Activated ERBB2/HER2 Licenses Sensitivity to Apoptosis upon Endoplasmic Reticulum Stress through a PERK-Dependent Pathway

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Abstract
HER2/Neu/ERBB2 is a receptor tyrosine kinase overexpressed in approximately 20% of human breast tumors. Truncated or mutant isoforms that show increased oncogenicity compared with the wild-type receptor are found in many breast tumors. Here, we report that constitutively active ERBB2 sensitizes human breast epithelial cells to agents that induce endoplasmic reticulum stress, altering the unfolded protein response (UPR) of these cells. Deregulation of the ERK, AKT, and mTOR activities elicited by mutant ERBB2 was involved in mediating this differential UPR response, elevating the response to endoplasmic reticulum stress, and apoptotic cell death. Mechanistic investigations revealed that the increased sensitivity of mutant ERBB2-expressing cells to endoplasmic reticulum stress relied upon a UPR effector signaling involving the PERK–CHOP pathway, upregulation of the proapoptotic cell surface receptor TRAIL-R2, and activation of proapoptotic caspase-8. Collectively, our results offer a rationale for the therapeutic exploration of treatments inducing endoplasmic reticulum stress against mutant ERBB2-expressing breast tumor cells.

Introduction
In response to different environmental and physiologic stress conditions that increase the load of unfolded proteins in the endoplasmic reticulum, protein sensors located in the luminal face of the endoplasmic reticulum membrane activate the unfolded protein response (UPR; ref. 1). Activation of this signaling pathway leads to a reduction in the influx of unfolded proteins into the endoplasmic reticulum, activates protein degradation pathways, and increases the folding capacity of the endoplasmic reticulum (2). In vertebrates, 3 different types of endoplasmic reticulum stress transducers have been identified. Each type defines a distinct branch of the UPR that is mediated by protein kinase RNA–like endoplasmic reticulum kinase (PERK; ref. 3), inositol-requiring protein-1 (Ire1; ref. 4), or activating transcription factor-6 (ATF6; ref. 5). In each case, an integral membrane protein senses the protein-folding status in the endoplasmic reticulum lumen and transmits this information across the endoplasmic reticulum membrane to the cytosol and nucleus (2). These mechanisms allow adaptive and repair mechanisms that re-establish homeostasis. However, above a certain threshold, unresolved endoplasmic reticulum stress results in apoptosis (6).

As a major regulator of cell growth, metabolism, and survival, the mTOR pathway is commonly activated during oncogenesis (7). Thus, inactivating mutations or deletions of PTEN lead to Akt and mTOR activation and occur frequently in human cancers (8). Furthermore, loss of the upstream regulators of the mTOR pathway TSC1 and TSC2 leads to constitutive activation of mTORC1 and results in the development of tumors (9). Interestingly, tumor cells harboring an activated mTOR pathway are more sensitive to endoplasmic reticulum stress–induced cell death (10–12), although the mechanism underlying this cell death process remains to be elucidated.

ERBB2 is a member of the ERBB receptor family, which also includes the epidermal growth factor receptor (EGFR, ERBB1), ERBB3, and ERBB4. Ligand binding to the extracellular domains of EGFR, ERBB3, and ERBB4 leads to the formation of catalytically active homo- and heterodimers to which ERBB2 is recruited as a preferred partner (13). Activation of the ERBB receptors leads to receptor autophosphorylation of C-terminal tyrosines and recruitment to these sites of cytoplasmic signal transducers that activate several downstream signaling pathways, such as the extracellular signal-regulated kinase and the phosphoinositide-3-kinase/AKT/mTOR pathways (14). Amplification of a genomic region containing the ERBB2 gene on chromosome 17q12 has been observed in approximately 25% of...
invasive breast tumors (15). ERBB2 overexpression is frequently accompanied by the occurrence of truncated forms of the receptor that are characterized by enhanced oncogenic potential (16, 17). In addition, somatic mutations in the ERBB2 gene have been reported in a number of tumors, including breast carcinomas, some of which results in a gain-of-function compared with wild-type ERBB2 (18, 19).

Herein, we have addressed the issue of the sensitivity to endoplasmic reticulum stress of human breast epithelial cells expressing an activated form of the ERBB2 oncogene. We show that mutant ERBB2 expression in breast epithelial cells leads to an overactivation of the UPR and markedly sensitizes these cells to endoplasmic reticulum stress–induced apoptosis. Hyperactivation of the PERK/ATF4/CHOP pathway in mutant ERBB2 cells following endoplasmic reticulum stress upregulates TRAIL-R2 expression, resulting in the induction of a caspase–8–mediated and mitochondria–operated apoptotic pathway. Importantly, deregulation of the PERK/ATF4/CHOP/TRAIL-R2 pathway and sensitivity of mutant ERBB2 to endoplasmic reticulum stress is abrogated by inhibition of the mitogen–activated protein kinase (MAPK)/ERK, Akt, or mTOR activities. These results point at endoplasmic reticulum stress as a biochemical target by which tumor cells expressing gain-of-function ERBB2 mutants may be approached for therapeutic intervention.

