Activation of the Unfolded Protein Response Bypasses Trastuzumab-mediated Inhibition of the PI-3K Pathway

Sreekanth Kumandan, Navin R. Mahadevan, Kevin Chiu, Alexandra DeLaney, and Maurizio Zanetti
The Laboratory of Immunology, Department of Medicine and Moores Cancer Center, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0815

Abstract
HER2-positive breast cancer initially responds to trastuzumab treatment, but over time, resistance develops and rapid cancer progression occurs, for which various explanations have been proposed. Here we tested the hypothesis that induction of the unfolded protein response (UPR) could override HER2 inhibition by trastuzumab, leading to the re-activation of growth signaling and the activation of the downstream target Lipocalin 2 (LCN2). Trastuzumab significantly inhibited the basal expression of LCN2 in HER2+ SKBr3 human breast cancer cells. The induction of the UPR completely abrogated trastuzumab-mediated LCN2 downregulation, and, in fact caused an increase in transcription and secretion of LCN2 over baseline. Reduction of the UPR using 4-phenyl butyric acid (PBA) a chemical chaperone that ameliorates ER stress, restored trastuzumab-mediated inhibition. Inhibition of the PI3K/AKT signaling pathway in trastuzumab-treated/UPR-induced SKBr3 cells partially reduced the upregulation of LCN2. These results suggest that the UPR is a possible way to override the effect of trastuzumab in HER2+ cancer cells.

Keywords
ER stress; Lipocalin 2; tumorigenesis

1. Introduction
Breast cancer is the leading cancer in women and represents the second leading cause of cancer death among all women (American Cancer Society 2011 Report). Based on surface receptor expression, breast cancer is classified into three major categories: estrogen and progesterone receptor positive (~60% of cases), HER2 positive (~ 25% of cases), and triple negative (~10% of cases). HER2 is a 185 kDa transmembrane oncoprotein encoded by the HER2/neu gene member of the HER (or ErbB) growth factor receptor family.
Homodimerization of HER2 results in phosphorylation of the intracytoplasmic domain which in turn results in the recruitment of several signaling molecules including Phosphatidylinositol 3-kinases (PI3K)/AKT and mitogen activated protein kinase (MAPK) [1]. HER2-mediated transformation of mammary epithelial cells activates the PI3K/AKT survival pathway [2]. HER2+ breast cancer is characterized by being a particularly aggressive form, and one that is unresponsive to traditional hormone therapy due its persistent PI3K/AKT activity [3; 4; 5].

The current standard of care for HER2+ breast cancer is passive therapy with the humanized monoclonal antibody trastuzumab [6], which targets the C-terminal portion of domain IV of HER2 [7]. Binding of trastuzumab to HER2 disables homodimerization of this receptor, consistent with the observation that trastuzumab has preferential activity against tumors in which HER2 dimerization drives tumor growth [8]. Binding of trastuzumab to HER2 also promotes the inhibition of the PI3K/AKT signaling pathway (add more refs consistent with rebuttal letter) [9; 10; 11; 12]. However, trastuzumab becomes ineffective over time in a significant number of patients (trastuzumab resistance) [11; 13]. Mechanisms of resistance include steric hindrance of HER2 binding, upregulation of HER2 downstream signaling pathways via compensatory upregulation of HER3, signaling through an alternate growth receptors (e.g., IGF-1R), lack of the trastuzumab-binding epitope, and mutational alterations in the PI3K/AKT pathway [14; 15; 16].

