Modulation of nuclear factor-kappa B activation by the endoplasmic reticulum stress sensor PERK to mediate estrogen-induced apoptosis in breast cancer cells

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Abstract
Stress responses are critical for estrogen (E2)-induced apoptosis in E2-deprived breast cancer cells. Nuclear factor-kappa B (NF-κB) is an important therapeutic target to prevent stress responses in chronic inflammatory diseases including cancer. However, whether E2 activates NF-κB to participate in stress-associated apoptosis in E2-deprived breast cancer cells is unknown. Here, we demonstrated that E2 differentially modulates NF-κB activity according to treatment time. E2 initially has significant potential to suppress NF-κB activation; it completely blocks tumor necrosis factor alpha (TNFα)-induced activation of NF-κB. We found that E2 preferentially and constantly enhances the expression of the adipogenic transcription factor CCAAT/enhancer binding protein beta (C/EBPβ), which is responsible for the suppression of NF-κB activity by E2 in MCF-7:5C cells. Interestingly, NF-κB p65 DNA-binding activity is increased when E2 is administered for 48 h, leading to the induction of TNFα and associated apoptosis. Blocking the nuclear translocation of NF-κB can completely prevent the induction of TNFα and apoptosis induced by E2. Further examination revealed that protein kinase RNA-like endoplasmic reticulum kinase (PERK), a stress sensor of unfolded protein response (UPR), plays an essential role in the late activation of NF-κB by E2. This modulation between PERK and NF-κB is mainly mediated by a stress responsive transcription factor, transducer and activator of transcription 3 (STAT3), independently of the classic canonical IκBα signaling pathway. Thus, inhibition of PERK kinase activity completely blocks the DNA binding of both STAT3 and NF-κB, thereby preventing induction of NF-κB-dependent genes and E2-induced apoptosis. All of these findings suggest that PERK is a key regulator to convey stress signals from the endoplasmic reticulum to the nucleus and illustrate a crucial role for the novel PERK/STAT3/NF-κB/TNFα axis in E2-induced apoptosis in E2-deprived breast cancer cells.

Introduction
Targeting the estrogen receptor (ER) with a selective estrogen receptor modulator (SERM) or inhibiting synthesis of estrogen (E2) with an aromatase inhibitor are successful therapeutic strategies to treat or prevent ER-positive breast cancer¹. However, acquired resistance to anti-hormone therapies will inevitably occur for the majority of treated patients. Paradoxically, the discoveries that physiological levels of E2 can induce regression of SERM-resistant breast tumors in athymic mice², ³ and induce apoptosis in E2-deprived breast cancer cells⁴, ⁵ have resulted in a novel therapy in breast cancer patients following exhaustive anti-hormone therapy⁶. This was the scientific rationale behind the use of E2 to treat aromatase inhibitor-resistant breast cancer in clinical trials with 30% benefit for patients⁷. Furthermore, hormone replacement therapy (HRT) with only E2 in postmenopausal women in their 60s has a reduced
incidence of breast cancer and mortality\textsuperscript{8} because of E\textsubscript{2}-induced apoptosis\textsuperscript{8}, whereas classic HRT with E\textsubscript{2} plus medroxyprogesterone acetate (MPA) increases the risk of breast cancer.\textsuperscript{8} This is because MPA acts like a glucocorticoid to block E\textsubscript{2}-induced apoptosis.\textsuperscript{8-10} All of these clinically relevant findings encouraged us to investigate the mechanism underlying E\textsubscript{2}-induced apoptosis and identify the key checkpoints involved, so that the therapeutic effects of E\textsubscript{2} in anti-hormone therapy-resistant breast cancer can be enhanced.