Materials and Methods

Cell culture

MCF10A cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 5% donor horse serum (Gibco), 2 mM L-glutamine, 20 ng of epidermal growth factor (EGF)/mL, 10 μg of insulin/mL, 100 ng of cholera toxin/mL, 0.5 μg of hydrocortisone/mL, 50 U of penicillin/mL, and 50 μg of streptomycin/mL at 37°C in a 5% CO2–humidified, 95% air incubator. The human tumor cell line MDA-MB231 was maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U of penicillin/mL, and 50 μg of streptomycin/mL. SKBR3 and BT-474 cell lines were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 U of penicillin/mL, and 50 μg of streptomycin/mL.

Retroviral vectors and virus production

pbabe-NeuN vector (wild-type ERBB2) for stable gene expression has been described previously (20). Constitutively active ERBB2 mutant (pbabe-NeuT) was kindly provided by D. Carroll (Harvard Medical School, Boston, MA). pbabe-Bcl-xL was a gift from Dr. C. Muñoz (IDIBELL, Barcelona, Spain). Retroviruses for protein overexpression were produced by transfection of HEK293-T cells by the calcium phosphate precipitation assay buffer. Protein content was measured with the Bradford reagent (Bio-Rad Laboratories), before adding Laemmli sample buffer. Proteins were resolved on SDS-polyacrylamide minigels and detected as described previously (22). Tubulin and glyceraldehyde-3-phosphate dehydrogenase were used as protein loading controls.

Reverse transcriptase and PCR assays

Total RNA was isolated from MCF10A cells with the TRIzol reagent (Life Technologies) as recommended by the supplier. Total RNA was used as a template for cDNA synthesis using a reverse transcriptase (RT)-PCR Kit (Perkin-Elmer). PCRs were carried out using specific primers (Supplementary Materials). RT-PCR product of β-actin was used as a control for mRNA input.

Real-time PCR

mRNA expression was analyzed in triplicate by RT-qPCR on the ABI Prism7500 sequence detection system using predesigned assay-on-demand primers and probes (Applied Biosystems). Hypoxanthine-guanine phosphoribosyltransferase (HPRT1 Hs01003267_m1) was used as an internal control and mRNA expression levels of CHOP, TRAIL, and TRAIL-R2 were given as fraction of mRNA levels in control cells. Primers and probes used were: ATF4 (Hs00909568_g1), CHOP (Hs01909850_m1), TRAIL (Hs00921974_m1), and TRAIL-R2 (Hs00366278_m1).

RNA interference

siRNAs against TRAIL-R2, Bid, Ire1, ATF4, ATF6, caspase-8, Raptor, Rictor, Noxa, Bim, and nontargeting scrambled control (Supplementary Materials) were synthesized by Sigma. Flexitube siRNAs against PERK, CHOP, and TRAIL, were purchased from Qiagen. Cells were transfected with siRNAs using DharmaFECT-1 (Dharmacon) as described by the manufacturer. After 6 hours, transfection medium was replaced with regular medium and cells were further incubated for 48 hours before further analysis.

Statistical analysis

All data are presented as the mean ± SE of at least three independent experiments. The differences among different groups were determined by the Student t test. P < 0.05 was
considered significant. *, P < 0.05; **, P < 0.01; ***, P < 0.001. n.s., not statistically significant.

