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Anticipatory UPR Activation: A Protective Pathway and Target in Cancer

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Abstract

The endoplasmic reticulum (EnR) stress sensor, the unfolded protein response (UPR), plays a key role in regulating intracellular protein homeostasis. The extensively studied reactive mode of UPR activation is characterized by unfolded protein, or other EnR stress, triggering UPR activation. Here we focus on the emerging anticipatory mode of UPR activation in which mitogenic steroid and peptide hormones and other effectors pre-activate the UPR and anticipate a future need for increased protein folding capacity. Mild UPR activation in breast cancer can be protective and contributes to antiestrogen resistance. Hyperactivation of the anticipatory UPR pathway in cancer cells with a small molecule converts it from cytoprotective to cytotoxic, highlighting its potential as a therapeutic target in estrogen receptor positive breast cancer.

Keywords

Estrogen; Cancer; Unfolded Protein Response; BHPI

The Reactive UPR Pathway

Protein folding homeostasis and quality control is maintained by the sensor system for **endoplasmic reticulum** (EnR) stress (see Glossary), the **unfolded protein response** (UPR) [1–4]. The UPR consists of three main branches that together balance the synthesis of new proteins, with the availability of chaperones and other proteins to help fold and transport proteins within cells. EnR stress activates the three main arms of the UPR (Figure 1). The transmembrane kinase protein kinase RNA-like endoplasmic reticulum kinase (PERK) is activated by autophosphorylation. P-PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), resulting in transient inhibition of most protein synthesis [5]. UPR activation also results in proteolytic cleavage and activation of activating transcription factor 6 α (ATF6α). Activated ATF6α (p50-ATF6α) enters the nucleus and regulates expression of UPR targets. Upon activation by oligomerization and autophosphorylation, the third UPR sensor, inositol-

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requiring enzyme 1 α (IRE1α), alternatively splices inactive XBP1 mRNA (X-box binding protein 1) producing active spliced XBP1 (sp-XBP1). IRE1α and ATF6α activation leads to induction of the chaperone BiP/GRP78/HSPA5 (binding immunoglobulin protein/glucose regulated protein 78 kDa/heat shock protein A5) and other chaperones that increase protein-folding capacity, and to altered mRNA decay and translation. Simultaneously, degradation of misfolded protein is increased.

In this “reactive” mode, EnR stress resulting from accumulation of unfolded or misfolded protein, or other stresses, triggers UPR activation. How are the UPR sensors activated? IRE1α contains domains that bind unfolded proteins leading to structural alterations that might result in oligomerization and autophosphorylation [6–9]. Whether the other UPR sensors are activated by binding unfolded proteins is unclear. The EnR membrane contains ATP-dependent SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) pumps that maintain a high concentration of calcium in the lumen of the EnR. The SERCA pump inhibitor, thapsigargin, and the ionophore, ionomycin, activate the UPR by depleting EnR calcium. The calcium-dependent chaperone BiP binds to the 3 UPR sensors, inhibiting their activation. Unfolded protein or low calcium may remove BiP from UPR sensors, allowing sensor oligomerization and UPR activation [2, 10].

Reactive UPR activation is the initial response of cells to EnR stress. Recently, a different mode of UPR activation, termed “anticipatory” activation [1] has emerged as a hormone-activated pathway and a therapeutic target [11]. In this pathway, cells pre-activate the UPR in anticipation of a future requirement for increased protein folding capacity due to protein secretion (Box 1) or hormone-stimulated cell proliferation. Here we focus on the **anticipatory UPR** pathway, its likely role in hormone-dependent cancer and therapeutic targeting of this pathway.

A Largely Common Pathway for Anticipatory Activation of the UPR

The biological functions of steroid and peptide hormones are mediated through direct interaction with hormone-specific receptors. Mitogenic hormones, including the steroid hormones estrogen (17β-estradiol: E_2), androgen (dihydrotestosterone, DHT) and **ecdysone** (Ec) bind to their respective receptors (**ERα**, AR, and EcR) and peptide hormones **epidermal growth factor** (EGF) and **vascular endothelial growth factor** (VEGF) bind to their plasma membrane receptors (EGFR, VEGFR). The mitogenic human steroid and peptide hormones E_2, DHT, EGF and VEGF contribute to the pathology of several types of cancer and are important therapeutic targets. Estrogens and androgens act via their receptors to play pivotal roles in breast and prostate cancer, respectively [12, 13]. Endocrine therapies based on inhibitors that target the synthesis of estrogens and androgens and antagonists that inhibit hormone binding to their respective receptors are mainstays in the treatment of breast and prostate cancer. EGF activates progrowth and antapoptotic signaling pathways in several types of cancer [14]. Tumor growth and metastases depends on growth of a new vascular network. VEGF acting through VEGF receptors, promotes cell viability and angiogenesis, the formation of new blood vessels [15]. The EGF and VEGF pathways are major therapeutic targets in many types of cancer.

Steroid hormones act in the nucleus to regulate gene expression. While activation of genomic steroid hormone regulated gene expression programs initiates quickly, they play out over many hours. In addition, a diverse set of rapid extranuclear actions of steroid receptors, often initiated at or near the plasma membrane both modulate the genomic programs and influence many cell functions [16]. In contrast to the well-studied effects of steroid and peptide hormones on established signaling pathways, until recently rapid anticipatory activation of the UPR by steroid and peptide hormones was largely unexplored.

