

Salubrinal-Mediated Upregulation of eIF2 α Phosphorylation Increases Doxorubicin Sensitivity in MCF-7/ADR Cells

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Eukaryotic translation initiation factor 2 alpha (eIF2 α), which is a component of the eukaryotic translation initiation complex, functions in cell death and survival under various stress conditions. In this study, we investigated the roles of eIF2 α phosphorylation in cell death using the breast cancer cell lines MCF-7 and MCF-7/ADR. MCF-7/ADR cells are MCF-7-driven cells that have acquired resistance to doxorubicin (ADR). Treatment of doxorubicin reduced the viability and induced apoptosis in both cell lines, although susceptibility to the drug was very different. Treatment with doxorubicin induced phosphorylation of eIF2 α in MCF-7 cells but not in MCF-7/ADR cells. Basal expression levels of Growth Arrest and DNA Damage 34 (GADD34), a regulator of eIF2 α , were higher in MCF-7/ADR cells compared to MCF-7 cells. Indeed, treatment with salubrinal, an inhibitor of GADD34, resulted in the upregulation of eIF2 α phosphorylation and enhanced doxorubicin-mediated apoptosis in MCF-7/ADR cells. However, MCF-7 cells did not show such synergic effects. These results suggest that dephosphorylation of eIF2 α by GADD34 plays an important role in doxorubicin resistance in MCF-7/ADR cells.

INTRODUCTION

Despite the fact that the development of novel antitumor drugs has enhanced the strategy for cancer chemotherapeutics, drug resistance makes it difficult to treat many cancers. Some features of drug-resistant cancer cells are modulation of the drug concentration by increased efflux and decreased influx, impairment of cell death pathways, enhancement of cell survival pathways, alternation of drug metabolism, and mutations in cell cycle pathways (Holohan et al., 2013). For example, increased

expression of the ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp)/MDR1, MDR2, and several multidrug-resistance-associated proteins (MRPs), elevated drug efflux and allowed cancer cells to elude chemotherapy by reducing the cellular anticancer-drug concentration (Gottesman et al., 2002). Some P-gp inhibitors used to reverse multidrug resistance could enhance chemosensitivity in resistant cells but cause cytotoxicity and a number of complications (Gottesman et al., 2002; Minderman et al., 2004).

Cancer development and chemoresistance is tightly related to malfunction of the cellular signaling pathways that control cell proliferation, survival, or death (Hanahan and Weinberg, 2011). There are many reports regarding the association of mitogen-activated protein kinases (MAPKs) with oncogenesis and chemoresistance (McCubrey et al., 2007; Ranganathan et al., 2006; Wang et al., 2012). Upregulation of antioxidant signaling also interferes with reactive oxygen species (ROS)-mediated apoptosis in cancer cells (Tsuruo et al., 2003). The unfolded protein response (UPR) pathway, an ER homeostasis-associated signaling pathway, is known to control cell survival and death under ER stress conditions in normal cells as well as in various diseases (Wang and Kaufman, 2012). Many studies showed that the elevated expression of the UPR genes, such as X-box binding protein 1 (XBP1), GRP78/BiP, and activating transcription factor 4 (ATF4), is associated with chemoresistance (Armstrong et al., 2010; Jiang et al., 2009; Lee et al., 2006). The importance of the ER protein-folding environment in cancer development has been described (Wang and Kaufman, 2014). Therefore, investigation of the roles of the UPR in cancer development and the acquisition of multidrug resistance is warranted.