Results
Enhanced sensitivity of mutant ERBB2-expressing cells to endoplasmic reticulum stress
Recent findings have demonstrated that the presence of ERBB2 somatic mutations is an alternative mechanism to activate ERBB2 in breast cancer (19). Different somatic mutations of ERBB2 enhance the tyrosine kinase activity and the oncogenic potential of this protein (18, 19, 23). One of the consequences of ERBB2 activation is the deregulated activation of the MAPK/ERK and PI3K/Akt/mTOR pathways, which promotes cell growth, proliferation, increased metabolism, and motility (13). Given the reported cross-talk between the UPR and mTOR signaling pathways (10–12, 24), we investigated the impact of ERBB2 activation on the sensitivity of breast epithelial cells to endoplasmic reticulum stress. Results depicted in Fig. 1A indicate that human breast epithelial cells MCF10A expressing a constitutively active ERBB2 (NeuT; ref. 20) showed constitutive phosphorylation of ERBB2 at Tyr1248 and were markedly more sensitive to the endoplasmic reticulum stress inducer thapsigargin than control cells (pbabe) or cells expressing wild-type ERBB2 (NeuN). Time course experiments further confirmed the increased sensitivity of NeuT cells to endoplasmic reticulum stress (Supplementary Fig. S1A). Tunicamycin, another endoplasmic reticulum stress inducer, also activated apoptosis differentially in NeuT cells (Supplementary Fig. S1B). Furthermore, results shown in Fig. 1B demonstrate that the ERBB2 tyrosine kinase inhibitor lapatinib markedly reduced thapsigargin-induced apoptosis in NeuT cells, strongly suggesting that sensitivity to endoplasmic reticulum stress is a result of the constitutive activation of ERBB2. In agreement with the data of the lower sensitivity of NeuN cells, breast tumor cell lines overexpressing either naturally (BT-474, SkBr3) or ectopically (MDA-MB231) the wild type form of ERBB2 showed a reduced sensitivity to endoplasmic reticulum stress (Fig. 1C). Evaluation of sensitivity to endoplasmic reticulum stress in breast tumor cells ectopically overexpressing the NeuT oncogene was not possible because these cells underwent premature senescence (Supplementary Fig. S1C), as it has been previously reported (25).

![Graphs and images representing results](image-url)

Figure 1. Apoptotic response of ERBB2-expressing cells to endoplasmic reticulum stress. A, cells were treated with the indicated doses of thapsigargin for 30 hours and apoptosis was determined as described in Materials and Methods. Inset shows the expression of total ERBB2 and p-ERBB2 (Tyr1248) in the different cell lines tested. Error bars, SD from three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. B, pbabe and NeuT cells were incubated with or without lapatinib (5 μmol/L) for 48 hours and then treated with thapsigargin (100 nmol/L) for 30 hours in the presence or the absence of lapatinib. Apoptosis was determined as described in Materials and Methods. Inset shows the expression of p-ERBB2 (Tyr1248) after treatment with or without lapatinib (5 μmol/L) for 48 hours. Error bars, SD from three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. C, indicated cell lines were treated with 100 nmol/L thapsigargin during 30 hours and apoptosis (left) was measured as described in Materials and Methods. Right panel shows the expression of p-ERBB2 (Tyr1248) and total ERBB2 in the different cell lines tested. Error bars, SD from three independent experiments. *, P < 0.05 and **, P < 0.01 comparing the various cell lines with NeuT cells.
Role of the UPR branches in endoplasmic reticulum stress–induced apoptosis in mutant ERBB2-transformed cells

To investigate the mechanism of the enhanced sensitivity of NeuT cells to endoplasmic reticulum stress, we examined the role of the UPR branches, which are known to regulate cell fate upon endoplasmic reticulum stress (2). Thus, we determined the processing by Ire1α of the mRNA encoding the transcription factor X box–binding protein 1 (XBP1) to generate the active transcription factor XBP1s. In control cells, initial Ire1α signaling was substantially reduced upon prolonged endoplasmic reticulum stress (Fig. 2A), as previously reported in other cell systems (26). In contrast, in NeuT cells Ire1α activity remained elevated as indicated by the sustained expression of XBP1s upon endoplasmic reticulum stress. However, Ire1α knockdown did not result in the abrogation of endoplasmic reticulum stress–induced apoptosis (Supplementary Fig. S2A). Likewise, ATF6 silencing did not reduce significantly apoptosis induced by thapsigargin (Supplementary Fig. S2B).

We next examined the activity of the PERK/ATF4/CHOP pathway in both control and NeuT cells upon thapsigargin treatment. We found no differences in the activation of PERK upon thapsigargin treatment, as determined by the phosphorylation of the eukaryotic initiation factor 2α (eIF2α; Fig. 2B). Likewise, inhibition of general protein synthesis following endoplasmic reticulum stress occurred to the same extent and with similar kinetics in both control and NeuT cells (Supplementary Fig. 2C). Interestingly, although we found no differences in ATF4 mRNA levels between control and NeuT cells following thapsigargin treatment (Supplementary