Several lines of evidence indicate that rapid hormone activation of the UPR occurs in the absence of EnR stress and accumulation of unfolded protein. VEGF did not alter EnR processing of a GFP-tagged secreted protein [17] and EGF activates the UPR in cells in which EGF does not induce cell proliferation [18]. How do hormones elicit rapid anticipatory activation of the UPR? The EnR is a closed membrane and the EnR lumen contains a very high concentration of calcium relative to the cell body. As discussed above for the reactive UPR pathway, loss of EnR calcium activates the UPR. Since the concentration of EnR calcium is high relative to the cell body, opening calcium channels in the EnR membrane results in rapid efflux of calcium from the lumen of the EnR into the cell body. Although there are several classes of calcium channels in the EnR membrane, all the known activators of the anticipatory UPR pathway use a common pathway that results in opening the EnR inositol triphophoshate receptor (IP_3R) calcium channels. The IP_3R calcium channels open on binding of inositol triphosphate (IP_3). IP_3 is produced enzymatically by activated phosphorylated phospholipase C γ (PLCγ). All the hormones that elicit anticipatory activation of the UPR activate PLCγ (Figure 2) [17–20]. The receptors for EGF and VEGF are tyrosine kinases that directly phosphorylate and activate PLCγ. The receptors for E_2 and ecdysone lack tyrosine kinase activity. Binding of E_2 to ERα and of ecdysone to EcR induces a conformational change that ultimately alters the activity of a tyrosine kinase that in turn activates PLCγ. Importantly, **BHPI**, a recently described ERα biomodulator with anticancer activity, binds ERα at a different site than estrogens and induces a conformational change in ERα that leads to strong and sustained activation of the anticipatory UPR pathway [20]. While the mitogens E_2, EGF and VEGF and the small molecule BHPI all activate the rapid anticipatory UPR pathway, DHT does not [21]. Instead, DHT activates the UPR after many hours [22]. Supporting the proposed pathway, inhibition and knockdown of PLCγ blocks UPR activation by all four rapid activators [17–20]. Moreover, E_2, EGF and BHPI all increased IP_3 levels [18–20]. For each activator, inhibition of the IP_3Rs by 2-aminoethoxydiphenyl borate (2-APB) abolishes increases in cytosol calcium [17–20]. Moreover, for EGF, E_2 and BHPI simultaneous knockdown of the three EnR IP_3R calcium channels blocked the increase in cytosol calcium and abolished UPR activation [18–20]. In contrast, other findings indicate that VEGF mediated UPR activation does not involve calcium depletion; the UPR remained activated after co-treatment with VEGF and the IP_3R inhibitor 2-APB. Instead, a complex between VEGF-activated PLCγ and mTORC was proposed as triggering UPR activation [17]. This raises the possibility of multiple pathways for hormone-mediated anticipatory activation of the UPR.

Additional experiments show that VEGF, EGF, E_2 and BHPI induce PLCγ-dependent anticipatory activation of two UPR arms via p50-ATF6α and p-IRE1α (Figure 1), resulting in increased activated sp-XBP1 and induction of BiP mRNA and protein [17–20]. Is the protein synthesis inhibiting PERK arm activated as part of these mitogenic programs? VEGF, EGF and E_2 each induce a mild and transient activation of the PERK arm of the UPR and transient appearance of p-eIF2α [17–19]. Detailed studies show E_2 induces a transient increase in phosphorylation of PERK and eIF2α and a transient and mild inhibition of protein synthesis [19]. These studies suggest mitogenic hormones initiate anticipatory activation of the chaperone-inducing, pro-proliferation arms of the UPR without impacting the supply of new protein needed for cell proliferation.

Rapid Anticipatory Activation of the UPR is Important for Hormone Induction of Cell Proliferation

The observations discussed above raise several questions. Does rapid UPR activation by VEGF, EGF and E_2 enhance their ability to induce cell proliferation? If so, what UPR-related molecules and signals facilitate cell proliferation? For all three hormones, PLCγ knockdown strongly inhibited hormone-induced cell proliferation [17–19]. Upon activation of the PLCγ-UPR pathway, intracellular calcium levels increase very rapidly. Several hours later BiP chaperone levels increase. Elevated intracellular calcium is a proliferation signal and is associated with aggressive tumors [23, 24]. Elevated BiP is associated with cancer cell proliferation, a poor prognosis and therapy resistance [25–28]. Knockdown experiments were employed to test the role of different pathway components in cell proliferation. Knockdown of eIF2α or ATF6α reduced VEGF-mediated endothelial cell survival by ~50% [17]; knockdown of ATF6α and XBP1 reduced EGF-stimulated cell proliferation 30–40% [18]. IP_3R knockdown strongly inhibited E_2-ERα induced proliferation of breast cancer cells, while XBP1 and ATF6α knockdown moderately inhibited proliferation. As expected, since estrogen had minimal effects on the PERK arm of the UPR, PERK knockdown had no effect [19]. Since XBP1 and ATF6α act after the increase in cytosol calcium, both elevated intracellular calcium and UPR induced chaperones play a role in hormone-induced cell proliferation.

PLCγ-IP_3R-mediated Elevated Intracellular Calcium is Important for Hormone-regulated Gene Expression

The role of anticipatory activation of the UPR in the mitogen-regulated signaling pathways and gene expression programs that underlie their effects on cell proliferation was explored. In addition to promoting survival of endothelial cells, activation of the UPR plays a major role in VEGF induced blood vessel formation. In a sophisticated matrigel plug angiogenesis assay, knockdown of PLCγ, eIF2α or ATF6α nearly abolished neovascularization [17]. Solid tumors are subject to hypoxia and hypoxia in turn activates a signaling pathway involving hypoxia inducible factor 1α (HIF-1α) and VEGF. UPR activation may impact induction of VEGF. Emerging data suggests the possible existence of a feed-forward regulatory loop in which VEGF activates the UPR, up-regulating XBP1. The XBP1 then works together with HIF-1α to induce VEGF [29]. In ERα negative breast cancer, elevated

XBP1 is associated with a poor prognosis [30]. Tumor progression may be driven by this HIF-1α-XBP1 axis [31].