The activated UPR regulates the eukaryotic translation initiation factor 2 α (eIF2 α), which is a regulator of translation through phosphorylation at serine 51. The phosphorylation of eIF2 α is caused by four kinases that are activated under different stress conditions. These are the general control nonrepressible 2 (GCN2), dsRNA-activated protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK) and heme-regulated inhibitor kinase (HRI). Although eIF2 α phosphorylation stops translation of general proteins, certain mRNAs, such as ATF4, are translated due to small ORFs in their 5' untranslated region (5' UTR) (Wek et al., 2006). Increased ATF4 proteins, in turn, induce the expression of Growth Arrest and DNA Damage 34 (GADD34) (Hollander et al., 1997) forming the GADD34-protein phosphatase 1 (PP1) complex

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which induces dephosphorylation of eIF2 α (Novoa et al., 2001). Therefore, ATF4 proteins switch on the negative feedback loop of eIF2 α phosphorylation. Many studies have shown that eIF2 α phosphorylation plays opposite roles in cytoprotection and cell destruction, depending on the conditions (Kusio-Kobialka et al., 2012; Morrow et al., 2013; Schewe and Aguirre-Ghiso, 2009; Wek et al., 2006). For example, upregulated eIF2 α phosphorylation inhibited imatinib-mediated apoptosis in chronic myeloid leukemia cells, while inhibition of eIF2 α dephosphorylation enhanced bortezomib-mediated apoptosis in multiple myeloma cells (Kusio-Kobialka et al., 2012; Schewe and Aguirre-Ghiso, 2009).

In this study, we investigated the role of eIF2 α phosphorylation in doxorubicin-resistant MCF-7/ADR breast cancer cells. Doxorubicin, an anthracycline agent, is an effective anti-cancer drug that has been applied to various malignancies (Gewirtz, 1999). It induces apoptosis by causing DNA damage, inhibiting topoisomerase II and producing free radicals (Minotti et al., 2004). MCF-7/ADR cells have characteristics of multi-drug resistant cancer cells, such as high expression levels of p-glycoprotein, anionic glutathione-s-transferase (GST), tissue transglutaminase, N-cadherin, CD44, Slug, Twist, as well as downregulation of Bcl-2, BRCA1/2 and wild-type p53 (Cowan et al., 1986; Fairchild et al., 1987; Tsou et al., 2015). Unlike MCF-7 cells, MCF-7/ADR cells express full-length functional caspase-3 and a mutant form of p53 (Mehta et al., 2002; Tsou et al., 2015). Previously, conflicting results were reported regarding the role of eIF2 α phosphorylation in doxorubicin-treated breast cancer cells (Bennett et al., 2012; Peidis et al., 2011). In this study, we demonstrate the pro-apoptotic role of eIF2 α phosphorylation in MCF-7/ADR cells.

MATERIALS AND METHODS

Chemicals

Doxorubicin and thapsigargin were purchased from Sigma (USA). Salubrinal was purchased from Calbiochem (USA). The anti-p-eIF2 α and anti-eIF2 α antibodies were obtained from Cell

Signaling Technology (USA). The anti-PARP, anti-caspase-7, anti-GAPDH, anti- β -actin and anti-ATF4 antibodies were obtained from Santa Cruz Biotechnology (USA).

Cell culture

MCF-7 and MCF-7/ADR cells were generously provided by Dr. Hwa Jeong Lee (Ewha W. University, Seoul) (Chung et al., 2005). Cells were cultured in DMEM as described previously (Jeong et al., 2009).

Cell survival and proliferation assays

Cells were seeded in 48-well plates and incubated for 16 h. Cells were treated with various concentrations of doxorubicin for 24 h or 48 h. Cell survival was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich). Briefly, 5 mg/ml MTT in PBS was diluted in media to 0.5 mg/ml and incubated in a CO₂ humidified chamber for 2 h. The medium was aspirated from each well and 200 μ l DMSO were added to dissolve the formazan crystals. The absorbance at a wavelength of 570 nm was measured using a UVM 340 plate reader. Cell proliferation was measured using the BrdU cell proliferation assay kit (Calbiochem, Germany).

Immunoblot analysis

Immunoblot analysis was performed as described previously (Jeong et al., 2014).