Unlike rapid chemotherapy-induced apoptosis, E\textsubscript{2} induces apoptosis in a delayed manner, with initial cellular proliferation in response to E\textsubscript{2} exposure in E\textsubscript{2}-deprived breast cancer cells\textsuperscript{11,10}. Our recent investigations revealed that accumulation of stress responses, including endoplasmic reticulum, oxidative, and inflammatory stresses, results in E\textsubscript{2}-induced apoptosis\textsuperscript{12,11}. The endoplasmic reticulum is a crucial regulatory site for stress responses\textsuperscript{12,11}. Three stress sensors of unfolded protein response (UPR), protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring protein 1 alpha (IRE1\textalpha{}), and activating transcription factor 6 (ATF-6) are initially activated by E\textsubscript{2} as an adaptation response to maintain homeostasis in the endoplasmic reticulum\textsuperscript{15,11,14}. PERK phosphorylates eukaryotic initiation factor 2 alpha (eIF2\textalpha{}) to attenuate protein translation\textsuperscript{15,16}, which is identified as an important mediator of E\textsubscript{2}-induced apoptosis\textsuperscript{11,12}, whereas ATF-6 and IRE1\textalpha{} are involved in endoplasmic reticulum-associated protein degradation (ERAD) of phosphoinositide 3-kinase (PI3K)/Akt/mTOR-related pathways\textsuperscript{13}. Additionally, a variety of stress- and inflammation-responsive genes, such as tumor necrosis factor alpha (TNF\textalpha{}), lymphotoxin alpha (LTA), lymphotoxin beta (LTB), and interleukin-6 (IL-6), are activated to create a special inflammatory microenvironment after E\textsubscript{2} exposure\textsuperscript{12,11}. Among these inflammatory factors, the function of TNF\textalpha{} has been confirmed to be an important factor to induce apoptosis with higher levels of cleaved PARP and caspase 9 in MCF-7:5C cells\textsuperscript{11}. Induction of TNF\textalpha{} by E\textsubscript{2} reaches a peak at 3 days in MCF-7:5C cells, whereas the highest levels of TNF\textalpha{} occur after 9–12 days of E\textsubscript{2} treatment in MCF-7:2A cells\textsuperscript{11,12}. In line with the time point of TNF\textalpha{} induction, E\textsubscript{2}-induces apoptosis in MCF-7:5C cells within 1 week, while apoptosis is delayed to 2 weeks after exposure to E\textsubscript{2} in MCF-7:2A cells\textsuperscript{11,12}. Nevertheless, how E\textsubscript{2} induces TNF\textalpha{} and why a delay occurs still need to be explained.

It is well known that TNF\textalpha{} is a nuclear factor-kappa (NF-kB)-dependent gene; on the other hand, TNF\textalpha{} is also a strong inducer for NF-kB\textsuperscript{18}. However, it remains unknown whether E\textsubscript{2} induces TNF\textalpha{} via activation of NF-kB in E\textsubscript{2}-deprived breast cancer cells. There is cross-talk existing between ER and NF-kB, but the latter is repressed by ER in breast cancer cells\textsuperscript{20}. Nonetheless, NF-kB can be activated in the setting of endoplasmic reticulum stress even though the mechanisms are poorly understood\textsuperscript{19}. Furthermore, compelling evidence indicates that NF-kB and lipid metabolism-associated transcription factor C/EBP\textbeta{} can interact to modulate endoplasmic reticulum stress and inflammatory responses\textsuperscript{22,21}. Thus, NF-kB has proven to be a viable therapeutic target for diseases related to endoplasmic reticulum stress, such as neurodegenerative diseases and diabetes\textsuperscript{23,24}. In most quiescent cells, NF-kB binds to the inhibitory I\textkappa{}B proteins in the cytosol and forms an inactive complex. A common canonical pathway that activates NF-kB is modulated by the degradation of I\textkappa{}B proteins, which leads to the release of NF-kB from the complex and translocation to the nucleus where NF-kB-dependent genes, such as TNF\textalpha{} and LTB, are activated\textsuperscript{19}. In addition, post-translational modifications of NF-kB protein subunits by kinases play important roles in the activation of the NF-kB response\textsuperscript{25}. Under endoplasmic reticulum stress, a downstream signal of PERK, eIF2\textalpha{} has been reported to be sufficient to activate NF-kB DNA binding through decreasing levels of I\textkappa{}B\textalpha{}, but does not affect the stability of the protein\textsuperscript{25}. We observed that E\textsubscript{2} activates PERK/eIF2\textalpha{}\textsuperscript{11}, but there are no reports concerning the interaction between PERK and the activation of NF-kB in modulation of E\textsubscript{2}-induced apoptosis in E\textsubscript{2}-deprived breast cancer cells.