EGF binding to EGFR rapidly activates the pro-proliferation ERK and AKT pathways [32]. This is quickly followed by initiation of the immediate early gene expression program. Immediate early gene expression is important for EGF-stimulated cell proliferation [33–35]. Since the ERK inhibitor, UO126, blocks EGF induction of immediate early genes, ERK activation is essential for immediate early gene expression. Because immediate early genes Fos and EGR1 were induced in 20 minutes, the IP_3R inhibitor, 2-APB, was unlikely to exhibit off-target and secondary effects. Blocking the increase in intracellular calcium with 2-APB completely blocked the rapid EGF-EGFR induction of Fos and EGR1 mRNAs [18]. Since 2-APB also blocked EGF-EGFR-mediated down regulation of gene expression, this is a specific action of EGF-EGFR mediated through the EnR IP_3Rs and is not the result of global inhibition of transcription. Surprisingly, 2-APB had no effect on the rapid EGF-EGFR activation of the ERK and AKT signaling pathways [18]. Since 2-APB did not block EGF activation of the ERK pathway, the PLCγ-IP_3R-mediated increase in intracellular calcium is a previously undescribed independent regulator of EGF-induced gene expression that works together with ERK activation to regulate immediate early gene expression.

ERα is an intensively studied ligand-regulated transcription factor. Blocking opening of the EnR IP_3R calcium channels with 2-APB, or chelating intracellular calcium with BAPTA, strongly inhibited E_2-ERα regulated gene expression [19]. A significant body of early work described a functional interaction between the calcium sensor calmodulin (CaM) and ERα in E_2-ERα regulation of gene expression [36–39]. The PLCγ-IP_3R pathway couples rapid extranuclear E_2-ERα regulation of calcium levels to nuclear regulation of gene expression. Future studies will doubtless explore mechanisms by which these increased calcium levels selectively regulate the E_2-ERα gene expression program.

The Anticipatory UPR Pathway in Therapy Resistant Breast Cancer

Activation of the UPR protects cells against stress. The important role of the UPR in protecting cancer cells against stress has been the subject of excellent recent reviews [3, 4, 40, 41], as has the more focused subject of the UPR in therapy-resistant **ERα positive** breast cancer [40]. Here, we focus primarily on anticipatory activation of the UPR in cancer with limited consideration of the widely studied role of the reactive UPR pathway.

Endocrine therapy using antiestrogens that compete with E_2 for binding to ERα and **aromatase inhibitors** that block estrogen synthesis is a mainstay in treatment of the ~70% of breast cancers that are ERα positive. Although most tumors respond initially, ~50% will develop acquired resistance to endocrine therapy. Resistance mechanisms are diverse and have been recently reviewed [42]. Recently, ERα mutations have been the focus of intense interest as a likely cause of resistance to endocrine therapy. In ~1/3 of patients with metastatic therapy-resistant breast cancer, integrative sequencing has revealed ERα mutations thought to be constitutively active [43–47]. The ERα mutation, K303R, is reportedly common in tumors, and is undetectable by standard DNA sequencing [48, 49]. Also, up-regulation of the UPR and cross-talk of the UPR with autophagy can play an important role in allowing these tumors to proliferate in nutrient-deprived, hypoxic environments and resist therapy [40, 42, 50]. In the reactive pathway, resistance to therapy is likely driven by selection and outgrowth of cancer cells that survive in part because they activated the UPR in response to hypoxia, nutritional deprivation and therapy-induced stress.

Since breast cancers likely undergo hypoxic stress throughout tumor progression, it is difficult to dissect out the contributions to UPR activation of the estrogen-driven anticipatory pathway and of reactive activation of the UPR in response to hypoxia and nutritional deprivation. Two factors suggest the anticipatory pathway plays a significant role in UPR activation early in tumor outgrowth. There is a strong correlation between elevated expression of a UPR gene signature, consisting of UPR sensors and downstream targets of UPR activation, and expression levels of E_2-ERα regulated genes [19]. Moreover, compared to normal mammary epithelial cells, the UPR is activated in premalignant mammary cells [19]. It is likely that the extent of activation of the anticipatory UPR pathway plays an important early role in selection of tumors that grow and progress; as tumors grow, hypoxia, nutritional deprivation and therapy-induced stress probably play an increasing role in UPR activation.

The UPR gene signature has been used to explore whether UPR activation at diagnosis predicts subsequent clinical outcome. Bioinformatic analysis of data from ~1,000 ERα positive breast cancer patients showed that elevated expression of the UPR gene signature at diagnosis was tightly correlated with subsequent resistance to **tamoxifen** therapy, reduced time to recurrence and reduced survival [19]. This supports a role for the anticipatory UPR in selection and progression of breast cancers. UPR activation can play an important role in protection of cancer cells from subsequent therapeutic insult. Weakly activating non-toxic concentrations of the UPR activator tunicamcyin elicit an adaptive stress response that increases EnR chaperones, and renders cells resistant to subsequent exposure to an otherwise lethal concentration of tunicamycin [51, 52]. Prior exposure of breast cancer cells to E_2, or to a low concentration of tunicamycin, each elicited an ~10 fold increase in the concentration of tunicamycin required to induce apoptosis [19]. This suggested that E_2-induced anticipatory activation of the UPR might both facilitate tumor proliferation and protect ERα positive breast tumors against subsequent stress due to hypoxia, nutritional deprivation and therapy.