Quantitative real-time PCR

Total RNA was obtained from cells using RNAiso plus (TAKARA, Japan) according to the manufacturer's protocols. The cDNA was synthesized using 2 μ g RNA and Improme II (Promega, USA). Quantitative real time PCR was performed with the SYBR[®] premix Ex Taq[™] II (TAKARA, Japan) according to the manufacturer's protocols.

Statistical analysis

Values are expressed as means \pm S.D. The figures in this study are representative of at least three independent experiments.

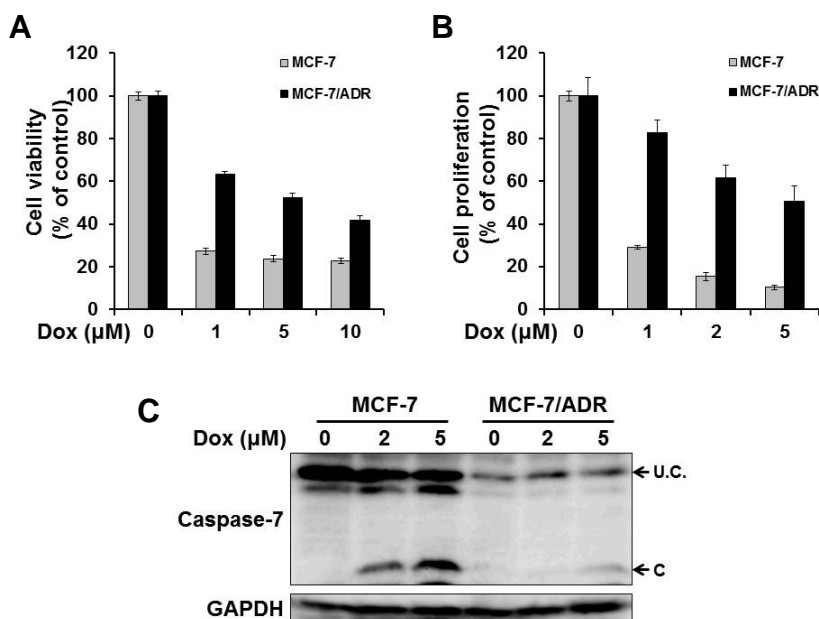


Fig. 1. MCF-7/ADR cells showed resistance to doxorubicin. (A) MCF-7 cells and MCF-7/ADR cells were treated with the indicated concentrations of doxorubicin for 48 h and cell viability was measured by MTT assay. (B, C) Both cell lines were treated with the indicated concentrations of doxorubicin for 24 h. Cell proliferation was measured by BrdU assay (B) and immunoblot analyses were performed using specific antibodies to caspase-7 and GAPDH (C). U.C, uncleaved; C, cleaved.

