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Tamoxifen-induced cytotoxicity in breast cancer cells is mediated by glucose-regulated protein 78 (GRP78) via AKT (Thr308) regulation

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ABSTRACT

Glucose regulated protein 78 (GRP78) has recently been suggested to be associated with drug resistance in breast cancer patients. However, the precise role of GRP78 in drug resistance and the involved signaling pathways are not clearly understood. In the present study, we show that among a panel of drugs, namely Paclitaxel (TAX), Doxorubicin (DOX), 5-fluorouracil (5-FU), UCN-01 and Tamoxifen (TAM) used, TAM alone up-regulated the expression of GRP78 significantly and induced apoptosis in MCF-7 and MDA-MB-231 cells. Interestingly, inhibition of GRP78 by a specific pharmacological inhibitor, VER-155008 augmented TAM-induced apoptosis, and overexpression of GRP78 rendered the cells resistant to TAM-induced cell death suggesting a role for GRP78 in TAM-induced cytotoxicity. Mechanistically, the expression of phosphorylated AKT as determined by Western blot analyses revealed that TAM selectively upregulated phosphorylation of AKT at Thr308 but not at Ser473, and siRNA silencing of GRP78 resulted in inhibition of AKT phosphorylation at Thr308 but not at Ser473. Further, a GRP78 inhibitor, VER155008 inhibited TAM-induced phosphorylation of GSK3β, a downstream substrate of AKT. These results, thus suggests a role for GRP78 in TAM-induced AKT activation. Additionally, co-localization studies by immunofluorescence, and immunoprecipitation experiments demonstrated a complex formation of AKT and GRP78. Furthermore, in glucose-free medium, the cells were sensitized to TAM-induced cell death that was associated with reduced AKT phosphorylation at Thr308, thus strengthening the association of AKT regulation with drug response. Collectively, our findings identify a role of GRP78 in AKT regulation in response to TAM in breast cancer cells.

1. Introduction

The glucose regulated protein 78 (GRP78), originally discovered as a chaperon protein in the endoplasmic reticulum induced under glucose starvation (Lee 2001), plays a major role in maintenance of cellular homeostasis(Hendershot 2004). Recently, GRP78 has been documented to be upregulated in tumors to help in the adaptation of tumors to the micro-environmental stress (Li and Li 2012). Several studies have shown that GRP78 is overexpressed in tumors like glioma (Pyrko et al., 2007), leukemia (Uckun et al., 2011), prostate cancer (Daneshmand et al., 2007) and breast cancer (Scriven et al., 2009) that have become refractory to therapy. Although there are reports that the receptor-mediated activation of the AKT/PI3 K pathway is involved in this process (Roller and Maddalo 2013), the detailed mechanism of GRP78-promoted resistance of cancer to chemotherapy is not clearly understood.

AKT is a serine/threonine protein kinase and it regulates a wide array of cellular processes such as survival, proliferation, protein translation and metabolism (Manning and Cantley 2007). AKT contains a pleckstrin homology (PH) domain which binds to PIP3 [phosphatidylinositol (3,4,5)-trisphosphate] in the plasma membrane (Franke et al., 1997). AKT can be phosphorylated at Ser473 by the mTOR complex 2 (mTORC2) and at Thr308 by 3-phosphoinositide dependent protein kinase 1 (PDK1) (Alessi et al., 1996; Alessi et al., 1997; Feng et al., 2004; Sarbassov et al., 2005). However, maximal AKT activity is dependent on the phosphorylation status of both Thr308 and Ser473 residues (Alessi et al., 1997). Endoplasmic reticulum stress has been shown to differentially reg-

Abbreviations: GRP78, glucose-regulated protein 78; UPR, unfolded protein response; TAX, paclitaxel; DOX, doxorubicin; 5-FU, 5 fluorouracil; TAM, tamoxifen; ER, estrogen receptor.

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ulate AKT phosphorylation by increasing Ser473 phosphorylation and suppressing Thr308 phosphorylation (Yung et al., 2011). GRP78 has also been reported to regulate AKT phosphorylation during cisplatin resistance in lung cancer cells (Lin et al., 2011), and to induce differential phosphorylation of AKT under prolonged glucose starvation (Gao et al., 2014).

Tamoxifen is a potent estrogen receptor (ER) antagonist derived from non-steroid triphenylethylene and is used extensively to treat ER-positive breast cancer (Ring and Dowsett 2004). Tamoxifen has also been shown to exert its effect in ER-independent manner by inhibiting the protein kinase C activity (Gundimeda et al., 1996). However, despite its effectiveness, long-term efficacy of TAM is limited due to associated side-effects (Yeh et al., 2014), relapse of disease and development of resistance (Ring and Dowsett 2004). Therefore, there is a need to circumvent these problems by use of combination therapy that will enhance the effectiveness of TAM, and also elucidate the molecular mechanism involved in the development of resistance.