Two UPR-regulated proteins, BiP chaperone and XBP1 are strongly implicated in antiestrogen resistance [40, 53–56]. As mentioned above, elevated BiP is associated with a poor prognosis [27]. BiP overexpression confers antiestrogen resistance and BiP knockdown resensitizes ERα positive breast cancer cells to antiestrogens [28]. Similarly, XBP1 knockdown restores sensitivity to antiestrogens, and XBP1 overexpression elicits antiestrogen resistance [28, 30, 57, 58]. Similar to E_2, tamoxifen and ICI 182780/fulvestrant can activate pro-survival signaling through the UPR [58]. Notably, in women more than 5 years post menopause, whose tumors grow in a low estrogen environment, which can lead to elevated ERα levels [59, 60], or after long-term treatment with SERMs, removal of SERMs, and treatment with high dose estrogens slows tumor growth and induces tumor regression [61, 62]. Both UPR activation and increased cellular reactive oxygen species (ROS) appear to play a role in high dose estrogen-induced apoptosis [63].

Mild activation of the UPR that primarily results in chaperone induction and does not strongly activate the PERK arm of the UPR is protective. However, robust and sustained activation of the UPR that leads to strong activation of the PERK arm of the UPR is toxic. Supporting the idea that UPR activation is an attractive therapeutic strategy, the ability of proteasome inhibitors to induce apoptosis of cancer cells is largely based on their ability to increase levels of unfolded proteins and thereby strongly activate the UPR [64–66]. Thus, the UPR in cancer is a finely titrated system, where stress that exceeds a threshold tips the scales from protective to lethal (Figure 3). In the anticipatory UPR pathway, hormones, such as estrogen induce a moderate and transient increase in intracellular calcium that results in weak activation of the UPR while BHPI induces a much larger and more sustained increase in intracellular calcium leading to strong and toxic activation of the PERK arm of the UPR [19, 20].

The Preclinical Anticancer Drug, BHPI, Induces Lethal Hyperactivation of the UPR

BHPI was the most effective biomodulator to emerge from an unbiased high throughput screen for small molecules that inhibit E_2-ERα induction of a luciferase reporter gene [20, 67]. Surprisingly, BHPI elicited rapid, near quantitative inhibition of protein synthesis, a seemingly unlikely action for a small molecule inhibitor of E_2-ERα regulated gene expression. It was therefore important to test whether BHPI works through ERα. BHPI gains the ability to inhibit protein synthesis when ERα is stably transfected into ERα negative MCF10A cells and loses the ability to inhibit protein synthesis when ERα is knocked down or degraded with ICI 182,780. Moreover, increasing levels of ERα elicit progressively increasing inhibition of protein synthesis, and BHPI only inhibits protein synthesis in ERα positive cell lines. Supporting BHPI acting through ERα, and not through the estrogen binding protein GPR30, BHPI has no effect on protein synthesis in ERα negative HepG2 cells, which contain functional GRP30 [68], and activating GPR30 with G1 did not inhibit protein synthesis. Consistent with binding to ERα, BHPI alters the intrinsic fluorescence emission pattern of ERα and alters the ERα digestion pattern in partial protease digestion studies [20]. Moreover, at early times, when inhibition of protein synthesis and UPR activation do not inhibit E_2-ERα-induced gene expression, BHPI non-competitively inhibits E_2-ERα induction and repression of gene expression and reduces binding of E_2-ERα to gene regulatory regions [20]. Taken together, these data indicate that BHPI binds ERα and that BHPI binding induces a conformational change in ERα.

The PERK arm of the UPR was identified as the pathway responsible for rapid BHPI inhibition of protein synthesis (Figure 1). This led to the finding that BHPI works by hyperactivating the anticipatory UPR pathway. Compared with E_2, BHPI more strongly activates PLCγ, producing much higher IP_3 levels, calcium release from the EnR, and UPR activation. BHPI potently inhibits protein synthesis by inducing rapid and robust phosphorylation of PERK and eIF2α. Supporting the role of the PERK arm of the UPR in inhibiting protein synthesis, knockdown and inhibition of ERα, PLCγ, the IP_3Rs, and PERK blocked rapid BHPI inhibition of protein synthesis [20].

How does BHPI convert the normal transient and protective activation of the UPR into a sustained toxic UPR activation? The substantial level of IP_3 produced by strong BHPI activation of PLCγ binds to and opens the EnR IP_3Rs, resulting in rapid efflux of calcium stored in the lumen of the EnR into the cytosol. To restore EnR calcium, the cell activates SERCA pumps, which catalyze ATP-dependent transfer of calcium from the cytosol into the lumen of the EnR. Since the IP_3R calcium channels are still open, the calcium pumped into the EnR leaks back out creating a futile cycle that rapidly depletes cell ATP. Depleting intracellular ATP activates the important metabolic sensor, AMP kinase (AMPK). Supporting this model, thapsigargin, which inhibits the SERCA pumps, blocks the BHPI-mediated decline in ATP levels and AMPK activation. Together, AMPK activation and elevated intracellular calcium activate the eukaryotic elongation factor 2 kinase (CAMKIII/eEF2K). Activated eEF2K phosphorylates eEF2, inhibiting protein synthesis at a second site. Inhibiting protein synthesis at a second site prevents synthesis of BiP and other chaperones and $p58_{IPK}$ and GADD34 (growth arrest and DNA damage-inducible protein 34) that normally reverse PERK activation [20]. Working together, several actions of BHPI, including long-term inhibition of protein synthesis, sustained UPR activation, ATP depletion and AMPK activation likely contribute to BHPI's ability to block proliferation and often kill ERα positive cancer cells. At nanomolar concentrations BHPI blocked growth, and killed diverse ERα positive breast and endometrial cancer cells. In a mouse xenograft model of breast cancer, BHPI stopped tumor growth and induced rapid and substantial regression of large tumors [20].