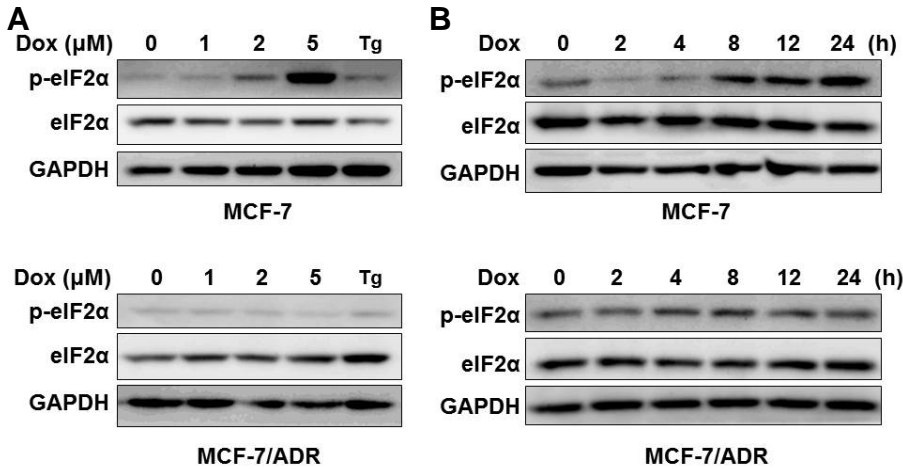


Fig. 2. Treatment of doxorubicin induced phosphorylation of eIF2 α only in MCF-7 cells. (A) MCF-7 cells (top) and MCF-7/ADR cells (bottom) were treated with the indicated concentrations of doxorubicin for 24 h and immunoblot analyses were performed using specific antibodies to the phosphorylated form of eIF2 α (p-eIF2), total eIF2 α (eIF2), and GAPDH. (B) MCF-7 cells (top) and MCF-7/ADR cells (bottom) were treated with 5 μ M doxorubicin for the indicated periods and immunoblot analyses were performed using antibodies to the phosphorylated form of eIF2 α (p-eIF2 α), total eIF2 α (eIF2 α), and GAPDH.

Statistical analysis of the data was performed using Student's *t*-test or Mann-Whitney U-test. Values of *P* < 0.05 were considered to indicate statistical significance.

RESULTS

MCF-7/ADR cells showed resistance to doxorubicin-mediated apoptotic cell death

In an attempt to elucidate the molecular mechanisms of multi-drug resistance, we investigated the characteristics of doxorubicin-resistant MCF-7/ADR breast cancer cells. First, we tested whether MCF-7/ADR cells showed resistance to doxorubicin, as reported previously. Treatment with an increasing dose of doxorubicin for 48 h markedly reduced the viability of MCF-7 cells, but not MCF-7/ADR cells. The viability of MCF-7/ADR cells was about twofold higher than that of MCF-7 cells (Fig. 1A). MCF-7/ADR cells treated with various concentrations of doxorubicin for 24 h showed significantly increased proliferation compared with MCF-7 cells (Fig. 1B). Under this condition,

MCF-7/ADR cells showed lower expression levels of both pro-caspase-7 and the cleaved form of caspase-7 compared to MCF-7 cells, suggesting that a reduction in caspase-7-mediated apoptosis is a characteristic of doxorubicin resistance in MCF-7/ADR cells (Fig. 1C).

eIF2 α Phosphorylation was induced by doxorubicin in MCF-7, but not MCF-7/ADR, cells

Because doxorubicin-induced eIF2 α phosphorylation plays opposite roles in cell death depending on the cell type, we investigated eIF2 α phosphorylation. Treatment of MCF-7 and MCF-7/ADR cells with various concentrations of doxorubicin for 24 h followed by immunoblotting resulted in an increased level of the phosphorylated form of eIF2 α in MCF-7 cells, but not MCF-7/ADR cells (Fig. 2A, top and bottom). Similar results were obtained when cells were treated with 5 μ M doxorubicin for various periods (Fig. 2B, top and bottom). These results suggest that the absence of doxorubicin-mediated phosphorylation of eIF2 α is related to doxorubicin resistance in MCF-7/ADR cells.