We sought here to further investigate how PERK kinase activates NF-kB/TNF\textalpha{} axis to affect E\textsubscript{2}-induced apoptosis. Our data demonstrate that E\textsubscript{2} initially suppresses activation of NF-kB and effectively prevents TNF\textalpha{} from activating NF-kB. We note a preferential elevation of C/EBP\textbeta{} expression by E\textsubscript{2} that is responsible for the suppression of NF-kB. However, NF-kB is subsequently activated by PERK via activation of transcription factor STAT3. This is independent of eIF2\textalpha{} phosphorylation and the canonical I\textkappa{}B\textalpha{} signaling pathway. Blockade of PERK phosphorylation and treatment with a NF-kB inhibitor produce equivalent effects in preventing E\textsubscript{2} from inducing TNF\textalpha{} expression and apoptosis. In addition to regulating the function of STAT3 and NF-kB, PERK modulates multiple stress responsive transcription factors, including nuclear factor erythroid-derived 2-like 2 (Nrf2), hypoxia-inducible factor 1-alpha (HIF-1\textalpha{}), and ERO-target genes, after E\textsubscript{2} treatment. This suggests that PERK is critical to convey stress signals from the endoplasmic reticulum to the nucleus with multiple stress-responsive transcription factors involved. Together, this study provides an important rationale for the exploration of targeting the stress responses in breast cancer patients undergoing exhaustive anti-hormone therapy.
Results

NF-κB is constitutively activated in MCF-7:5C cells

Many genes are activated during the stress response caused by E2 deprivation\textsuperscript{13}. To determine whether E2 deprivation affects the basal levels or activation of NF-κB, we compared the E2-deprived breast cancer cell lines MCF-7:5C and MCF-7:2A with parental MCF-7 cells. MCF-7:5C cells had lower expression levels of NF-κB than did the other two cell lines. MCF-7:2A and MCF-7 cells had similar NF-κB expression levels (Fig. 1a). However, MCF-7:5C had higher levels of constitutively activated NF-κB than did the other cell lines (Fig. 1b). We detected this difference using electrophoretic mobility shift assays (EMSA), an appropriate way to assess the nuclear activation of NF-κB via direct NF-κB p65 DNA binding\textsuperscript{26}. The higher levels of active NF-κB in MCF-7:5C cells resulted in higher basal expression levels of NF-κB-dependent genes, e.g. TNF family members (TNFα, LTA, and LTβ) and the chemokine receptor CXCR4 (Fig. 1c, d, e, f). Treatment with the selective NF-κB inhibitor JSH-23 effectively prevented nuclear translocation of NF-κB and completely blocked NF-κB p65 DNA binding induced by TNFα, which is a strong inducer of NF-κB activation (Fig. 1g). JSH-23 also effectively reduced the expression of TNFα and LTβ in a time- and dose-dependent manner (Fig. 1h, i and Supplementary Fig. S1A). Importantly, it also markedly inhibited the proliferation of the three breast cancer cell lines after different concentrations treatment (Supplementary Fig. S1C–1E). These results indicated that long-term E2 deprivation may alter the activation of NF-κB, which acts as a growth signal in breast cancer cell lines.

E2 rapidly suppresses nuclear activation of NF-κB in E2-deprived breast cancer cells

To further determine whether E2 regulates the activation of NF-κB, we first treated MCF-7:5C cells with E2 for different periods, using cells treated with TNFα as positive controls. As expected, TNFα quickly increased NF-κB phosphorylation after 5 min of treatment. Simultaneously, the classic canonical signaling was activated, demonstrating increased levels of phosphorylated IκBα and higher degradation rate of IκBα, as compared with control. E2 had a significant potential for completely suppressing TNFα-induced activation of NF-κB and IκBα (Fig. 2a). Unlike TNFα treatment, E2 alone did not significantly change these signaling pathways within 1 h (Fig. 2b). In agreement with this, expression of NF-κB target gene CXCR4 was suppressed by E2 in MCF-7:5C cells (Fig. 2c), as well as in both MCF-7 and MCF-7:2A cells (Fig. 2d). Treatment with E2 also reduced the expression levels of NF-κB in MCF-7:5C cells, but not in the other two cell lines (Supplementary Fig. S1B). We detected a weak elevation of phosphorylated NF-κB p65 in the cytosol of MCF-7:5C cells when treated with E2 for more than 48 h (Fig. 2e), while we did not observe elevated phosphorylation or detectable degradation of IκBα in parallel (Fig. 2e), indicating that other mechanisms are involved in the regulation of NF-κB activation by E2.

