The integrated stress response and proteotoxicity in cancer therapy

David J. McConkey
Director, Johns Hopkins Greenberg Bladder Cancer, Institute and Professor, Brady Urological Institute, 600 North Wolfe Street, Baltimore, MD, 21287

Abstract
A variety of different forms of cellular stress can cause protein misfolding and aggregation and proteotoxicity. The cytoprotective response to proteotoxicity is termed the integrated stress response and involves 4 distinct serine/threonine protein kinases that converge on the translation initiation factor eIF2α, resulting in phosphorylation at S51, cell cycle arrest, and a general inhibition of global protein synthesis. Phosphorylation of eIF2α also promotes translation of ATF4 and the expression of ATF4 target genes that ameliorate proteotoxic stress but can also promote apoptosis. This mini review provides a general overview of these mechanisms and discusses how the inter-tumor heterogeneity that involves them affects sensitivity and resistance to proteasome inhibitors, a new class of cancer therapeutics that promotes tumor cell killing via proteotoxic stress.

Keywords
ER stress; unfolded protein response; PERK; HRI; translation; protein aggregates; autophagy

Introduction
Proteotoxic stress occurs when misfolded protein accumulation overwhelms cellular protein quality control mechanisms involving the proteasome and autophagy. Interest in defining the molecular mechanisms involved has increased over the past decade because of the recognition that protein aggregation plays critical roles in most neurodegenerative diseases and because of the development of proteasome inhibitors for cancer therapy. The overall goal of ongoing research is to exploit these mechanisms to enhance protein aggregate degradation to delay or prevent cytotoxicity in the former and to enhance proteotoxic stress to promote cytotoxicity in the latter.

Members of the HSP70 family are the first lines of defense in the cellular response to proteotoxic stress. Distinct members of the family localize to the cytosol, mitochondria, and endoplasmic reticulum (ER). They interact with hydrophobic regions within their
clients\textsuperscript{10}, promoting protein folding and transmembrane transport. Heavy metals, heat shock, and oxidative stress can damage proteins and cause exposure of these hydrophobic domains that are normally buried within their interiors, which makes them prone to aggregation\textsuperscript{3}. Members of the HSP70 family therefore play crucial roles in preventing protein aggregation by binding tightly to these exposed hydrophobic regions in damaged proteins as soon as they emerge to promote refolding, and if proteotoxic stress becomes overwhelming, transporting damaged proteins and protein aggregates to the proteasome or autophagosomes for degradation\textsuperscript{3}.

\textbf{eIF2\alpha\ kinases control the response to proteotoxic stress}

Of the different types of proteotoxic stress, most information is available for the molecular mechanisms underlying the responses of cells to endoplasmic reticular (ER) stress\textsuperscript{11,12}. When misfolded proteins accumulate with the ER-Golgi network, glucose-related protein 78 (Grp78), a HSP70 family member that is localized to the ER, releases three constitutive client proteins (PERK, IRE1, and ATF6) that serve as upstream activators of a coordinated signal transduction system known as the unfolded protein response (UPR)\textsuperscript{13}. PERK is a protein serine/threonine kinase whose only known substrate is eukaryotic translation initiation factor-2 alpha (eIF2\alpha), which it phosphorylates on serine 51 (S52 in mice)\textsuperscript{13}. This causes a near complete global shutdown of translation accompanied by a redirection of eIF2\alpha to mRNA targets that encode proteins that alleviate ER stress, including protein chaperones and endoplasmic reticular structural proteins\textsuperscript{12}. One protein that is particularly dependent on ongoing translation for its expression is cyclin D\textsuperscript{14}, which functions as a critical regulator of the G1 to S transition. Consequently, eIF2\alpha phosphorylation causes rapid cell cycle arrest\textsuperscript{14}. PERK exerts these effects by promoting translation of the transcription factor, ATF4, which in turn promotes expression of another transcription factor, DDIT3 (also known as GADD153 or CHOP)\textsuperscript{15}. In parallel, release of Grp78 from IRE1 enables the latter to promote splicing and activation of the transcription factor XBP1, and release of Grp78 from the transcription factor ATF6 enables it to translocate to the Golgi, where it is proteolytically processed and activated\textsuperscript{13}. In summary, the release of Grp78’s constitutive clients that is induced by its tight binding to misfolded proteins serves the general mechanism that initiates the entire UPR.

PERK is one of 4 related protein kinases that are activated by different upstream signals but all phosphorylate the same site on eIF2\alpha\textsuperscript{3}. Protein kinase R (PKR) is activated by double-stranded RNA and plays a central role in the innate immune response to viruses\textsuperscript{16}. General control nonderepressible 2 (GCN2) is activated by uncharged amino acids that accompany amino acid pool depletion\textsuperscript{17}, and heme-related eIF2\alpha kinase (HRI) is activated by heavy metals, heat shock, and proteasome inhibition\textsuperscript{18}. By phosphorylating eIF2\alpha, all of them attenuate proteotoxic stress by downregulating protein synthesis, thereby reducing the upstream input that would serve to increase protein aggregation\textsuperscript{19}. Phosphorylation of eIF2\alpha also promotes disposal of protein aggregates via autophagy\textsuperscript{20}.