Figure 2. Role of the PERK/ATF4/CHOP pathway in endoplasmic reticulum stress–induced apoptosis. pbabe and NeuT cells were treated with thapsigargin (100 nmol/L) for the indicated times. Following these treatments, XBP1 splicing was analyzed by RT-PCR (A); phosphorylation of eIF2α (B) and ATF4 induction (D) was assessed by Western blotting; protein synthesis was measured by the incorporation of [3H]leucine (10 Ci/mL) into acid-precipitable material (C) and CHOP mRNA levels were determined by RT-qPCR (E). Quantification of RT-PCR and Western blot signals was performed by densitometry with ImageQuant TL software after scanning the films on ImageScanner II (GE Healthcare). The relative expression of proteins was normalized to that of loading controls. Results are representative of three independent experiments. NeuT cells were transfected either with a scrambled oligonucleotide (Scr) or with siRNAs targeting PERK (F), ATF4 (G), or CHOP (H) for 48 hours. Cells were then treated with or without thapsigargin (100 nmol/L) for 30 hours and apoptosis was determined. Error bars, SD from three independent experiments. *, P < 0.05 and **, P < 0.01. ATF4 knockdown was determined by quantitative PCR. CHOP levels were determined by RT-qPCR.
Fig. S2C), expression of ATF4 was significantly enhanced at the protein level in NeuT cells as compared with control cells upon endoplasmic reticulum stress (Fig. 2D). In addition, expression of the ATF4 target gene CHOP (27) was also markedly upregulated in NeuT cells as compared with control cells, upon endoplasmic reticulum stress (Fig. 2E). Regarding sensitivity to endoplasmic reticulum stress, a significant inhibition of endoplasmic reticulum stress-induced apoptosis was observed in NeuT cells following PERK knockdown (Fig. 2F). Further evidences for the role of the PERK pathway in endoplasmic reticulum stress, a significant inhibition of endoplasmic reticulum stress (Fig. 2E). Regarding sensitivity to endoplasmic reticulum stress, a significant inhibition of endoplasmic reticulum stress-induced apoptosis was observed in NeuT cells following PERK knockdown (Fig. 2F). Further evidences for the role of the PERK pathway in endoplasmic reticulum stress-induced apoptosis in mutant ERBB2-transformed cells were obtained in experiments silencing the expression of downstream effectors of this pathway. As shown in Fig. 2G, ATF4 knockdown caused a marked inhibition of CHOP expression and apoptosis upon thapsigargin treatment. Similarly, silencing CHOP expression resulted in an important inhibition of thapsigargin-induced apoptosis in NeuT cells (Fig. 2H).

**TRAIL-R2–mediated apoptosis in NeuT cells upon endoplasmic reticulum stress**

We next investigated the role of caspases and the mitochondria in this cell death process. The pan-caspase inhibitor Z-VAD-fmk completely abrogated cell death induced by thapsigargin (Fig. 3A). Furthermore, caspase-9 and caspase-3 processing was also clearly enhanced in NeuT cells as compared with control cells (Fig. 3B). Strikingly, in Bcl-xL–overexpressing NeuT cells thapsigargin-induced apoptosis was clearly inhibited (Fig. 3C), which demonstrated that the increased sensitivity of NeuT cells to endoplasmic reticulum stress was because of the enhanced activation of a mitochondria-operated apoptotic pathway.

Transcriptional induction of BH3-only proteins by CHOP plays a role in endoplasmic reticulum stress–induced apoptosis in different systems (28, 29). We determined by reverse transcriptase–multiplex ligation-dependent probe amplification the mRNA levels of twenty members of the Bcl-2 family, including 10 BH3-only members. Although small differences were found between control and NeuT cells in the mRNA expression levels of Noxa and Bim upon endoplasmic reticulum stress (Supplementary Fig. S3A), knockdown of these BH3-only proteins did not inhibit thapsigargin-induced apoptosis in NeuT cells (Supplementary Fig. S3B). Collectively, these results suggested that activation of the mitochondrial pathway of apoptosis in NeuT cells by endoplasmic reticulum stress was independent of the upregulation of BH3-only or downregulation of anti-apoptotic Bcl-2 proteins expression.