Preferential elevation of C/EBPβ expression induced by E2 is responsible for suppression of NF-κB activity

Cross-talk between NF-κB and C/EBPβ affects each other’s function\textsuperscript{27}. We found that E2-deprived MCF-7:5C and MCF-7:2A had higher basal levels of C/EBPβ mRNA expression than did wild-type MCF-7 cells (Supplementary Fig. S2A). Treatment with the endoplasmic reticulum stress inducer tunicamycin (Tu) further increased expression of C/EBPβ in MCF-7:5C cells (Supplementary Fig. S2B). E2 started to upregulate C/EBPβ expression after 1 h of treatment (Fig. 3a) and continually elevated the expression of C/EBPβ in MCF-7:5C cells over levels in wild-type MCF-7 cells (Fig. 3b). This elevated C/EBPβ expression, induced by E2, was abolished by 4-hydroxytamoxifen (4-OHT), suggesting an ERα-dependent mechanism (Supplementary Fig. S2C). Treatment with estrogen dendrimer conjugate, a synthetic macromolecule that activates membrane-associated ERα\textsuperscript{28}, could not activate C/EBPβ in MCF-7:5C cells (Supplementary Fig. S2D), confirming that elevation of C/EBPβ expression was mainly mediated by the nuclear ERα. We further used a specific siRNA to effectively knock down C/EBPβ protein expression in MCF-7:5C cells (Fig. 3c). This increased NF-κB mRNA expression, which was further elevated after E2-based treatment (Fig. 3d). In line with this result, expression of NF-κB-dependent gene TNFα was upregulated after knockdown of C/EBPβ (Fig. 3e). E2 was synergistic with C/EBPβ siRNA in increasing TNFα and LTβ expression (Fig. 3e, f). Reduction of C/EBPβ expression by siRNA also elevated the expression of the apoptotic markers Bim and HMOX1 in MCF-7:5C cells, which increased more when C/EBPβ siRNA was combined with E2 (Supplementary Fig. S2E and S2F). Since the mTOR signal pathway has been observed to regulate the function of C/EBPβ in response to inflammation\textsuperscript{29}, the mTOR inhibitor, rapamycin was used to effectively block phosphorylation of the downstream signal p70S6 (Fig. 3g). Similar to C/EBPβ siRNA, blocking mTOR signal was synergistic with E2 to increase the expression of NF-κB-dependent genes TNFα and LTβ (Fig. 3h and Supplementary Fig. S2G). Additionally, C/EBPβ is well known to be a transcription factor that regulates IL-6 expression\textsuperscript{30}. Of note, IL-6/IL-6R expression increased after knockdown of C/EBPβ and further with E2 treatment (Fig. 3i, j), indicating a suppressive effect on the IL-6/IL-6R pathway by C/EBPβ. Contrary to the repressive effects of C/EBPβ on NF-κB, inhibition of nuclear translocation of NF-κB reduced the basal expression of C/EBPβ and completely blocked the induction of C/EBPβ by E2 (Fig. 3k). This suggested a complex interaction existing between C/EBPβ and NF-κB in the settings of stress.
Late activated NF-κB by E2 induces TNFα expression and causes apoptosis

The TNF family members are inflammatory factors induced by E2 to trigger apoptosis in two E2-deprived breast cancer cell lines. Our results demonstrated that treatment with E2 did not induce TNFα or LTB expression in MCF-7:5C cells within 24 h. However, E2 treatment began to increase the expression of TNFα and LTB after 48 h, peaking at 72 h (Fig. 4a, b). Other members of the TNF family, such as LTA, had similar dynamic changes in expression (Supplementary Fig. S3A). Treatment with the NF-κB inhibitor JSH-23 markedly reduced the basal expression of TNFα and LTB and effectively prevented E2 from inducing expression of TNFα (Fig. 4c), LTB (Fig. 4d), and LTA (Supplementary Fig. S3B). Similarly, E2 started to induce TNFα after 6 days of treatment and reached a peak after 12 days of exposure to E2 in another E2-deprived breast cancer cells, MCF-7:5C.