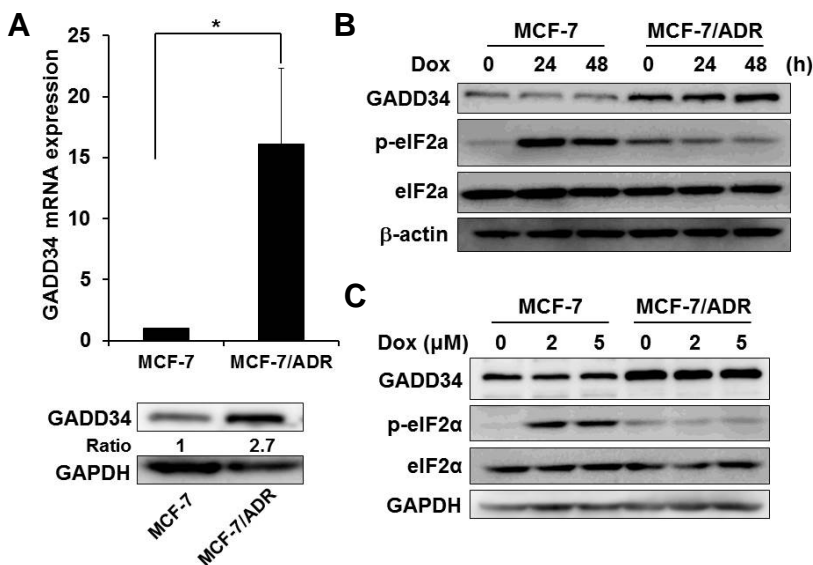


Fig. 3. The expression level of GADD34 was higher in MCF-7/ADR cells than in MCF-7 cells. (A) Basal GADD34 mRNA and protein levels were determined by real-time PCR (top) and immunoblot analyses (bottom) in MCF-7 cells and MCF-7/ADR cells. The quantitated ratio of GADD34 to GAPDH protein levels is indicated. GADD34 mRNA data represent the means \pm S.D. of three independent experiments. Statistical significance was calculated by Mann-Whitney U-test with **p* < 0.01. (B, C) MCF-7 cells and MCF-7/ADR cells were treated with 5 μ M doxorubicin for various periods (B) or with various concentrations of doxorubicin for 24 h (C) and immunoblot analyses were performed using specific antibodies.

GADD34 expression was higher in MCF-7/ADR cells than in MCF-7 cells

To evaluate eIF2 α phosphorylation levels in the two cell lines, we determined the expression levels of GADD34, which functions as a negative regulator of eIF2 α by facilitating its dephosphorylation. First, the basal GADD34 expression level was determined by real-time PCR and immunoblot analyses. GADD34 expression was several fold higher in MCF-7/ADR cells compared to MCF-7 cells (Fig. 3A, top and bottom). Following treatment of the cells with 5 μ M doxorubicin for various time periods, the expression of GADD34 was very slightly decreased in MCF-7 cells, but not in MCF-7/ADR cells (Fig. 3B and unpublished results). Similar results were obtained when the cells were treated with various concentrations of doxorubicin for 24 h (Fig. 3C). Under these conditions, phosphorylation of eIF2 α was clearly increased in MCF-7 cells, but not in MCF-7/ADR cells (Figs. 3B and 3C). These results suggest that the differences in the expression levels of GADD34 might explain

the differences in eIF2 α phosphorylation in the two cell lines. However, this cannot be the sole reason for induction of eIF2 α phosphorylation in MCF-7 cells because GADD34 expression levels were not significantly decreased by doxorubicin treatment (Figs. 3B and 3C).

Increased phosphorylation of eIF2 α induced by salubrinal treatment enhanced doxorubicin-mediated apoptosis in drug-resistant breast cancer cells

To determine whether the absence of eIF2 α phosphorylation in doxorubicin-treated MCF-7/ADR cells is directly involved in drug resistance, we used salubrinal, a selective inhibitor of eIF2 α dephosphorylation that inhibits the constitutive (CREP, a homolog of GADD34) and ER stress-induced (GADD34-PP1) phosphatase complexes (Boyce et al., 2005; Jousse et al., 2003). The viability of MCF-7/ADR cells pretreated with 10 μ M salubrinal for 1 h prior to treatment with various concentrations of doxorubicin for 24 h was markedly decreased compared with