Aromatase inhibitors and competitor antiestrogens such as tamoxifen and ICI work by preventing E_2-ERα action, and are therefore ineffective in ERα positive cancer cells that do not require estrogens and ERα for growth. Although ~50% of ovarian cancers are ERα positive [69], ovarian cancers do not depend on estrogens or ERα for growth and endocrine therapy is therefore ineffective [70]. In contrast, BHPI retains full effectiveness in ERα positive ovarian, breast and endometrial cancer cells that do not depend on estrogens or ERα for growth [17]. BHPI is effective in these cells because it is a noncompetitive biomodulator that uses ERα to block cell growth by hyperactivating the UPR. Moreover BHPI was highly effective in multiple breast and ovarian cell-based models for tamoxifen and ICI resistance. BHPI's impressive effectiveness demonstrates the therapeutic potential of small molecules that target the anticipatory UPR pathway.

Concluding Remarks and Future Perspectives

It remains to be established whether all of the known activators of PLCγ (Figure 2), and perhaps activators of other PLC family members, activate the UPR. Calcium efflux through the EnR IP_3R channels is regulated by interaction with multiple proteins. Consistent with IP_3R modulator proteins influencing responses to E_2 and BHPI, RACK1 is an E_2-ERα induced gene that is overexpressed in aggressive breast tumors [71] and RACK1 increases calcium efflux through the EnR IP_3R channels [72–74]. This suggests that overexpression in tumors of proteins that enhance the calcium efflux through the IP_3R calcium channels may contribute to the selectivity of BHPI for cancer cells. How the diverse regulators of the IP_3R channels influence the anticipatory UPR pathway is unexplored.

Whether non-hormonal activators of ERα, such as the metals calcium [75, 76] and cadmium [77], activate the UPR has not been investigated. E_2 increases cytosol calcium in ~1 min. suggesting this may be the initial response of a cell to estrogen [19]. The protein complex in which E_2-ERα activates a tyrosine kinase that phosphorylates and activates PLCγ is as yet unidentified. Progress in identifying the tyrosine kinase linked to E_2-ERα activation of the UPR may shed light on how other PLCγ activators that are not tyrosine kinases activate the UPR.

Anticipatory activation of the UPR induces an extremely rapid increase in intracellular calcium and after several hours, increased levels of BiP and other chaperones. How these signals from activation of the UPR pathway couple to and control the EGF-EGFR, VEGF-VEGFR and E_2-ERα gene expression and cell proliferation programs will be important areas for future study.

Studies to date have largely focused on effects of anticipatory activation of the UPR by VEGF, EGF and E_2 on gene expression and cell proliferation in cancer. However, estrogens protect against stress in pancreatic islet cells in diabetes [78, 79] and in the nervous system [80–82] and important roles for the UPR have been described in these systems [78, 83, 84]. Whether some of the well-known protective effects of estrogen in the nervous system and in diabetes are due to anticipatory activation of the UPR remains to be explored.

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Glossary

Aromatase inhibitor

a class of anticancer drugs that work by inhibiting the enzyme aromatase (CYP19A1), which converts precursors into estrogen.

BHPI

a potent non-competitive small molecule ERα biomodulator that selectively blocks proliferation of ERα positive breast, ovarian and endometrial cancer cells by hyperactivating the unfolded protein response.

Ecdysone

a steroidal pro-hormone produced by the prothoracic gland of insects that regulates molting and stimulates metamorphosis.

Endoplasmic reticulum (EnR)

a eukaryotic membrane organelle. Protein folding and processing occur in the interior, or lumen, of the EnR. (Since estrogen receptor is abbreviated ER, to abbreviate endoplasmic reticulum, we use EnR, rather than the more common ER.)

Epidermal growth factor (EGF)

a polypeptide growth factor with diverse biological effects including stimulating proliferation and differentiation of mesenchymal and epithelial cells.

ER positive breast cancer

a type of breast cancer that grows in response to estrogen and contains estrogen receptor α. At diagnosis, about 70% of breast cancers are ERα positive.

Estrogen receptor α (ERα)

a nuclear steroid hormone receptor that is activated by estrogen. Upon estrogen binding, ERα undergoes a conformational change and carries out both nuclear and extranuclear actions.

Reactive and anticipatory activation of the unfolded protein response (UPR)

in reactive activation of the UPR, sensors respond to unfolded proteins, or other forms of stress, by activating the UPR. The anticipatory UPR is activated in the absence of endoplasmic reticulum stress, in anticipation of future increased need for protein folding capacity.

Src

a membrane-associated non-receptor tyrosine kinase. It plays a role in diverse cellular pathways including cell survival, angiogenesis, cell proliferation and invasion.

Tamoxifen

a prodrug used to treat ER positive breast cancer. It is a competitive inhibitor of estrogen binding to ERα and inhibits growth of breast cancer cells. *In vivo,* tamoxifen is converted to the more active, 4-hydroxytamoxifen.

Unfolded protein response (UPR)

when unfolded or misfolded proteins accumulate in the lumen of the EnR, the UPR is activated, resulting in induction of molecular chaperones, decreased translation and increased degradation of misfolded proteins.

Vascular endothelial growth factor (VEGF)

it was originally isolated from tumor cells and was initially referred to as ‘tumor angiogenesis factor’. VEGF stimulates development of new vascular networks that provide oxygen and nutrients to tumors.