Proteasome inhibitors are commonly used to model proteotoxic stress. Most early studies focused on the effects of proteasome inhibitors on PERK activation and ER stress\textsuperscript{21,22}, but it now appears that ER stress plays a major role mostly in cells with very high secretory
capacities, and that the other eIF2α kinases play more important roles in cells that do not. Cells with high secretory capacities are particularly prone to ER stress and require ongoing proteasome activity to remove the misfolded proteins that are produced as a normal byproduct of translation. In these cells proteasome inhibitors cause robust PERK activation, and PERK mediates eIF2α phosphorylation and downstream effects. Other cell types require basal proteasome activity to maintain amino acid pool levels, perhaps because they exhibit particularly high rates of global protein synthesis. In these cells proteasome inhibition activates GCN2, and GCN2 mediates eIF2α phosphorylation. Finally, HRI appears to generally mediate eIF2α phosphorylation in response to cytosolic protein aggregation induced by heat shock, and we have found that proteasome inhibitors are potent activators of HRI in most human cancer cell lines and that in these cells HRI mediates eIF2α phosphorylation (M. White, manuscript under revision). Therefore, any attempt to specifically modulate an eIF2α kinase to produce a desired biological effect in a given cell type would require some knowledge of the specific protein synthesis-related functions of that cell.

Mechanisms of proteotoxicity-associated cell death

Misfolded or denatured monomeric polypeptides are recognized by ubiquitin ligases and targeted to the proteasome for degradation. However, the proteasome’s cap complex can only accommodate protein monomers that must be further unwound prior to insertion into its narrow catalytic core, so larger protein aggregates must be redirected to autophagy for degradation. When both systems are overwhelmed protein aggregates build up in the cytosol and/or within organelles, and the appearance of these protein aggregates is typically followed by cell death. The molecular mechanisms involved have been under active investigation for over a decade, but they appear to be complex and highly cell type-dependent.

Active mechanisms that mediate cell death

In addition to promoting expression of protein chaperones and other cytoprotective proteins, CHOP has also been shown to directly or indirectly induce the expression of canonical proapoptotic proteins, including death receptor-5 (DR5) and PUMA, a proapoptotic BH3-only member of the BCL2 family. In addition, CHOP indirectly induces transcriptional activation of the BH3-only protein, NOXA, via translational activation of ATF5. PUMA is a broad spectrum BH3 protein that interacts with all of the anti-apoptotic members of the BCL2 family, whereas Noxa specifically interacts with and inhibits MCL. Importantly, these effects may “prime” cells for apoptotic death, but execution of apoptosis probably requires a more direct cytotoxic stimulus.

One of CHOP’s primary transcriptional targets is GADD34, a protein phosphatase that dephosphorylates S51 in eIF2α. The physiological function of GADD34 induction is to restore protein translation after proteotoxic stress is resolved. However, GADD34 induction may also serve as a “timer” to initiate apoptosis if proteotoxic stress is not resolved quickly enough. By restoring translation in the face of continuing proteotoxic stress, GADD34 induction exacerbates protein aggregation to promote cell death. This
mechanism probably coordinates the active mechanisms described above with the passive mechanisms described below.

**Passive mechanisms that mediate cell death**

There is abundant circumstantial evidence implicating protein aggregation as the most upstream cytotoxic mechanism in proteotoxicity-induced cell death. However, precisely how protein aggregates cause cell death is still not clear. One attractive possibility is that, by inhibiting flux through the proteasome and autophagy degradation pathways, proteotoxicity inhibits disposal of depolarized and damaged mitochondria, which are normally removed by a specialized form of autophagy known as “mitophagy”\(^{33}\). As a consequence, accumulation of these damaged mitochondria produce reactive oxygen species (ROS) and cause oxidative stress leading to cytochrome c release, caspase activation, and apoptosis. Consistent with this hypothesis, many studies have demonstrated that proteasome inhibitors induce early ROS production\(^{34,35}\) and that this ROS production is blocked by inhibiting protein synthesis (and therefore protein aggregation) with cycloheximide (M. White, manuscript under revision). Antioxidants such as N-acetyl cysteine (NAC) also block proteasome inhibitor-induced ROS production and cell death\(^{34,35}\). In addition to inducing ROS, proteasome inhibitors also disrupt intracellular Ca\(^{2+}\) homeostasis\(^{22}\). Together, ROS and Ca\(^{2+}\) probably promote activation of the intrinsic pathway of apoptosis by inducing cytochrome c release and apoptosome formation\(^{36}\).