Fig. 1 NF-κB is constitutively activated in MCF-7:5C cells. a MCF-7 cells were transferred to E2-free medium for 3 days. Cells were harvested similarly to that with the E2-deprived cell lines MCF-7:5C and MCF-7:2A. NF-κB expression was quantitated by RT-PCR. *P < 0.05 compared with MCF-7 cells. b NF-κB DNA-binding activity. MCF-7 cells were cultured in parallel in E2-replete or E2-depleted medium for 3 days. Next, cells were harvested similarly to that with MCF-7:5C and MCF-7:2A for EMSA. c-f Basal mRNA expression levels of TNFα (c), LTA (d), LTB (e), and CXCR4 (f) in three cell lines were quantitated by RT-PCR as in a. *P < 0.05 and **P < 0.001 compared with MCF-7 cells. g The NF-κB inhibitor blocked NF-κB/DNA-binding. MCF-7:5C cells were treated with TNFα (0.1 nM), the NF-κB inhibitor JSH-23 (20 µM), or a combination of them for 30 min for EMSA. h MCF-7:5C cells were treated with JSH-23 (10 µM) for 24, 48, and 72 h. TNFα expression levels were quantitated by RT-PCR. *P < 0.05 compared with control. i MCF-7:5C cells were treated with different concentrations (5, 10, 15, 20 µM) of JSH-23 for 48 h. TNFα expression levels were quantitated by RT-PCR. *P < 0.05 compared with control.
7:2A. JSH-23 mildly increased TNFα expression levels after 12 days administration, but it significantly blocked TNFα induction by E2 after combination treatment (Supplementary Fig. S3C). Additionally, inhibition of NF-κB activity reduced expression of the apoptotic marker Bim and completely blocked induction of Bim by E2 (Fig. 4e). Further biological experiments demonstrated that treatment with the NF-κB inhibitor effectively reduced cleavage of PARP activated by E2 (Fig. 4f). To confirm the roles of NF-κB in the induction of TNF family members, NF-κB was effectively knocked down by a specific siRNA (Fig. 4g). Consistently, depletion of NF-κB decreased the expression of TNFα and LTB and attenuated that of TNFα and LTB induced by E2 in MCF-7:5C cells (Supplementary Fig. S3D and S3E). More importantly, blockade of nuclear translocation of NF-κB by JSH-23 completely inhibited E2-induced apoptosis (Fig. 4h and Supplementary Fig. S3F). These results suggested that NF-κB participates in E2-induced apoptosis via mediation of the TNF family member-associated inflammatory factors.

**NF-κB is lately activated by PERK after E2 treatment**

PERK activation is a key event in E2-induced apoptosis. A question was raised whether PERK kinase could activate NF-κB after E2 treatment. E2 transiently activated the PERK downstream signal eIF2α in MCF-7:5C cells after 6 h of treatment (Fig. 5a) and continuously phosphorylated eIF2α after 24 h. The phosphorylation of eIF2α could be blocked by a PERK inhibitor (Fig. 5b). Consistent with these findings, the downstream PERK genes, i.e. C/EBP-homologous protein (CHOP) and ATF4 expression were effectively blocked by the PERK inhibitor (Fig. 5b).
the IκBα protein, E2 caused downregulation and could decrease IκBα amount further when combined with the PERK inhibitor (Fig. 5e). This result suggested that neither E2 nor PERK modulates the function of NF-κB through the canonical pathway. It is well known that NF-κB is a DNA-binding protein that transcriptionally regulates its target genes. In the present study, MCF-7:5C cells had constitutively activated NF-κB exhibiting high basal levels of DNA binding. E2 significantly suppressed NF-κB DNA binding within 24 h, but this binding capacity returned after 48 h of exposure to E2 (Fig. 5f). Unexpectedly, the PERK inhibitor completely blocked the basal constitutive activation of NF-κB in MCF-7:5C cells and further activation by E2 (Fig. 5f). This inhibitor also effectively abolished induction of the NF-κB-dependent genes TNFα, LTB, Bim, and CXCR4 by E2 (Fig. 5g, i, and Supplementary Fig. S4A) and partially attenuated E2-induced IL-6 mRNA expression in MCF-7:5C cells (Supplementary Fig. S4B). Interestingly, inhibition of PERK kinase downregulated NF-κB mRNA expression (Supplementary Fig. S4C). In contrast, further depletion of PERK by an siRNA (Supplementary Fig. S4D) did not alter NF-κB mRNA expression (Supplementary Fig. S4E), indicating a transcriptional regulation of NF-κB via PERK.

**Fig. 3 E2 preferentially elevates C/EBPβ expression to suppress the function of NF-κB.** a MCF-7:5C cells were treated with E2 (1 nM) for the indicated period. C/EBPβ expression levels were quantitated by RT-PCR. *P < 0.05 and **P < 0.001 compared with control. b MCF-7 cells were transferred to E2-free medium for 3 days, then MCF-7 and MCF-7:5C cells were treated with E2 for the indicated periods. C/EBPβ expression levels were quantitated by RT-PCR. *P < 0.05 and **P < 0.001 compared with control. c Knockdown of C/EBPβ by siRNA. MCF-7:5C cells were transfected with different C/EBPβ siRNAs for 72 h, and cells transfected with pooled or single scrambled siRNAs were used as controls. Cell lysates were harvested for immunoblot assay. d–f, i, j MCF-7:5C cells were transfected with C/EBPβ siRNA #8 for 72 h, and cells transfected with single scrambled siRNA were used as control. Next, transfected cells were treated with vehicle control or E2 (1 nM) for 72 h. NF-κB (d), TNFα (e), LTB (f), IL-6R (i), and IL-6 (j) expression levels were quantitated by RT-PCR. *P < 0.05 and **P < 0.001 compared with scrambled siRNA vehicle control. g MCF-7:5C cells were treated with vehicle control (0.1% DMSO) or rapamycin (20 nM) for 48 h. Phosphorylated p70S6 was measured using western blotting. h MCF-7:5C cells were treated with E2 (1 nM), rapamycin (20 nM), or a combination of them for 72 h. TNFα expression levels were quantitated by RT-PCR. **P < 0.001 compared with control. k MCF-7:5C cells were treated with E2 (1 nM), JSH-23 (20 µM), or a combination of them for 72 h. C/EBPβ expression levels were quantitated by RT-PCR. *P < 0.05 and **P < 0.001 compared with control.
kinase. Together, these findings suggested that PERK specifically modifies the nuclear function of NF-κB by increasing its DNA-binding capacity.