CHOP involvement in endoplasmic reticulum stress–induced apoptosis has been previously linked to upregulation of TRAIL-R2 expression and a potential CHOP-binding site has been identified in the 5′-flanking region of the TRAIL-R2 gene (30). Time course analysis of TRAIL-R2 levels indicated that CHOP-dependent TRAIL-R2 upregulation following endoplasmic reticulum stress treatment was enhanced in NeuT cells compared with pbabe cells (Fig. 4A and Supplementary Figs. S4, S5A, and S5B). Moreover, TRAIL-R2 upregulation induced by endoplasmic reticulum stress was accompanied by a marked decrease in FLIP expression (Fig. 4B). Notably, caspase-8 activation was only observed in NeuT cells treated with thapsigargin (Fig. 4C). As FLIP downregulation was observed in both cell lines, these results suggested that the enhanced upregulation of TRAIL-R2 in NeuT cells may be an important event in endoplasmic reticulum stress–induced apoptosis in these cells. In this respect, TRAIL-R2 knockdown markedly reduced endoplasmic reticulum stress–induced apoptosis (Fig. 4D and Supplementary Fig. S5C). Furthermore, silencing caspase-8 expression markedly inhibited endoplasmic reticulum stress–induced apoptosis in different systems (28, 29). We determined by reverse transcriptase–multiplex ligation-dependent probe amplification the mRNA levels of twenty members of the Bcl-2 family, including 10 BH3-only members. Although small differences were found between control and NeuT cells in the mRNA expression levels of Noxa and Bim upon endoplasmic reticulum stress (Supplementary Fig. S3A), knockdown of these BH3-only proteins did not inhibit thapsigargin-induced apoptosis in NeuT cells (Supplementary Fig. S3B). Collectively, these results suggested that activation of the mitochondrial pathway of apoptosis in NeuT cells by endoplasmic reticulum stress was independent of the upregulation of BH3-only or downregulation of anti-apoptotic Bcl-2 proteins expression.

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**Figure 3.** Endoplasmic reticulum stress activates caspase-dependent cell death through a mitochondria-operated apoptotic pathway. A, apoptosis was determined in pbabe and NeuT cells treated for 30 hours with thapsigargin (100 nmol/L) in the presence or absence of z-VAD-fmk (10 μmol/L). B, pbabe and NeuT cells were treated with 100 nmol/L thapsigargin for the indicated times. Activation of caspase-9 and caspase-3 was assessed by Western blotting. Results are representative of two independent experiments. C, apoptosis was assessed in Bcl-xL–overexpressing NeuT cells treated with thapsigargin (100 nmol/L) for 30 hours. Error bars, SD from three independent experiments. P < 0.001. Bcl-xL overexpression was measured by Western blotting.
apoptosis in NeuT cells (Fig. 4E and Supplementary Fig. S5C), revealing a pivotal role of caspase-8 activation in endoplasmic reticulum stress–induced apoptosis in these cells. Activation of caspase-8 leads to the processing of the BH3-only substrate Bid generating a 15-kDa fragment that translocates to mitochondria to promote the release of apoptogenic factors (31). In NeuT cells, silencing Bid expression significantly reduced apoptosis induced by endoplasmic reticulum stress (Fig. 4F). Collectively, these results and data shown in Fig. 3C suggest a differential activation of a TRAIL-R2–mediated, mitochondria-operated apoptotic pathway in NeuT cells upon endoplasmic reticulum stress.

To determine the role of potentially secreted TRAIL in the apoptosis induced by endoplasmic reticulum stress, we used a recombinant TRAIL-R2/Fc chimeric protein that has been shown to potently neutralize the apoptotic activity of exogenous TRAIL (32). However, at doses 5 times higher than those required to inhibit apoptosis by exogenously added TRAIL (Supplementary Fig. S6A, right), TRAIL-R2/Fc did not inhibit apoptosis induced by thapsigargin (Supplementary Fig. S6A, left). Furthermore, silencing TRAIL expression before endoplasmic reticulum stress treatment did not inhibit apoptosis by thapsigargin (Supplementary Fig. S6B), which suggested the lack of involvement in apoptosis induction.

Figure 4. Involvement of TRAIL-R2 and caspase-8 in endoplasmic reticulum stress–induced apoptosis in NeuT cells. Cells were treated with 100 nmol/L thapsigargin for the indicated times. Following these treatments, TRAIL-R2 induction (A) was determined by RT-qPCR and Western blotting. FLIPL levels (B) and caspase-8 activation (C) were examined by Western blotting. Results are representative of two independent experiments. D–F, NeuT cells were transfected either with a scrambled oligonucleotide or siRNA oligonucleotides targeting TRAIL-R2 (D), caspase-8 (E), or Bid (F) for 48 hours as described in Materials and Methods. Cells were then incubated in the presence or absence of thapsigargin (100 nmol/L) for 30 hours. Protein knockdown and apoptosis were determined as previously described. Error bars, SD from three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
of endogenous TRAIL in endoplasmic reticulum stress–induced apoptosis.