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BOX 1: Anticipatory Activation of the UPR Across Cell Types and Species

B-cell Differentiation Triggers Anticipatory Activation of the UPR

Anticipatory activation of the UPR was first observed in differentiating immune cells. Following exposure to an antigen, B-cells differentiate into plasma cells, which ultimately synthesize and secrete massive amounts of immunoglobulins. Anticipating the need to expand protein-folding capacity, the UPR is activated prior to increased immunoglobulin secretion [85, 86]. Importantly, differentiating mutant B cells that cannot make immunoglobulin still activate the UPR [85]. Although the full pathway has not been elucidated, B-cell antigen receptor (BCR), acting through tyrosine kinases (Figure 2) likely initiates the pathway [87]. Thus a wide range of stimuli can activate the anticipatory UPR pathway,

The Anticipatory UPR Pathway in Insects: Ecdysone

One factor that suggests a pathway is important is its conservation across species. The steroid hormone, ecdysone, acting through the ecdysone receptor (EcR), plays an important role in insect metamorphosis. Recent studies suggest that the anticipatory UPR pathway plays an important role in ecdysone action. The anticipatory UPR pathway is activated when inositol triphosphate receptor (IP3R) calcium channels in the EnR membrane are opened; this allows high concentrations of calcium stored in the lumen of the EnR to flow into the cytosol. IP3R calcium channels open following binding of inositol triphosphate (IP3). IP3 is produced by the enzyme reaction catalyzed by activated phospholipase C γ (PLCγ) (Figure 1). Consistent with activation of the anticipatory UPR pathway, in the lepidopteran insect Helicoverpa armigera, (the cotton bollworm) 20-hydroxyecdysone-EcR acts through G-protein coupled receptors (GPCRs) and Src (proto-oncogene Sarcoma) tyrosine kinase to phosphorylate and activate PLCγ, increasing cytosol calcium. Several lines of evidence support a role in ecdysone action for the early steps in the anticipatory UPR pathway. Inhibition or knockdown of PLCγ blocked 20-hydroxyecdysone induced pupation and caused larval death. Moreover, an IP3R inhibitor blocked the increase in cytosol calcium [88], and blocking the increase in cytosol calcium inhibited 20-hydroxyecdysone induced transcription [88, 89]. These data suggest ecdysone activates an evolutionarily conserved insect anticipatory UPR pathway.

Outstanding Questions

How do ERα and other steroid receptors couple to tyrosine kinases to activate PLCγ and initiate the anticipatory UPR pathway?

Does activation of other isoforms of PLC also trigger activation of the anticipatory UPR pathwway?

What is the role of the anticipatory UPR pathway in the brain and nervous system in which estrogens are known to mitigate stress?

Increased intracellular calcium released by opening endoplasmic reticulum calcium channels is important for initiating hormone regulated gene expression programs. How is the calcium signal read at the level of transcription and how is selectivity maintained?

Using ERα to elicit cytotoxic hyperactivation of the UPR shows impressive therapeutic potential. Can new approaches be identified for selective hyperactivation of the PLCγ-IP$_3$R anticipatory UPR pathway in ERα negative cancer cells?

Trends

Diverse signaling molecules including peptide hormones, steroid hormones, immunoreceptors and GPCRs signal through phospholipase C γ (PLCγ) to activate the anticipatory UPR pathway.

Rapid activation of the anticipatory UPR pathway is important for subsequent hormone-regulated gene expression and cell proliferation.

The anticipatory and reactive UPR pathways work together to enable antiestrogen resistance in ERα positive breast cancer.

Targeting the anticipatory UPR, converting it from protective to toxic holds significant therapeutic promise.

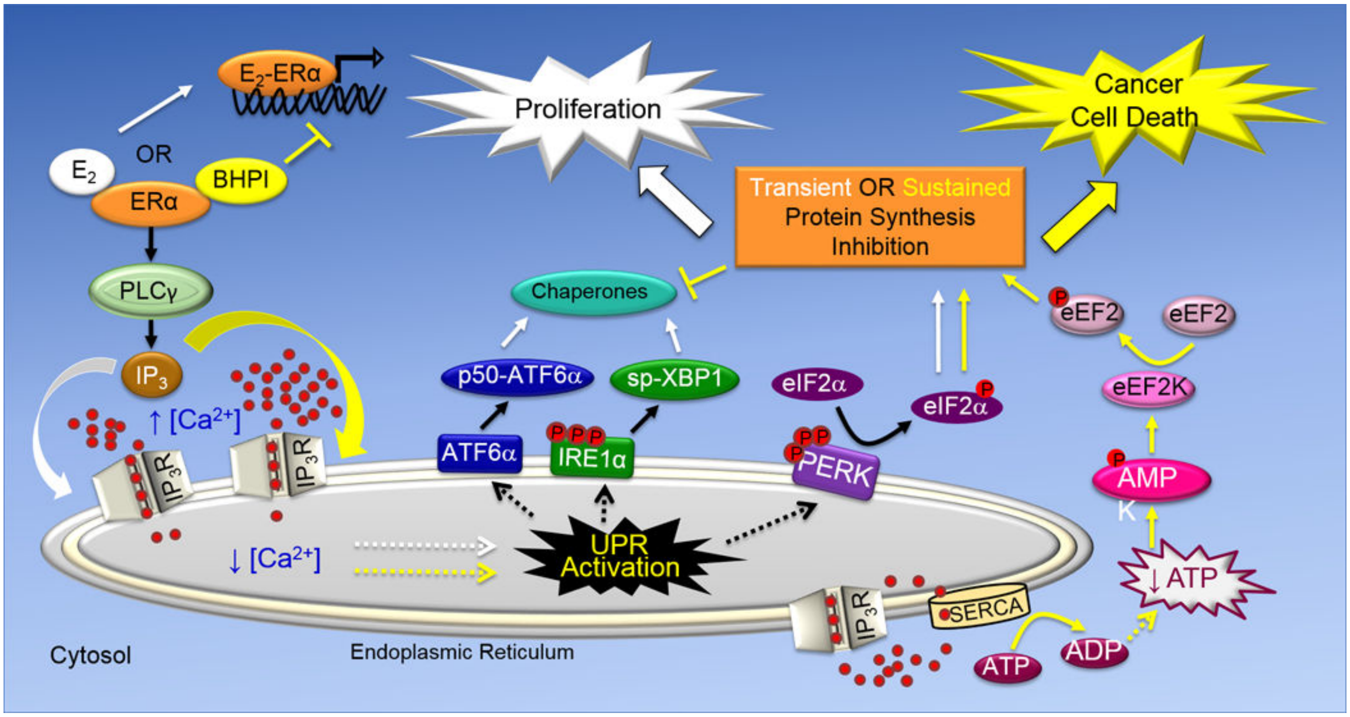

Fig. 1. Schematic Representation of the Cytoprotective and Cytototxic Anticipatory UPR Pathways