**PERK transcriptionally modulates the interaction between NF-κB and STAT3**

Based on the results in Fig. 5 that PERK kinase mainly increased NF-κB DNA-binding (Fig. 5e, f), it indicates some other molecules are involved to mediate the responses between the endoplasmic reticulum and the nucleus. NF-κB and STAT3 are two closely related transcription factors in the regulation of inflammatory pathway. To determine how PERK kinase modulates the nuclear function of NF-κB, we found that STAT3 was activated by E2 after 24 h of treatment, which was completely blocked by 4-OHT (Fig. 6a). Further inhibition of PERK kinase effectively blocked the phosphorylation of STAT3 (Fig. 6b) and STAT3 DNA-binding activity induced by E2 (Fig. 6c), demonstrating that STAT3 is a downstream signal of PERK. The STAT3 inhibitor, Stat3 inhibitor, effectively blocked nuclear translocation of STAT3 (Fig. 6d), but almost did not affect the phosphorylation of
STAT3 in MCF-7:5C cells (Fig. 6e). Notably, STAT3 DNA binding was increased by E2 in a time-dependent manner, and Stattic was far more effective to block STAT3 DNA binding than prevent nuclear translocation (Fig. 6e, f). Furthermore, the STAT3 inhibitor effectively blocked the DNA-binding activity of NF-κB (Fig. 6g) and the induction of NF-κB-dependent genes TNFα and LTB (Fig. 6h and Supplementary Fig. S5A and S5B). This finding suggested that STAT3 DNA binding is essential to affect the nuclear activation of NF-κB. In addition to regulating the function of STAT3 and NF-κB by PERK kinase, Nrf2 is a critical transcription factor that maintains redox homeostasis. Treatment with E2 elevated Nrf2 expression in MCF-7:5C cells, whereas that with the PERK inhibitor reduced basal Nrf2 expression and effectively blocked the upregulation of Nrf2 expression by E2 (Fig. 6i). Consequently, inhibition of PERK kinase completely prevented expression of the oxidative stress indicator HMOX1 (Fig. 6j). A similar regulatory pattern was observed in stress-responsive transcription factor HIF-1α, which was upregulated by treatment with E2, but reduced by that with the PERK inhibitor. This inhibitor also blocked induction of HIF-1α expression by E2 (Fig. 6k). Since STAT3 acts as a downstream signal of PERK, inhibition of STAT3 also effectively blocked upregulation of HMOX1 and Nrf2 in MCF-7:5C cells treated with E2 (Fig. 6i). Intriguingly, the PERK inhibitor reduced expression of ERα-targeted genes pS2, c-Myc, and FOXO3 in MCF-7:5C cells (Supplementary Fig. S5F–S5H), despite the fact that E2 activates PERK via ERα. Therefore, in addition to attenuating protein translation, PERK acts as a central regulator that modulates the function of multiple nuclear transcription factors in response to stress (Fig. 7).
Discussion

Estrogen-induced apoptosis has clinical relevance for the treatment of aromatase inhibitor-resistant breast cancer. And it is a mechanistic interpretation for the decrease in breast cancer incidence and mortality of the E2 alone trial of the Women’s Health Initiative.6–8. Endoplasmic reticulum stress precedes E2-induced apoptosis, which involves the activation of the three stress sensors with different functions in the modulation of cellular homeostasis.11–13. Furthermore, PERK has been found as a pivotal stress sensor to regulate E2-induced apoptosis in E2-deprived breast cancer cells.12, 11 However, the mechanism whereby PERK interacts with transcription factors and integrally modulates E2-induced apoptosis is obscure. We have now deciphered the mechanism precisely. E2 suppresses the activation of NF-kB by preferentially upregulating C/EBPβ expression whereas PERK acts as a major driver to transcriptionally activate NF-kB/TNFα axis-linked apoptotic pathways in a delayed manner via STAT3.