**Signaling pathways regulation upon endoplasmic reticulum stress in control and NeuT cells**

To further investigate the mechanism of endoplasmic reticulum stress–induced apoptosis in NeuT cells, we first examined the activation of Akt and ERK in both pbabe and NeuT cells treated with thapsigargin. Basal levels of phosphorylated Akt were markedly different in pbabe and NeuT cells (Fig. 5A), as expected from the constitutive activation of the PI3K/Akt pathway by the mutant ERBB2 protein. As previously reported in other cell types (33), there was an acute activation of Akt in both pbabe and NeuT cells upon thapsigargin treatment, which was followed by a decrease in phosphorylated Akt to levels below the basal level in pbabe cells. In contrast, levels of phosphorylated Akt protein remained elevated after the initial peak of activation in NeuT cells treated with thapsigargin (Fig. 5A). Similarly to what was observed for Akt, basal levels of activated ERK were highly elevated in NeuT cells as compared with pbabe cells (Fig. 5B). Furthermore, in pbabe cells, endoplasmic reticulum stress caused a marked inhibition of ERK phosphorylation starting at 7 hours after the addition of thapsigargin (Fig. 5B). Remarkably, in NeuT cells we observed a sustained activation of ERK upon endoplasmic reticulum stress that remained phosphorylated for up to 20 hours of thapsigargin treatment (Fig. 5B). The observed correlation between the sustained activation of the Akt and ERK pathways and the increased apoptosis of NeuT cells upon endoplasmic reticulum stress prompted us to assess the effect of specific inhibitors of these pathways on the apoptosis induced by thapsigargin. Incubation of NeuT cells either with an Akt (GSK990693) or a MEK1 (U0126) inhibitor partially inhibited apoptosis (Fig. 5C). Strikingly, in the presence of both inhibitors apoptosis was markedly inhibited, suggesting that the sustained activation of the Akt and ERK pathways observed upon endoplasmic reticulum stress played a key role in the induction of apoptosis.

TSC2 phosphorylation by either Akt or ERK/BSK leads to disruption of the TSC1/TSC2 complex, a negative regulator of mTOR activity (34, 35). Moreover, a number of evidences have revealed the existence of a bidirectional crosstalk between endoplasmic reticulum stress and mTOR signaling (24). In addition, a link between ERBB2 overexpression and activation of the Akt/mTOR/4E-BP1 pathway has been reported in breast cancer progression (36). As expected, basal levels of phosphorylated p70(S6K), a major mTORC1 substrate, was higher in NeuT than in control cells (Fig. 6A). Upon endoplasmic reticulum stress there was an acute activation of the mTORC1 pathway in both cell lines, with maximal activity at 3-hour post-thapsigargin addition. Thereafter, phosphorylated p70(S6K) returned to levels below the basal level in control cells. On the contrary, in NeuT cells the initial peak of p70(S6K) phosphorylation was followed by a sustained mTORC1 activity, which remained elevated for up to 20 hours (Fig. 6A). We next examined the impact of sustained mTORC1 activity in the sensitivity of NeuT cells to endoplasmic reticulum stress. Intriguingly, at a dose that strongly inhibits p70(S6K) phosphorylation (Fig. 6B, right) the mTORC1 inhibitor rapamycin did not significantly reduce endoplasmic reticulum stress–induced apoptosis (Fig. 6B, left). We also tested the effect of torin1, a highly potent and selective ATP-competitive mTOR inhibitor that, unlike rapamycin, fully inhibits mTORC1 and mTORC2 complexes (37). Torin1 completely inhibited both rapamycin-sensitive and -insensitive mTORC1 activities in NeuT cells (Fig. 6B, right). Furthermore, torin1 also efficiently inhibited mTORC2 activity as indicated by the complete inhibition of AktSer473 phosphorylation (Fig. 6B, right). Strikingly, we observed an almost complete inhibition of apoptosis by torin1 (Fig. 6B, left), suggesting an important role of mTOR in this cell death process. The role of mTORC1 and mTORC2 activities in the sensitivity of NeuT cells to

Figure 5. Signaling pathways activated in cells undergoing endoplasmic reticulum stress. A and B, cells were treated with 100 nmol/L thapsigargin for the indicated times. A, Akt activation (p-Akt(Ser473)). B, Erk activation (p-ERK) was assessed by Western blotting. Results are representative of three independent experiments. C, cells were treated with thapsigargin (100 nmol/L) for 30 hours in the presence or absence of GSK6909693 (10 μmol/L), U0126 (10 μmol/L), or both inhibitors, and apoptosis was determined. Error bars, SD from three independent experiments. **, P < 0.01; †††, P < 0.001. The efficacy of the inhibitors was assessed by Western blotting.
endoplasmic reticulum stress was further studied in experiments silencing Raptor or Rictor expression with siRNA. Interestingly, Raptor knockdown significantly reduced thapsigargin-induced apoptosis in NeuT cells (Fig. 6C, left). In contrast, silencing Rictor expression did not result in a significant inhibition of endoplasmic reticulum stress–induced apoptosis (Fig. 6C). Furthermore, a double Raptor/Rictor knockdown did not further reduce endoplasmic reticulum stress–induced apoptosis over the effect of Raptor siRNA (Fig. 6C). In view of these results, although inhibition of apoptosis by Raptor knockdown was not as strong as that observed with torin1, it is possible that the residual Raptor protein may be sufficient to sensitize the cells to thapsigargin (Fig. 6C, right). They also suggest that inhibition of rapamycin-insensitive mTORC1 activities may be responsible for the observed abrogation of endoplasmic reticulum stress–induced apoptosis by Torin1.