The UPR consists of three arms, ATF6α, IRE1α and PERK that are named after proteins embedded in the membrane of the endoplasmic reticulum. While activation of the ATF6α and IRE1α arms of the UPR leads to induction of chaperones that increase protein folding capacity, activation of the PERK arm of the UPR leads to reduced protein production. ATF6α activation leads to translocation of the transmembrane protein p90-ATF6α from the EnR to the Golgi apparatus, where it encounters proteases that liberate the N-terminal fragment of ATF6α (p50-ATF6α). p50-ATF6α is a transcription factor that increases the protein-folding capacity of the EnR by inducing EnR-resident chaperones, including BiP. The transmembrane protein IRE1α is a nuclease. Oligomerization and phosphorylation activates IRE1α. Activated IRE1α removes an intron from full-length XBP1 mRNA, producing spliced (sp)-XBP1 mRNA. sp-XBP1 is a transcription factor that increases the protein-folding capacity of the EnR and turnover of misfolded proteins by inducing EnR resident-chaperones. Oligomerization and autophosphorylation activate the transmembrane kinase PERK. Activated p-PERK phosphorylates eukaryotic initiation factor 2α (eIF2α), leading to inhibition of protein synthesis and a reduction in the endoplasmic reticulum protein folding load. E_2 and BHPI bind at distinct sites on ERα. In the anticipatory UPR (shown on the left), E_2 and BHPI act via ERα to activate a pathway that results in opening of EnR IP_3R calcium channels allowing efflux of calcium stored in the lumen of the EnR into the cytosol. This activates the UPR. In ERα containing cancer cells, strong and sustained opening of the EnR IP_3R calcuim channels by BHPI sets up a futile cycle of ATP-dependent pumping of calcium into the EnR by SERCA pumps and leakage back into the cytosol through the open IP_3R channels (lower right). This depletes intracellular ATP, activating the energy sensor AMPK (AMP kinase). Elevated intracellular calcium and activated AMPK phosphorylate and activate eukaryotic elongation factor 2 (eEF2) kinase.

eEF2 kinase phosphorylates and inactivates eEF2. This inhibits protein synthesis at a second site, resulting in strong and sustained toxic inhibition of protein synthesis by BHPI.