A novel finding is how E2 modulates activation of NF-kB by alternate mechanisms to determine the fate of E2-deprived breast cancer cells. NF-kB is an inducible transcription factor that mediates numerous biological functions to regulate proliferation or apoptosis depending on the cellular context and inflammatory microenvironment.35–37. Constitutive activation of NF-kB is one of the stress responses required for adaptation to long-term E2 deprivation, which initially acts as a growth signal in E2-deprived breast cancer cells as in other antiestrogen-resistant breast cancer cell lines3.38. However, E2 exposure completely alters the biological functions of NF-kB in
E2-deprived breast cancer cells. Nuclear ERα, but not membrane-associated ERα, is known to be activated by E2 to induce apoptosis, which preferentially elevates expression of the lipid metabolism-associated transcription factor C/EBPβ to suppress the activation of NF-κB. This repressive potential of E2 is enough to compete with a strong inducer, TNFα, to prevent NF-κB activation. In line with our findings, Hayakawa et al. demonstrated a suppressive interaction between C/EBPβ and NF-κB in mesangial cells under endoplasmic reticulum stress. C/EBPβ and NF-κB belong to distinct families of transcription factors that have functional and physical associations between their DNA-binding domains or through protein–protein interactions. Although mechanisms whereby C/EBPβ suppresses NF-κB are unclear, it is very likely that predominant activation of C/EBPβ in settings of endoplasmic reticulum stress precedes NF-κB to occupy DNA-binding regions or form protein complexes with the majority of NF-κB, subsequently preventing NF-κB DNA binding. Furthermore, C/EBPβ is an important adipogenic transcription factor with a function that is closely associated with the mTOR transduction pathway that develops in response to stresses. The phospholipid-associated pathways PI3K/Akt/mTOR are activated early by E2 in MCF-7:5C cells, whereas these signals are degraded by IRE1 and ATF-6-mediated ERAD after 48 h of E2 action. Therefore, attenuation of the mTOR signal by UPR might dissociate the inhibitory interactions between C/EBPβ and NF-κB, which results in the activation of NF-κB at a later time.

The mechanism by which PERK kinase modulates NF-κB DNA binding provides an in-depth understanding of E2-induced apoptosis in E2-deprived breast cancer cells. A central biological function of PERK is to reduce the protein burden in the endoplasmic reticulum by activating eIF2α kinase. Deng et al. reported that eIF2α kinase decreases IκBα expression which further blocks NF-κB activity. These
data suggest that eIF2α phosphorylation is not required to activate NF-κB by PERK. Consistent with our results, Cullinan et al. found that PERK regulates nuclear translocation of the redox homeostasis modulator Nrf2 without requirement of eIF2α phosphorylation. Our evidence also indicates that PERK does not regulate the canonical IkBα signal pathway to affect NF-κB DNA binding. Of note, STAT3 is identified as a target transcription factor that is regulated by PERK to activate NF-κB DNA binding and NF-κB-dependent genes. Furthermore, our finding demonstrates that STAT3 DNA binding, rather than phosphorylation can significantly affect NF-κB DNA binding, indicating an essential interaction between STAT3 and NF-κB in DNA. There are many signals that might be involved in the modulation of STAT3 by PERK. For instance, PERK activates cytokine IL-6 which is a strong inducer for STAT343. Additionally, STAT3 by PERK. For instance, PERK activates cytokine signals that might be involved in the modulation of NF-κB between STAT3 and NF-κB. How NF-κB expresses higher levels of TNFRSF18, TNFRSF19, and MCF-7:2A cells; while MCF-7:2A cells have been found to have higher basal levels of TNFα, LTA, and LTB than those in MCF-7:5C cells18. How NF-κB is the initial site for E2 to induce apoptosis in MCF-7:5C cells, the function of many other transcription factors with short half-lives, such as c-Fos in E2-deprived cells. These have high potential to cause an accumulation of aberrant unfolded proteins in the endoplasmic reticulum. Consequently, determining how E2 produces unfolded proteins to trigger endoplasmic reticulum stress-associated apoptosis is essential to find the mechanisms underlying E2-induced apoptosis. Our recent observations demonstrate that E2 preferentially and consistently activates some transcription factors with short half-lives, such as c-Fos in E2-deprived cells. These have high potential to cause an accumulation of aberrant unfolded proteins in the endoplasmic reticulum. A rigorous investigation of the apoptosis triggering mechanism in human cancer cell models provides valuable insight into a vulnerability of endocrine resistant cancer. Future application of this knowledge will aid development of treatments to increase breast cancer patient survival.