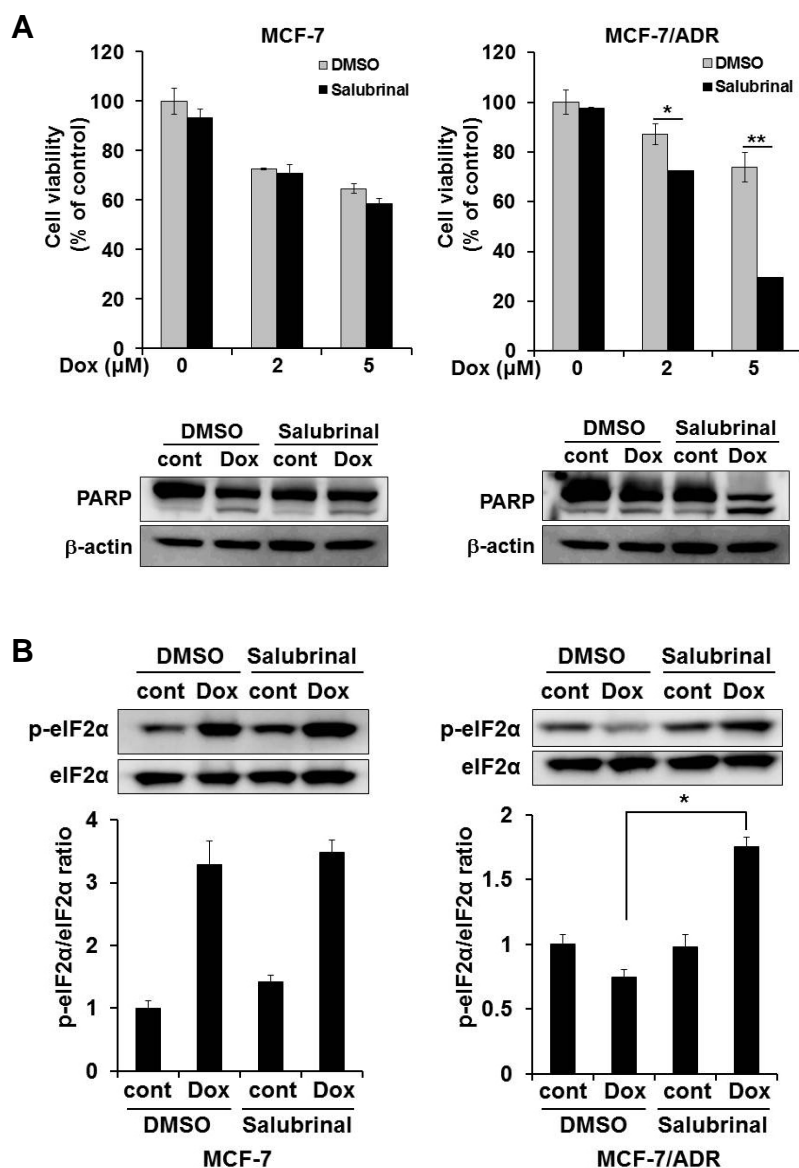


Fig. 4. Increased phosphorylation of eIF2 α by salubrinal treatment enhanced doxorubicin-mediated apoptosis in MCF-7/ADR cells. (A) MCF-7 or MCF-7/ADR cells pretreated with or without 10 μ M salubrinal for 1 h were treated with various concentrations of doxorubicin for 24 h (top) or with 5 μ M doxorubicin for 24 h (bottom). Cell viability was measured by MTT assay (top) and PARP cleavage was measured by immunoblot analyses (bottom). Statistical significance was calculated by Student's t-test with * $p < 0.05$, ** $p < 0.01$. (B) MCF-7 or MCF-7/ADR cells pretreated with 10 μ M salubrinal for 1 h were treated with 5 μ M doxorubicin for 8 h and immunoblot analyses were performed using specific antibodies (top). Histograms show the mean relative amount of p-eIF2 α to eIF2 α after normalization from three independent experiments (bottom). * $p < 0.01$.

the DMSO-treated controls (Fig. 4A, top right). Additionally, the level of the cleaved form of PARP was increased (Fig. 4A, bottom right). Unlike MCF-7/ADR cells, viability and PARP cleavage was not obviously affected in MCF-7 cells by salubrinal pre-treatment followed by doxorubicin treatment (Fig. 4A, top and bottom). The level of the phosphorylated form of eIF2 α was clearly increased in salubrinal and doxorubicin co-treated MCF-7/ADR cells (Fig. 4B, right). The phosphorylation of eIF2 α in doxorubicin-treated MCF-7 cells was not affected by salubrinal treatment (Fig. 4B, left). These results suggest that decreased phosphorylation of eIF2 α confers resistance to doxorubicin-induced apoptosis in MCF-7/ADR cells.