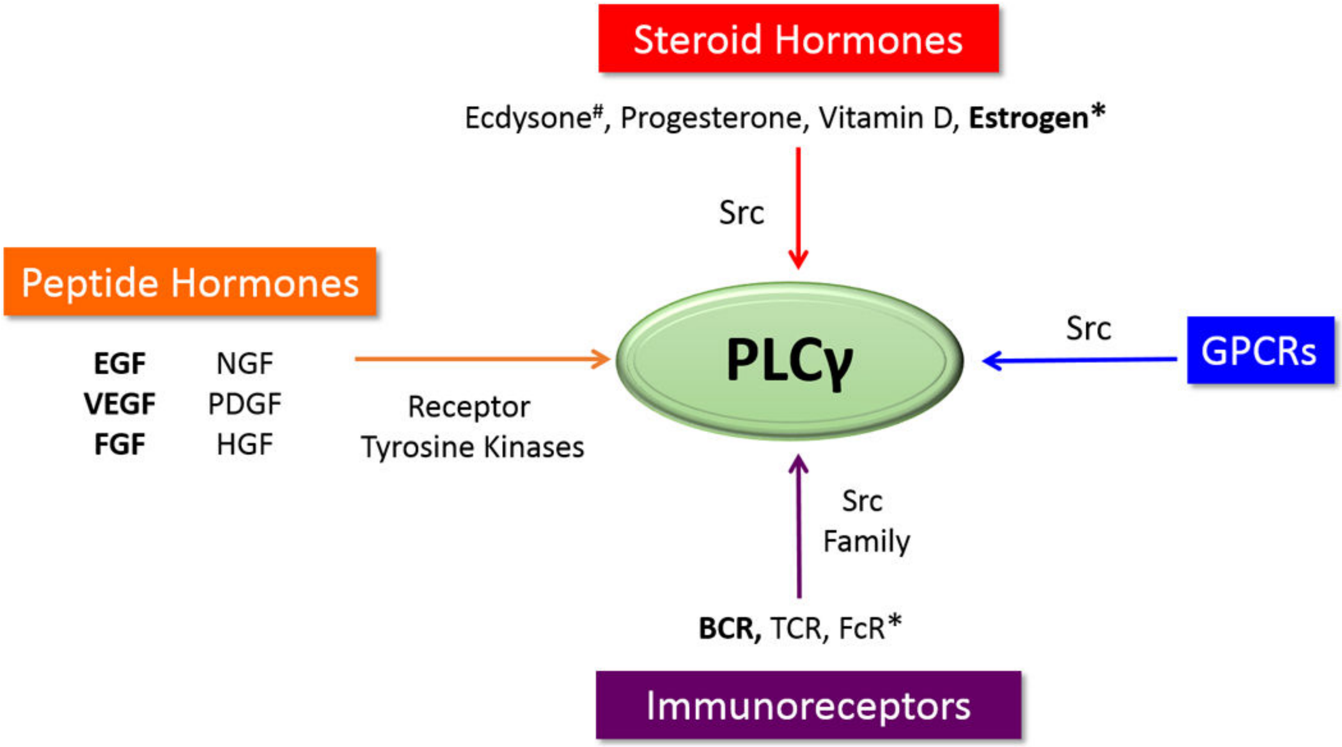

Fig. 2. The Diverse Activators of PLCγ

Activators of PLCγ known to activate the UPR are in bold. * indicates the activating tyrosine kinase has not been identified. #Ecdysone acts with a GPCR to open the IP_3R calcium channels, but activation of the UPR arms has not been investigated. Tyrosine kinases that activate PLCγ are expected to activate the anticipatory UPR pathway. Peptide hormone receptors that are membrane tyrosine kinases that activate PLCγ are shown on the left. Src kinase has also been shown to activate PLCγ. Several steroid hormones activate Src kinase and are likely activators of the anticipatory UPR pathway (center). Immunoreceptors, shown on the bottom, working through GPCRs and Src family kinases are not mitogenic hormones and instead activate the UPR to antcipate the increased protein folding load that accompanies antibody production.

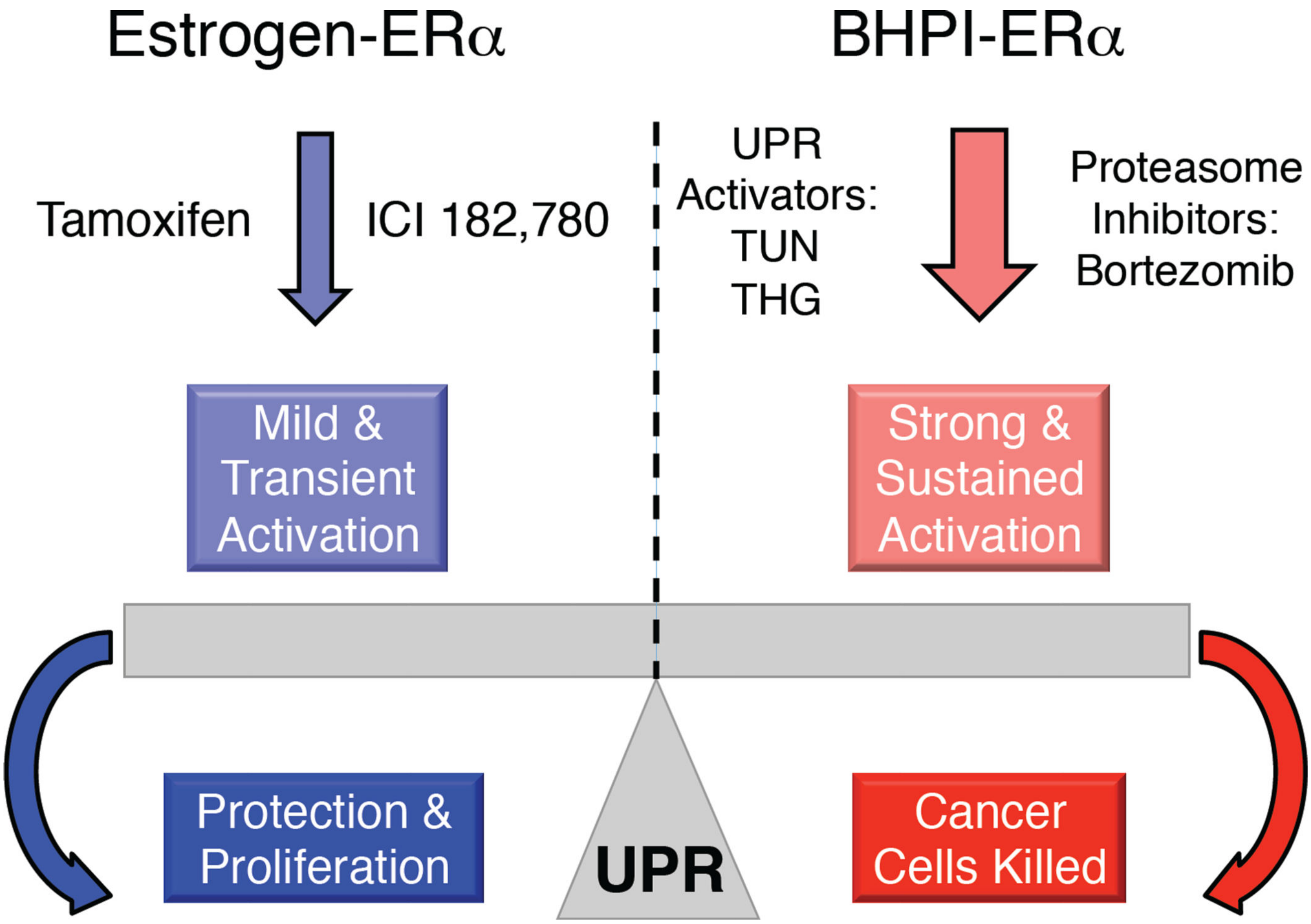

Fig. 3. The UPR is a Molecular Rheostat Responding to Different Levels of UPR Activation

The UPR is nearly off in normal non-malignant cells. In cells likely to initiate rapid proliferation, in antiestrogen-treated cancer cells, and in cells that will soon expand protein production and secretion, UPR activation is mild and protective. Strong and sustained UPR activation induced by BHPI, by proteasome inhibitors and by classical UPR activators, such as tunicamycin and thapsigargin, is toxic. Because the classical UPR activators are quite toxic, they have not found therapeutic application.