Materials and methods

Materials

Estrogen and rapamycin were purchased from Sigma-Aldrich. The NF-κB inhibitor, ISH-23 was purchased from CalBiochem. The PERK inhibitor, GSK797800 was obtained from Toronto Research Chemicals. The STAT3 inhibitor, Statick was purchased from Tocris. For western blotting, antibodies against NF-κB, phosphorylated-NF-κB, phosphorylated-IκBα, total-IκBα, phosphorylated-eIF2α, total-eIF2α, PERK, and PARP were purchased from Cell Signaling Technology. NF-κB, C/EBPβ, PERK and scrambled siRNAs were obtained from GE Dharmacon.
Cell culture conditions and cell line validation

MCF-7:WS8 cells were clonally selected from their parental counterpart MCF-7 (obtained from Dean Edwards, San Antonio, TX) for sensitivity to growth stimulation by E2, which were used in all experiments indicating MCF-7. MCF-7 cells were maintained in phenol red containing RPMI 1640 medium supplemented with 10% fetal bovine serum. MCF-7:5C and MCF-7:2A cells were cloned from E2-deprived MCF-7 cells and maintained in phenol red-free RPMI 1640 supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum. Three cell lines were validated by a single nucleotide polymorphism (SNP) based assay at MD Anderson’s Characterized Cell Line Core (CCLC) on January 31, 2017. The SNP ID pattern of three cell lines is consistent with the report of the CCLC standard MCF-7 cells (Supplementary Table S1).

Annexin V analysis of apoptosis

A FITC Annexin V Detection Kit I (BD Pharmingen) was used to quantify apoptosis of MCF-7:5C cells through flow cytometry according to the manufacturer’s instructions. In brief, MCF-7:5C cells were seeded in 10-cm dishes. The next day, the cells were treated with different compounds for different periods. Cells were suspended in 1× binding buffer and 1× apoptosis was performed as described previously11.

Subcellular fractionation and immunoblotting

Cytosolic extract of MCF-7:5C cells was prepared using cell lysis buffer. Cytosolic cell lysates were spun at 12,000 rpm for 2 min, and supernatants were collected in separate eppendorf tubes. Nuclear extracts were prepared using a nuclear extraction. Nuclear cell lysates were spun at 14,000 rpm for 10 min and supernatants were collected and kept at −80 °C. Total proteins were extracted in cell lysis buffer (Cell Signaling Technology) supplemented with Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail Set I and Set II (CalBiochem). Immunoblotting was performed as described previously11.

Electrophoretic mobility shift assays

To assess the nuclear activation of NF-κB and STAT3 in MCF-7:5C cells, EMSA of nuclear extracts from treated- and untreated-cells was performed as described previously43. In brief, MCF-7:5C cells (2 × 10⁶/mL) were treated with E2, a PERK inhibitor, or a STAT3 inhibitor for different periods. Nuclear extracts from the cells were incubated with a 32P end-labeled NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) (5′-TTGTGTA CAAAGGACTTTCCGCTGGGGAGTCCGGAGG CGTGG-3′, with NF-κB-binding sites) or two 32P-labeled high-affinity sis-inducible element (hSIE) STAT3 probes (5′-CTTCATTTCCGGTAAATCCCTAAAGCT-3′ and 5′-AGCTTTAGGGATTTACGGGAATGTA-3′)44 for 30 min at 37 °C. The resulting protein-DNA complex was separated from free oligonucleotides on 6.6% native polyacrylamide gels. The dried gels were visualized via exposure on X-ray films, and radioactive bands were analyzed.

Quantitative real-time reverse transcription-PCR

Total RNA isolated from cells using an RNeasy Micro kit (Qiagen) was converted to first-strand cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystem). Quantitative real-time PCR assays were performed with SYBR Green PCR Master Mix (Applied Biosystems) and a QuantStudio 6 Flex real-time PCR System (Applied Biosystems). All primers were synthesized in Integrated DNA Technologies. All data were normalized by 36B4.

Statistical analysis

All values are reported as means ± SEM. Statistical comparisons were carried out using a two-tailed Student’s t-test. Results were considered statistically significant if the P-value was less than 0.05.

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Competing interests

The authors declare that they have no competing interests.

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