Cancer cells are exposed to tumor microenvironmental noxae such as reduced nutrient supply, low pH, and hypoxia, or tumor-intrinsic stressors, such as oxidative stress, aberrant protein folding and glycosylation, viral infection, and defects in calcium homeostasis. Collectively, perturbation of cellular homeostasis caused by these stressors results in endoplasmic reticulum (ER) stress, which induces the activation of a conserved set of adaptive intracellular pathways known as the unfolded protein response (UPR) [17]. The UPR is initiated when the ER chaperone molecule, Grp78, which, under conditions of ER stress, disassociates from three ER membrane-bound sensors (IRE1a, ATF6, and PERK), inducing their activation. Downstream signaling cascades ameliorate ER stress via several mechanisms, including selective translation inhibition and upregulation of genes that encode enzymes that aid protein folding, maturation, and degradation [18]. UPR signaling is upregulated in tumors of several histological subtypes, and has been shown to be crucial tumor growth, adaptation, and resistance to chemotherapy [19; 20; 21; 22].

The Lipocalin 2 (LCN2) gene, which codes for a 25 kDa chaperone protein [23], is highly upregulated during the UPR in an NF-κB-dependent manner [24]. Because the UPR also activates the PI3K/AKT/NF-κB signaling axis [25], LCN2 is a likely common effector molecule downstream from the UPR and HER2/PI3K/AKT signaling pathways. LNC2 is upregulated in several solid cancers in human (for review see [26]), and elevated LCN2 levels have been found in breast cancer patients with accelerated progression and metastasis [27]. Studies in mice have linked LCN2 expression and secretion with an increased rate of mammary cancer progression, and deletion of LCN2 in the mammary tissue of tumor-prone mice significantly retards tumor growth [28; 29].
The work presented here tests the hypothesis that ER stress and the attendant UPR represent a possible alternative way through which PI3K/AKT growth signaling is reactivated during trastuzumab inhibition, ultimately leading to the upregulation of LCN2 in HER2+ breast cancer cells, hence potentially resulting in trastuzumab resistance.

2. Materials and Methods

2.1 Cell Culture and Treatments

Human SKBr3 cells were grown in RPMI containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone #SH3036.03), 1% non-essential amino acids, 1% sodium pyruvate, 1% HEPES, 1% penicillin/streptomycin/L-glutamine, and .005% β-ME. The timing of drug treatments is indicated in the figure legends. Trastuzumab (Herceptin, Genentech) was obtained from the Moores Cancer Center Pharmacy. Thapsigargin (Enzo Life Sciences #BML-PE180-0001) was used at 300 nM. 4-phenyl butyric acid (SIGMA #P21005-25G) was used at 30 mM. LY294002 (SIGMA #PHZ1144) was used at concentrations ranging from 5 μM to 20 μM. NVP-BEZ235 (Selleck Chemicals # S1009-5mg) was used at concentrations ranging from .1 μM to 5 μM.

2.2 RT-qPCR

RNA was isolated from cells using Nucleopsin II Kit (Machery-Nagel #740955.250). Concentration and purity of RNA was quantified the NanoDrop (ND-1000) spectrophotometer (Thermo Scientific) and analyzed with NanoDrop Software v3.8.0. cDNA was obtained using the High Capacity cDNA Synthesis kit (Life Technologies/ Applied Biosystems #4368813), and RT-qPCR was performed on an ABI StepOne system using TaqMan reagents for 50 cycles using universal cycling conditions. Target gene expression was normalized to β-actin, and analyzed using the −ΔΔCt relative quantification method. Validated FAM-labeled Human LCN2, HSPA5 (GRP78), DDIT3 (CHOP), and VIC-labeled Human β-actin TaqMan primer/probe sets (Life Technologies/Applied Biosystems, #4331182) were used.