Crosstalk between ERBB2-regulated signaling and the PERK/ATF4/CHOP/TRAIL-R2 pathway upon endoplasmic reticulum stress

To elucidate the step(s) in the PERK/ATF4/CHOP/TRAIL-R2 pathway that was affected by ERBB2–activated signaling in NeuT cells, we first studied the upregulation of ATF4 upon thapsigargin treatment. Of note, ATF4 induction by thapsigargin was reduced in the presence of GSK690693 and U0126 (Fig. 7A, left) or Torin1 (Fig. 7A, right). Likewise, results shown in Fig. 7B (left) demonstrated that the combination of GSK690693 and U0126 significantly inhibited CHOP expression upon endoplasmic reticulum stress. In addition, mTOR inhibition by Torin1 reduced CHOP mRNA induction by thapsigargin (Fig. 7B). Finally, we determined the effect of the various inhibitors on TRAIL-R2 expression in ER-stressed NeuT cells. Upregulation of TRAIL-R2 mRNA (Fig. 7B, right) and protein levels (Fig. 7C) by thapsigargin were considerably abrogated by the inhibitors. Interestingly, Raptor knockdown by siRNA markedly abrogated TRAIL-R2 upregulation upon thapsigargin treatment (Fig. 7D), further confirming the role of mTORC1 activity in endoplasmic reticulum stress–induced apoptosis (Fig. 6C). Overall, our results support the model that sustained activation by mutant ERBB2 of signaling pathways that converge in mTORC1 favors the transition from an adaptive response to an ATF4/CHOP/TRAIL-R2–mediated apoptotic process in human breast epithelial cells undergoing endoplasmic reticulum stress.

Discussion

ERBB2 activation leads to dysregulation of intracellular signaling pathways that control cell metabolism, growth, and proliferation (13, 14). A number of ERBB2 somatic mutations found in patients with ERBB2 gene amplification–negative breast cancer are activating mutations that likely drive...
tumorigenesis and may confer resistance to ERBB2 tyrosine kinase inhibitors (19). This study demonstrates that breast epithelial cells expressing a constitutively active form of ERBB2 are markedly sensitive to endoplasmic reticulum stress. Our work also identifies ERK, Akt and mTOR activities as responsible for the enhanced sensitivity of these cells to endoplasmic reticulum stress. Recent studies have reported that chronic activation of mTORC1 results in an increase in PERK activity and sensitivity to endoplasmic reticulum stress, although the molecular mechanism leading to cell death was not elucidated (11, 12, 38, 39). Moreover, there are marked differences between our results in mutant ERBB2-expressing cells and those reported in TSC1/2-deficient cells. Thus, TSC1/2-deficient cells showed elevated eIF2α phosphorylation upon endoplasmic reticulum stress and a truncated UPR in which induction of other endoplasmic reticulum stress markers was severely compromised (11). In contrast, we did not observe an increased PERK activity upon endoplasmic reticulum stress in ERBB2 cells compared with control cells. Interestingly, we have found an enhanced induction of ATF4 and CHOP and sustained activation of the Ire1α branch in mutant ERBB2-expressing cells. Although certain links between Ire1α and endoplasmic reticulum stress–induced apoptosis have been suggested (40, 41), our results indicate that Ire1α silencing did not reduce apoptosis in ER-stressed ERBB2 cells, excluding a role of the Ire1α pathway in death induced by endoplasmic reticulum stress in these cells.