Glucose regulated protein 78 has been demonstrated to be associated with chemoresistance in breast cancer, however the precise mechanisms and signaling pathways involved in the process are not clearly understood. In this study, we aimed to analyze the molecular mechanisms of GRP78 regulation during drug response in human breast cancer cells with a focus on AKT signaling. For this purpose, we examined the effect of a panel of chemotherapeutic drugs on GRP78 expression in MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) cell lines. We show that among the drugs tested, only TAM up-regulated the expression of GRP78 and induced apoptosis. Inhibition of GRP78 enhanced sensitivity to low-dose TAM via a decrease in the AKT (Thr308) phosphorylation and subsequent inhibition of GSK3β phosphorylation at Ser9. Our results thus demonstrate the involvement of AKT regulation in GRP78-mediated response to TAM in human breast cancer cells.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines MCF-7 and MDA-MB-231 were procured from American Type Culture Collection (ATCC Rockville, USA) and maintained in RPMI 1640 (Gibco, USA) supplemented with 10% heat inactivated fetal calf serum (FCS), 100 μg/ml streptomycin and 100units/ml penicillin at 37 °C in 5% CO_2 and 95% humidified air. The cells were split every alternate day.

2.2. Cell viability studies by MTT assay

The effect of various chemotherapeutic agents on viability of human breast cancer cell lines, MCF-7 and MDA-MB-231 was monitored by MTT assay. Cells were seeded in a 96 well plate at a density of 5000 cells/100 μl/well and treated with different doses (5–60 μg/ml) of various drugs namely, Tamoxifen (TAM), Paclitaxel (TAX), 5-fluorouracil (5-FU) and UCN-01(50 nM–1 μM) for 24 h. MTT (10 μl from a stock of 5 mg/ml in PBS) was added to each well and incubated at 37 °C for 3 h in 5% CO_2 and 95% humidified air. The formazan crystals formed were dissolved in the solubilizing reagent [10% SDS (w/v) in 0.01 N HCl] for 12 h, in dark at 37 °C. The absorbance was measured at 570 nm with a background subtraction at 650 nm using a microplate reader (Molecular Devices, USA). The percent viable cell number was calculated with respect to untreated controls considered as 100%. The cells treated with equal concentrations of DMSO were used as vehicle control.

To determine the effect of glucose starvation during response to TAM, the cells were glucose- and serum starved for 1 h followed by addition of insulin. They were then treated with TAM (5 and 10 μg/ml) for 24 h in the presence and absence of glucose and insulin. MTT assay was performed as mentioned above. The vehicle controls were exposed to equal concentration of DMSO.

2.3. Cell cycle analysis

Cells were treated with DMSO (control) or 10 μg/ml of different drugs namely, TAM, TAX, DOX and 5-FU for 24 h, washed and fixed in 70% chilled ethanol for 30 min at 4 °C followed by incubation with 50 μl Ribonuclease A (5 mg/ml in PBS, DNase free) for 10 min at room temperature and stained with Propidium Iodide (50 μg/ml in PBS). The DNA content was analyzed on the FL-2A channel of flow cytometer (FACS Calibur, BD Biosciences, USA) equipped with a 488 nm argon laser on a linear scale for cell cycle analysis. The data was analyzed by Cell Quest Pro software for determining the distribution of cells in different phases of cell cycle.

2.4. Immunofluorescence

For visualization of GRP78 expression *in situ*, cells were grown on coverslips followed by fixing with 4% paraformaldehyde. The cells were permeabilized with 0.2% Triton X-100 and non-specific signal was blocked by incubating the cells with blocking solution for 1 h. Cells were washed with 1X Tris-buffered saline (TBS) and then incubated with primary antibodies to GRP78, phospho AKT (Thr308) and total AKT (Santa Cruz Biotechnologies, USA) for 1 h and washed thrice with TBS. Cells were stained with Cy3-/FITC-labelled secondary antibody for 1 h and washed twice with 1X TBS. Nuclei were stained with DAPI. The coverslips were mounted on slides with mounting medium and the cells were viewed with Confocal Laser Scanning Microscope (Zeiss LSM 510, Germany) equipped with 488 nm and 560 nm Argon lasers.

2.5. Western blotting

Cells were lysed using RIPA lysis buffer (120 mM NaCl, 1.0% Triton X-100, 20 mM Tris–HCl, pH 7.5, 100% glycerol, 2 mM EDTA, protease inhibitor cocktail; Roche, Germany). Total soluble protein was electrophoresed on SDS-polyacrylamide gels and blotted onto PVDF membrane (Millipore, USA). After blocking with 5% BSA, the blots were probed with antibodies to GRP78, AKT (Ser473), AKT (Thr308), total AKT (Santacruz Biotechnology, USA) and GSK3β (Ser9) (Cell Signaling Technologies, USA) followed by HRP-labelled secondary antibodies (Sigma, USA). The bands were visualized by chemiluminescence using Super Signal West Femto Maximum Sensitivity Substrate (Pierce, USA). β-actin (MP Biomedicals, USA) was used as the loading control. The untreated control cells were exposed to equal concentrations of DMSO.

2.6. Co-immunoprecipitation studies

Protein (500 μg) from the whole cell lysate was mixed with 20 μl of immobilized anti-GRP78 antibody bead slurry and incubated overnight at 4 °C with gentle rocking. After extensive washing with lysis buffer to remove residual proteins, the beads were mixed with protein gel loading buffer and boiled for 10 min. The proteins were resolved by SDS-PAGE and immunoblotted with anti-GRP78 and anti-AKT antibodies.

2.7. Quantitative real-time PCR

Total cellular RNA was isolated from MCF-7 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed into cDNA according to the manufacturer's instructions (Promega, USA). Quantitative real-time PCR (Realplex real-time thermal cycler,