2.3 Western Blot Analysis

After treatment, SKBr3 cells were washed with ice cold PBS and suspended in the RIPA Lysis Buffer system: 1X RIPA buffer and cocktail of protease inhibitors (Santa Cruz Biotechnologies #sc-24948). Cell lysates were centrifuged at 4 °C at 16,000xg for 15 min and the supernatants were extracted. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific #23335). Samples were denatured and equal concentrations of protein (70–95 μg) were electrophoresed on a Bio-Rad 4–20% Mini-PROTEAN TGX Precast Gels (Bio-Rad #456-1094). The proteins were transferred at 4°C using the Novex XCell II Blot system (Invitrogen #EI0002) for 2 hrs at 30V onto 0.2 μM PVDF membrane. The membranes were probed with goat polyclonal anti-human Lipocalin-2/NGAL antibody (R&D Systems #AF1757), and rabbit monoclonal anti-human P-AKT (Ser473) and Pan-AKT antibodies (Cell Signaling #4060 and #4691, clones: D9E and C67E7), β-actin was used as a loading control and blots were probed with polyclonal goat anti-human β-actin (Abcam #ab8229). Blots were revealed using either HRP-conjugated donkey antibody to goat IgG (Santa Cruz Biotechnology #sc2020) or HRP-
conjugated goat antibody to rabbit IgG (Cell Signaling #7074). Bands were visualized using Pierce ECL Blotting Substrate (Thermo Scientific #32106). All cell lysate samples were normalized to β-actin. For secreted LCN2 analysis, serum-free or serum-containing supernatant from treated SKBr3 cells was extracted and concentrated ~45 fold. Sample volumes were normalized by the BCA concentrations of their respective cell lysates. Concentrated samples were then analyzed using the hLipocalin2 Quantikine ELISA Kit (R & D Systems, #DLCN20) or the Lipocalin-2/NGAL antibody by Western blot, as described above.

3. Results and Discussion

Treatment of the HER2+ human breast cancer cell line, SKBr3, with trastuzumab for 48 hrs decreased basal transcription (~40%) of LCN2, but did not affect the basal expression level of the UPR elements GRP78 and CHOP (Fig. 1A). We reasoned that the induction of a UPR could bypass inhibition by trastuzumab, thus increasing LCN2 transcription. Thapsigargin (Tg) is a canonical ER stress inducer via non-competitive inhibition of the sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) [30]. Addition of Tg to trastuzumab-treated SKBr3 cells for the last 18 hrs of the total 48 hr treatment with trastuzumab caused as expected, an increase in LCN2 transcription and secretion (Fig. 1B–C). GRP78 and CHOP were also upregulated confirming the induction of the UPR (Fig. 1B). Thus, UPR signaling readily rescues the production of LCN2 from trastuzumab-mediated suppression.

To confirm that UPR signaling was actually responsible for LCN2 rescue, experiments were repeated using 4-phenyl butyric acid (PBA), a chemical protein folding chaperone that ameliorates ER stress [31]. As expected, PBA treatment greatly diminished the magnitude of the UPR as reflected by decreased GRP78 and CHOP transcription. It also dramatically decreased LCN2 transcription levels (Fig. 2). Indeed, LCN2 transcription was decreased to levels below that of control cells, similar to those of cells treated with trastuzumab indicating that the UPR is primarily responsible for the increase in LCN2 transcription.

Prior findings have suggested that the UPR is capable of activating the PI3K/AKT signaling pathway. To confirm this, the phosphorylation of AKT was examined 2, 4, and 6 hours after treatment with Thapsigargin. Thapsigargin potently increased the phosphorylation of AKT in SKBr3 cells concurrently treated with trastuzumab (Fig. 3).

To probe the involvement of PI3K/AKT signaling in UPR-mediated LCN2 rescue from trastuzumab inhibition, Tg-treated SKBr3 cells were also treated with one of two inhibitors of AKT activation, LY294002 or NVP-BEZ235. These two small molecules inhibit activation of PI3K by binding to its ATP binding cleft, thus preventing the consequent phosphorylation of AKT [32; 33]. Compared to cells treated with Tg alone, Tg-treated SKBr3 cells underwent a 70% decrease in LCN2 expression following treatment with LY294002 and a 99% decrease in expression after co-treatment with NVP-BEZ235. Neither inhibitor completely restored the basal expression of LCN2, suggesting that UPR-mediated LCN2 rescue is only in part PI3K/AKT mediated. LCN2 secretion by Tg-induced SKBr3 cells treated with NVP-BEZ235 corroborated the RT-qPCR results (Fig. 5A). As expected, NVP-BEZ235 strongly
inhibited AKT activation, suggesting that the down regulation of LCN2 in cells treated with these two inhibitors is likely due to the loss of AKT activity (Fig. 5B). Interestingly, NVP-BEZ235 failed to completely inhibit Tg-induced UPR-mediated LCN2 secretion, corroborating our qPCR findings, suggesting that PI3K independent mechanisms may also be involved in the UPR-mediated upregulation of LCN2.

