]. However, neither of these mechanisms has been linked to lipotoxic ER stress, and there are other mechanisms by which FAs are known to repress insulin gene transcription more directly and independently of the UPR [79]. There is also evidence that improvements in ER protein folding can partially overcome impaired insulin biosynthesis due to glucotoxicity, but the relevance of this to lipotoxicity is unknown [80].

### *Is there a specific impairment of glucose-stimulated insulin secretion?*

A key feature of T2DM is the selective loss of secretory responsiveness to glucose, which occurs in the proximal pathways of stimulus-secretion coupling, independently of reductions in β cell mass and/or insulin content [81]. Although the latter are clearly linked to ER stress, this is less obvious for secretory dysfunction. Perhaps the strongest supportive evidence comes from a model of glucotoxicity, where *in vivo* administration of 4-phenylbutyrate (PBA) resulted in improved glucose-stimulated insulin secretion (GSIS) from islets *ex vivo* with little alteration of insulin content [82]. However, it cannot be excluded that there are indirect benefits on β cell function in this model due to systemic effects of PBA. Although there are also numerous *in vitro* studies purporting to show a restoration of GSIS following resolution of ER stress, this could more simply be explained by a repletion of insulin stores, as was observed in one of the few investigations where both insulin content and secretion were measured in parallel [80]. β cell dedifferentiation has also been linked to ER stress in mouse models [37,57,58] and to glucotoxic ER stress *in vitro* [83], but whether these alterations in mRNA expression equate to a functional loss of stimulus-secretion coupling has not been rigorously established. Stronger evidence for a link between ER stress and secretion revolves around a novel role for Wolfram syndrome 1 (Wfs1) [84]. This gene product normally represses the ATF6 arm of the UPR, such that naturally occurring mutations in WFS1 result in ER stress, β cell death, and a rare form of diabetes known as Wolfram syndrome [85]. The new study convincingly shows that glucose promotes the trafficking of Wfs1 from the ER to plasma membrane, where it positively regulates cAMP generation, an amplifying signal for both distal exocytosis and insulin gene expression [84]. The cAMP pathway was inhibited by thapsigargin (consistent with Wfs1 retention at the ER) but unaltered by resolution of moderate ER stress with the chemical chaperone PBA. It will be of interest to determine whether there are other potential pathways for reciprocal modulation of ER stress and insulin secretion.

### ER stress and ubiquitination

The ubiquitin-proteasome system (UPS) is believed to be the primary machinery for the degradation of misfolded protein during ER stress, via the ER-associated protein degradation (ERAD) process. Lipotoxicity appears to impact on the UPS, given that one of its key components, ubiquitin carboxyl-terminal esterase L1 (UCHL1), is essential for β cell survival under these conditions [86]. Moreover, a recent study demonstrated the downregulation of many UPS genes and reduction of proteasome activity in T2DM and palmitate-treated human islets [87]. This suggests that lipotoxicity amplifies or even initiates ER stress by directly compromising the UPS. The underlying molecular mechanisms remain unknown. Also poorly understood is the crosstalk between the UPS and autophagy, another means of degrading protein [88,89].

### ER stress and autophagy

Autophagy is triggered by ER stress in many cell types, but the molecular mechanisms, and means for targeting misfolded proteins, are not elucidated in detail [89]. Although autophagy clearly aids β cell survival in both animal and *in vitro* models of lipotoxicity [90–92], the links to ER stress have been less clear. Autophagy in response to FA treatment was only partially inhibited with the protein-folding chaperone, PBA [90] and, where measured, ER stress markers were reduced rather than enhanced in autophagy-deficient islets [92]. This is compounded by an ongoing controversy as to whether FAs stimulate or inhibit autophagy in β cells [90–93]. However, in some models in which β cells would be expected to be undergoing ER stress, such as ob/ob or Akita mice, there was a concomitant increase in autophagy markers [92,94]. In addition, ER stress was inversely modulated when autophagy was manipulated pharmacologically in Akita islets *in vitro* [94]. Thus, the existence of such a reciprocal relation in lipotoxic models of β cell failure needs to be investigated more fully.

## Concluding remarks and future perspectives

Elucidating the contributing roles of ER stress to the β cell dysfunction of T2DM remains a daunting but important task. Firstly, even within the context of the classic UPR, whether a β cell defaults to apoptosis depends on a complex balance between the adaptive and terminal pathways. Secondly, as outlined here, lipotoxic ER stress may exhibit features differing from those of the classic UPR, possibly relating to the manner or strength of its induction, or because of the capacity for FAs to impact (directly and indirectly) at multiple points in the cascade. Finally, it is difficult to establish whether a functional hierarchy exists between the various features of β cell failure that might be attributable to ER stress, such as disruptions in proinsulin processing and trafficking, loss of insulin content, and enhanced apoptosis, or whether these represent alternative or even adaptive responses.

As a practical plan to address these issues, we propose two broader strategic goals, and some key research questions. Firstly, better *in vivo* models are needed, given that the high-fat feeding protocol is less useful for studying ER stress than for inducing insulin secretory defects. As the metabolic inputs underlying lipotoxic ER stress are elucidated, it might be possible to engineer mice that recapitulate these proximal events. These would be invaluable in studying the downstream pathways and functional consequences of ER stress. Secondly, greater effort is required in extending findings from basic models to human T2DM. Although an enhanced UPR has been described in β cells obtained from postmortem tissue, more markers should be tested to clarify both underlying mechanisms and the course of the disease.

A more focused question is whether ER stress inhibits GSIS independently of reductions in insulin content. Given the importance of secretory failure to T2DM, the contribution of ER stress needs to be either confirmed or refuted definitively. Another goal would be to elucidate how FAs impact on ER stress by regulation of protein turnover. Some work has addressed autophagy, but less is known about the role of ERAD in β cell physiology. Finally, more needs to be known about the nexus between ER stress, protein trafficking, and pro-insulin processing. Although lipotoxicity appears to disrupt ER-to-Golgi protein trafficking, it is less clear whether this impacts universally or selectively on different secretory cargo, and especially whether pro-insulin transport and/or processing is thus affected.

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