DISCUSSION

In this study, we investigated the roles of eIF2 α in anticancer drug resistance using the breast cancer cell lines MCF-7 and MCF-7/ADR. As shown previously, MCF-7/ADR cells showed resistance to doxorubicin-induced apoptotic cell death. We showed that this resistance has a co-relationship with low phosphorylation levels of eIF2 α in MCF-7/ADR cells. The expression level of GADD34, a component of the eIF2 α phosphatase complex, was higher in MCF-7/ADR cells compared with MCF-7 cells. Indeed, the increase in eIF2 α phosphorylation caused by salubrinal treatment enhanced doxorubicin-mediated apoptosis in MCF-7/ADR cells.

It has been reported that doxorubicin-induced phosphorylation of eIF2 α functions in both cell death and survival pathways (Bennett et al., 2012; Peidis et al., 2011). Despite several reports of their characteristics, little is known about the roles of eIF2 α phosphorylation in doxorubicin-resistant breast cancer cells. Therefore, we investigated the effects of eIF2 α phosphorylation on viability and death in MCF-7/ADR cells. Our data showed that eIF2 α phosphorylation was induced by doxorubicin treatment in MCF-7 cells, but not in MCF-7/ADR cells (Fig. 2), suggesting that eIF2 α phosphorylation functions as a pro-death factor in these cells. Indeed, upregulation of eIF2 α phosphorylation by salubrinal treatment in MCF-7/ADR cells significantly increased doxorubicin sensitivity (Fig. 4). Since one of the main roles of eIF2 α phosphorylation is translation attenuation, one possible explanation for eIF2 α phosphorylation-dependent cell death is translational downregulation of pro-survival/anti-apoptotic genes that function in caspase-dependent apoptosis pathways, autophagy-dependent cell death pathways, the DNA repair system, the ROS scavenging system, and ER stress-induced cell death pathways. In that sense, inhibitor of apoptosis (IAP)-family proteins, such as XIAP, cIAP1/2, Apollon, Livin and Survivin, which are anti-apoptotic, are good candidates for translational downregulation (Hassan et al., 2014; Salvesen and Duckett, 2002; Silke and Vucic, 2014). Indeed, translation of the anti-apoptotic factor XIAP, which confers resistance to ER-stress-induced cell death, is downregulated by PERK and the phosphorylated form of eIF2 α (Hiramatsu et al., 2014). Therefore, it is likely that insufficient translational attenuation of the proteins that function in pro-survival/anti-apoptotic pathways confers doxorubicin resistance on MCF-7/ADR cells.

Bcl-2-targeted cancer therapy has been developed (Azmi et al., 2011), because upregulation of Bcl-2 protein protects cells from apoptotic cell death and is tightly related to chemoresistance in most cancer cells (Reed et al., 1994; Sedlak et al., 1995). Indeed, an increase in the ratio of Bax/Bcl-2 reduces cell viability in many doxorubicin-treated cancer cells; the same is true in MCF-7 cells (Leung and Wang, 1999). In MCF-7/ADR cells, however, basal expression of Bcl-2 is downregulated, and

the Bax/Bcl-2 ratio is not increased upon doxorubicin treatment (Calcabrini et al., 2006; Minko et al., 2005; Ogretmen and Safa, 1996). Therefore, resistance to doxorubicin-induced apoptosis in MCF-7/ADR cells does not seem to be dependent on the Bax/Bcl-2 ratio, despite some discrepancies among MCF-7/ADR sublines. Rather, the acquisition of doxorubicin resistance in MCF-7/ADR cells would be caused by the upregulation of membrane p-glycoprotein and many drug-metabolizing enzymes, either due to mutations or differential expression of the genes involved in cell death/survival pathways, drug metabolism, DNA repair, etc.