Our data suggest that a UPR, which in the tumor microenvironment is induced as a consequence of reduced nutrient supply, low pH, and hypoxia, oxidative stress, aberrant protein folding and glycosylation, viral infection, and defects in calcium homeostasis, can override inhibition of the oncogene LCN2 by trastuzumab in HER2-overexpressing breast cancer cells. Further, we show that rescue of PI3K/AKT signaling from trastuzumab-mediated inhibition is partially involved in UPR-mediated LCN2 production. In this context it is also worth noting that lapatinib-resistant SKBr3 cells upregulate members of the UPR signaling cascade, including GRP78, IRE1, PERK, and XBP-1s [34]. Since LCN2 is secreted at higher levels upon induction of the UPR with Tg, even during inhibition by trastuzumab, our data emphasizes the importance of the UPR in driving both transcription and secretion of LCN2 in breast cancer cells. We thus tentatively suggest that the initiation of a UPR may constitute an alternative way through which activation of the PI3K/AKT pathway and resistance to trastuzumab in HER2+ cancer cells can occur.

Based on the foregoing we propose a model where the UPR could contribute to the emergence of trastuzumab resistance. Our data suggest that the UPR can activate cell-intrinsic growth signaling pathways overriding external trastuzumab-mediated inhibition of HER2 signaling, including the downstream marker LCN2. Additionally, our work suggests that the upregulation of LCN2 by activation of the UPR may proceed via both PI3K/AKT-dependent [35] and independent mechanisms (Fig. 4). It is known that trastuzumab-mediated inhibition of HER2 also de-represses PTEN, enabling it to oppose the activation of PI3K/AKT signaling [16]. The relationship between PTEN, the UPR, and tumorigenesis is highlighted by experiments in which the conditional homozygous knockout of Grp78 in the Pten null prostates of mice protects against prostate cancer growth [19], suggesting that Pten null tumorigenesis requires the UPR. Thus, the UPR could directly activate PI3K/AKT signaling as shown here and by others [19; 25] and/or inhibit of PTEN [19], thus weakening its control of PI3K/AKT signaling. Notably, synergy between these two effects could result in UPR-borne uncontrolled PI3K/AKT signaling, a possibility relevant in tumors with PTEN loss or mutations, which account for ~50% of patients [36]. Accordingly, targeting the UPR may represent a new form of adjuvant therapy in HER2+ breast cancer.

In summary, here we show the importance of the UPR as a potential mechanism to override the effects of trastuzumab and initiate resistance. Because activation of the UPR can rescue the PI3K/AKT axis and cause the expression of downstream oncogenes, such as LCN2, the considerations emanating from this study could extend to other HER2+ cancers, such as prostate, lung, gastric, pancreatic, and endometrial cancers. Future work will need to determine if the UPR similarly reactivates key growth signaling pathways in these cancers.
Acknowledgments

This work was supported in part by a grant from the UCSD Academic Senate. N.R.M. acknowledges support from the UCSD Medical Scientist Training Program and NIDA T32 Training Grant DA007315-07A2.

References


Cancer Lett. Author manuscript; available in PMC 2014 July 10.