The PERK/ATF4/CHOP branch of the UPR has a dual role in cells undergoing endoplasmic reticulum stress. As part of the adaptive response to endoplasmic reticulum stress, the PERK/ATF4/CHOP pathway has been related to the activation of cytoprotective autophagy upon endoplasmic reticulum stress in different cellular models (42, 43). Although at present we could not exclude a role of autophagy in the different sensitivity of mutant ERBB2-expressing cells to endoplasmic reticulum stress, our data demonstrate that knockdown of any of the PERK/ATF4/CHOP axis proteins resulted in a significant inhibition of endoplasmic reticulum stress–induced apoptosis in cells expressing constitutively active ERBB2. The proapoptotic role of the PERK/ATF4/CHOP pathway in ER-stress–induced apoptosis has been previously demonstrated. Thus, downregulation of anti-apoptotic Bcl-2 family members and upregulation of proapoptotic BH3-only proteins by CHOP have been frequently reported in the activation of apoptosis upon endoplasmic reticulum stress (6, 29). However, our data do not support a similar mechanism in mutant ERBB2-expressing cells. However, a number of evidences support the involvement of death receptors and in particular TRAIL-R2 in the death of cells undergoing endoplasmic reticulum stress (30, 44, 45). In addition, TRAIL-R2 upregulation upon endoplasmic reticulum stress treatments has been suggested to play a prominent role in the sensitization of tumor cells to exogenous TRAIL by endoplasmic reticulum stress treatments (46). Furthermore, CHOP-dependent upregulation of TRAIL-R2 and a potential
binding site in the TRAIL-R2 promoter have been reported (30). However, the molecular mechanisms controlling TRAIL-R2 upregulation by the PERK/ATF4/CHOP pathway have not been fully elucidated. We found that dysregulated activation of the ERK, Akt, and mTORC1 in cells expressing a constitutively active form of ERBB2 critically enhances ATF4, CHOP, and TRAIL-R2 levels upon endoplasmic reticulum stress that lead to the activation of a caspase-8–dependent, TRAIL-independent apoptotic process. Ligand-independent assembly of the DISC has been demonstrated in the TNF family of death receptors, most likely because of the homotypic association of receptors mediated by the pre-ligand–binding assembly domain (47). Furthermore, ectopic TRAIL-R2 expression has been previously demonstrated to be sufficient to induce apoptosis in the absence of ligand (48). As the DISC components may colocalize in an intracellular membrane fraction in breast epithelial cells in the absence of TRAIL (22), the increased expression of TRAIL-R2 and downregulation of cFLIP induced by endoplasmic reticulum stress in ERBB2-expressing cells could result in the formation of a DISC containing TRAIL-R2, FADD, and procaspase-8 in which caspase-8 is activated. Our findings underscore the complexity of the mechanisms involved in the apoptosis elicited by endoplasmic reticulum stress in mutant ERBB2-expressing cells and warrant further investigation to characterize the site of caspase-8–activation in these cells.

Although we do not know the mechanism underlying the increased expression of ATF4 protein in mutant ERBB2-expressing cells upon endoplasmic reticulum stress, available evidence suggests that a critical step in the regulation of ATF4 protein levels is the ATF4 translational control at the 5′-leader of the ATF4 mRNA, a region containing 2 upstream open-reading frames that are well conserved among vertebrates (49). Furthermore, regulatory elements in the 5′-untranslated region located upstream of the translation start site of certain mRNAs are also key components of the translational response to mTOR activation (50). Alternatively, ATF4 is a short-lived protein with a half-life of less than 30 minutes, whose degradation by the proteasome depends on the interaction with the SCF/βTrCP E3 ubiquitin ligase (51). In addition to an increase in ATF4 levels, other important mechanisms for posttranslational regulation of ATF4 activity are phosphorylation or interaction with other transcription factors thus increasing its transcriptional activity (52). Whether or not these mechanisms are responsible for the enhanced ATF4 expression and activity in mutant ERBB2 cells is an issue that requires further investigation. In addition, further studies to elucidate the role of mTORC1 activity in the control of ATF4 levels would provide new insights into the molecular basis of the transition from an adaptive response to an apoptotic process in human breast epithelial cells undergoing endoplasmic reticulum stress.

In vivo tumor microenvironment is characterized by severe hypoxia, glucose deprivation, and acidosis. These combined factors lead to the accumulation of misfolded proteins in the endoplasmic reticulum that results in endoplasmic reticulum stress, triggering the UPR to facilitate tumor survival and growth. Chronic endoplasmic reticulum stress in tumor cells increases the expression of the endoplasmic reticulum chaperones that provides a survival advantage to tumor cells in an adverse microenvironment. Therefore, pharmacologic interference or knockdown strategies to abrogate chaperone expression or function may represent a potentially relevant strategy to sensitize these cells to different chemotherapeutic agents. Alternatively, our results suggest that overactivating the proapoptotic branches of the UPR in tumor cells that are prone to endoplasmic reticulum stress because of environmental conditions or constitutive activation of signaling pathways may modulate the expression of proteins of the TRAIL pathway (53) and activate a ligand-independent apoptotic program in these cells. This would be particularly relevant in tumor cells with activating mutations in the ERBB2 gene or expressing truncated p95ERBB2, which may be resistant to trastuzumab or tyrosine kinase inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Activated ERBB2/HER2 Licenses Sensitivity to Apoptosis upon Endoplasmic Reticulum Stress through a PERK-Dependent Pathway

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