It is well known that the UPR, especially the PERK/eIF2 α -P/ATF4/CHOP pathway, plays a pro-apoptotic role under severe ER stress conditions (Malhotra and Kaufman, 2007). CHOP, a downstream target of ATF4, is known as a pro-apoptotic transcription factor (Hetz, 2012) and its apoptosis-relevant targets are GADD34, TRAIL Receptor-2 (DR5), and endoplasmic reticulum oxidoreductase-1 (Ero1 α) (Li et al., 2009; Lu et al., 2014). CHOP also inhibits Bcl-2 transcription and induces Bim expression (McCullough et al., 2001; Puthalakath et al., 2007). CHOP and ATF4 are closely related to cell death by regulating a redox system, protein degradation, and expression of pro-apoptotic factors (Han et al., 2013; Hiramatsu et al., 2014; Wang and Kaufman, 2014). However, in drug-resistant cancer cells, the adaptive/death signal of the UPR is rarely, if ever understood. Indeed, the basal expression level and induction/activation of UPR-related genes under ER stress conditions differed markedly between MCF-7 and MCF-7/ADR cells. For example, neither treatment with doxorubicin nor the ER-stress inducer thapsigargin activated PERK in MCF-7/ADR cells (manuscript in preparation), suggesting that the two drug-mediated induction of the ER stress was not sufficient to activate the PERK. Therefore, it is unlikely that ER stress-mediated induction of PERK/eIF2 α -P/ATF4/CHOP branch plays important roles in apoptosis of doxorubicin-treated MCF-7/ADR cells.

The steady-state intracellular eIF2 α -P level is determined by the control of phosphorylation and dephosphorylation by eIF2 α kinases such as PERK, PKR, HRI, and GCN2, and phosphatases such as GADD34 and CReP. The negative feedback loop due to GADD34 activity plays a critical role in decreasing eIF2 α phosphorylation levels in GADD34-overexpressing cells (Novoa et al., 2001). In our experiment, salubrinal-mediated blocking of the binding of GADD34 to protein phosphatase 1 clearly increased both eIF2 α phosphorylation levels (Fig. 4B) and doxorubicin sensitivity (Fig. 4A) in MCF-7/ADR cells. Therefore, the reduced eIF2 α phosphorylation caused by high-level expression of GADD34 confers doxorubicin resistance on MCF-7/ADR cells. These results support the notion that eIF2 α phosphorylation serves as a pro-apoptotic factor in MCF-7/ADR cells. However, the abundant increase in eIF2 α phosphorylation in doxorubicin-treated MCF-7 cells cannot be explained by the low level expression of GADD34 alone because the expression of the UPR-related genes upon doxorubicin treatment was completely different between the two cell lines.

There are many reports regarding the roles of GADD34 in apoptosis and cytoprotection. As a pro-apoptotic protein, GADD34 induces cell death by attenuating TRAF6-mediated Akt activation; i.e., binding of GADD34 to TRAF6 prevents its interaction with Akt, resulting in the retention of Akt in the cytoplasm (Farook et al., 2013). As a cytoprotective protein, GADD34 is known to inhibit mammalian target of rapamycin (mTOR) signaling by dephosphorylating the tuberous sclerosis complex (TSC) 1/2 and activating cytoprotective autophagy (Hyrskyluoto et al., 2012; Watanabe et al., 2007). The

GADD34/PP1 complex inhibits translocation of pro-apoptotic calreticulin (CRT) to the cell surface, thus preventing immunogenic cell death (Obeid et al., 2007). Therefore, a strategy inducing synthesis of pro-apoptotic proteins by modulating GADD34 activity and eIF2 α phosphorylation, as well as reducing tolerance to protein translation will enable multidrug resistance in breast cancer cells to be overcome.

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