Figure 1. Downregulation of LCN2 by trastuzumab is overcome by the induction of the UPR

(A) SKBr3 cells were treated with trastuzumab or an isotype control antibody for 48 hrs, and then analyzed by RT-qPCR for LCN2 transcription and UPR activation. (B) SKBr3 cells were treated with trastuzumab or an isotype control antibody for 48 hrs. Tg was introduced during the final 18 hrs of treatment. Tumor cell mRNA was isolated and analyzed by RT-qPCR for LCN2 transcription and UPR activation. Columns indicate fold increase in expression levels (RQ) for each treatment condition. The value of the isotype control was set to 1. Error bars represent the SEM of biological replicates pooled from 5 or more independent experiments. *P<0.05, **P<0.01, ***P<0.001, NS = not significant (unpaired, two-tailed t test.) (C) SKBr3 cells were treated or not with trastuzumab for 38 hrs and Tg was introduced during the final 8 hrs of treatment. Cell-free concentrated supernatants were analyzed for secreted extracellular LCN2 protein by 2 site capture ELISA. Concentrations were then normalized for cell number.
Figure 2. ER stress amelioration restores trastuzumab sensitivity leading to downregulation of LCN2

SKBr3 cells were treated with trastuzumab or an isotype control antibody for 48 hrs. Tg and 4-phenyl butyric acid (PBA) were introduced during the final 18 hrs of treatment. Tumor cell mRNA was isolated and analyzed by RT-qPCR for LCN2 transcription and UPR activation. Columns indicate fold increase in expression levels (RQ) for each treatment condition. The value of the isotype control was set to 1. Error bars represent the SEM of biological replicates pooled from 4 or more independent experiments. *P<0.05, **P<0.01, ***P<0.001, NS = not significant, (unpaired, two-tailed t test.)

Cancer Lett. Author manuscript; available in PMC 2014 July 10.
Figure 3. Activation of the UPR by ER stress leads to the reactivation of AKT
SKBr3 cells were treated with or without trastuzumab for 36 hrs and Tg was introduced during the final 6 hrs of treatment. Cells from each condition were extracted at 32, 34, and 36 hrs, lysed, and then interrogated for protein expression by Western blot.
Figure 4. UPR-mediated rescue of LCN2 is partially PI3K/AKT dependent
SKBr3 cells were treated with trastuzumab or an isotype control antibody for 48 hrs. Tg and (A) LY294002 or (B) NVP-BEZ235 were introduced during the final 18 hrs of treatment. Tumor cell mRNA was isolated, and analyzed by RT-qPCR for LCN2 transcription and UPR activation. Columns indicate fold increase in expression levels (RQ) for each treatment condition. The value of the isotype control was set to 1. Error bars represent the SEM of biological replicates of 4–6 independent experiments. *P<0.05, **P<0.01, ***P<0.001, NS = not significant, (unpaired, two-tailed t test.)
Figure 5. The decrease in LCN2 expression by NVP-BEZ235 is mediated by PI3K/AKT signaling

(A) SKbr3 Cells were treated in serum-free media for 24 hours. Cell-free concentrated supernatants were analyzed for secreted extracellular LCN2 protein by Western blot. Sample volumes loaded were normalized by the BCA values of their respective cell lysates.

(B) SKBr3 cells were treated with or without trastuzumab for 34 hrs. Tg and NVP-BEZ235 was introduced during the final 6 hrs of treatment. Cells from each condition were extracted, lysed, and then interrogated for protein expression by Western blot.
Figure 6. Hypothetical Model for how the UPR overrides trastuzumab inhibition of HER2 in cancer cells

(A) HER2 homodimerization in unstressed cancer cells inhibits PTEN and activates PI3K/AKT signaling, which in turn leads to the activation of NF-kB, which drives the upregulation of LCN2. (B) Trastuzumab disables HER2 homodimerization inhibiting PI3K docking and derepressing PTEN. The net effect of trastuzumab inhibition is to stop tumor cell growth. (C) Upon induction of the UPR several events occur: (1) the reactivation of the PI3K/AKT axis; (2) the inhibition of PTEN; and (3) the activation of NF-κB. Cumulatively, these three events result in LCN2 upregulation, and reactivation of tumor cell growth despite continuous HER2 inhibition. Notably, UPR-activated NF-κB can also independently contribute to AKT phosphorylation, hence further contributing to LCN2 expression.