Suppressing apoptosis is usually an active process that requires ongoing expression of “survival proteins” that inhibit apoptosis\(^{37}\). Therefore, sustained inhibition of global translation will also contribute to passive cell death via this mechanism. These effects are probably exacerbated in GCN2-dependent cells by proteasome inhibition-associated amino acid pool depletion, which interferes with both survival protein translation and the synthesis of glutathione.

**Initiator caspase activation**

The presence of misfolded proteins is initially “sensed” by members of the HSP70 family. It would seem reasonable that persistent, overwhelming proteotoxic stress could also be “sensed” by some direct mechanism. So far there is no direct evidence for this, but it was reported that caspase-2 was activated rapidly and selectively by heat shock\(^{38,39}\), suggesting that it may be the most attractive candidate. Other studies implicated caspase-8\(^{34,40}\) or caspase-4 (which resides in the ER)\(^{41}\), but the precise mechanisms involved in their activation in response to proteotoxic stress were not identified, and their relative importance in mediating apoptosis remains unclear. In particular, recent work demonstrating that caspases 4, 5 and 11 (in the mouse) function as specific innate immune receptors for lipopolysaccharide (LPS)\(^{42-44}\) has cast doubt on the relative importance of caspase-4 in ER stress-mediated apoptosis.
Cancer heterogeneity and sensitivity to proteotoxic stress

As introduced above, proteasome inhibitors are now FDA approved for the treatment of patients with multiple myeloma\textsuperscript{45}. They are also being evaluated in combination with conventional chemotherapy in patients with solid tumors. Sensitivity to proteasome inhibitors in preclinical models of cancer and in patients is highly heterogeneous, so there is strong interest in defining the molecular determinants of sensitivity so that biomarkers can be developed that can be used to identify the groups of patients who will obtain the most clinical benefit from them.

Our group has identified two independent mechanisms that appear to contribute to sensitivity or resistance. We observed that many human cancer cell lines constitutively contain high levels of eIF2\textalpha phosphorylation at baseline\textsuperscript{26} (M. White, manuscript under revision). In these cells proteasome inhibitors fail to stimulate a further increase in eIF2\textalpha phosphorylation\textsuperscript{26}, and as a consequence, protein synthesis continues unabated and the cells rapidly accumulate protein aggregates. The molecular mechanisms that cause the high basal eIF2\textalpha phosphorylation probably vary according to cell type, but in our models knockdown of GCN2 decreased basal phosphorylation (M. White, manuscript under revision).

Therefore, we suspect that amino acid pool “stress” may be involved, possibly secondary to high rates of anabolic metabolism, as is observed in cells with high Myc pathway activation\textsuperscript{46}.

We have also identified an interesting pathway of resistance. We used whole genome mRNA expression profiling to compare and contrast patterns of gene expression in proteasome inhibitor-sensitive and–resistant human bladder cancer cell lines before and after exposure to the proteasome inhibitor, bortezomib (Velcade)\textsuperscript{47}. One of the top most differentially expressed genes was inducible HSP70, which increased markedly in the proteasome inhibitor-resistant cells but did not increase at all in the proteasome inhibitor-sensitive cells\textsuperscript{47}. Analyses of the molecular mechanisms involved demonstrated that proteasome inhibitors activated the upstream transcription factor, heat shock factor-1 (HSF-1)\textsuperscript{47}. Consistent with this hypothesis, methylation-specific PCR demonstrated that HSP70 was methylated in the resistant cells, strongly suggesting that DNA methylation was responsible for the silencing of the HSP70 gene in the sensitive cells. Whether or not HSP70 methylation provides some growth advantage to the cancer cells is unclear.

Conclusions

Proteotoxic stress appears to contribute to cell killing in neurodegenerative diseases, heavy metal toxicity, and proteasome inhibitor-induced apoptosis in cancer cells. The eIF2\textalpha kinases are attractive candidate targets for new therapies aimed at inhibiting proteotoxicity in neurodegenerative disease and promoting proteotoxicity in cancer. However, differences in baseline “wiring” of the integrated stress response in cancer cells makes the development of these drugs very challenging. In addition, on-target toxicity in normal cells may dramatically limit the utility of eIF2\textalpha kinase inhibitors in cancer\textsuperscript{48}. However, it seems likely that by focusing on basic mechanisms, opportunities to exploit our growing understanding of the
integrated stress response will produce effective therapies for neurodegenerative diseases, cancer, and other disorders in the future.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**References**


**Highlights**

- Kinases that phosphorylate the translation initiation factor eIF2α play central roles in the responses of cells to proteotoxic stress.
- Different kinases play dominant roles in different cell types based on the metabolic “wiring” of the cells.
- Phosphorylation of eIF2α is initially cytoprotective but can promote apoptosis if proteotoxic stress becomes overwhelming.
- Apoptosis induced by proteotoxic stress is mediated by reactive oxygen species and alterations in intracellular Ca2+ homeostasis.
- Some cancers constitutively express high levels of eIF2α phosphorylation, and these cancers may be particularly vulnerable to proteotoxic stress.