TSA protects H9c2 cells against thapsigargin-induced apoptosis related to endoplasmic reticulum stress-mediated mitochondrial injury

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ABSTRACT

Endoplasmic reticulum stress (ERS) activates an adaptive unfolded protein response (UPR) that facilitates cellular repair, however, under prolonged ER stress, the UPR can ultimately trigger apoptosis thereby terminating damaged cells. Recently, TSA has shown protective effects on ERS and its mechanisms related to ER pathway has been previously characterized. However, whether TSA exerts its protective role via metabolic events remain largely undefined.

Objectives: To explore the possible involvement of the metabolic changes during ERS and to better understand how TSA influence mitochondrial function to facilitate cellular adaptation.

Results: TSA is an inhibitor of histone deacetylase which could significantly inhibit H9c2 cell apoptosis induced by Thapsigargin (TG). It also intervene the decrease of mitochondrial membrane potential. By immunofluorescence staining, we have shown that GRP78 was concentrated in the perinuclear region and co-localized with ER. However, treatments with TG and TSA could let it overlap with the mitochondrial marker MitoTracker. Cellular fractionation also confirmed the location of GRP78 in mitochondrion. Conclusions: TSA decreases ERS-induced cell apoptosis and mitochondrial injury may related to enhance the location of GRP78 in mitochondrion.

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1. Introduction

Endoplasmic reticulum (ER), a multifunctional membranous organelle, controls almost every aspects of cellular survival (Braakman and Bulleid, 2011; Arbabian et al., 2011). Under physiological or pathophysiological conditions, the perturbed Ca2+ homoeostasis and accumulation of unfolded or mis-folded proteins in the ER lumen have been noted. These conditions in the ER are known as endoplasmic reticulum stress (ERS). To combat the deleterious effects of ERS, cells have evolved a variety of protective strategies including the unfolded protein response (UPR) (Csala et al., 2012; Travers et al., 2000). As reported previously, cellular recovery and apoptosis are two conflicting outcomes related to UPR (Sykes et al., 2016; Abbas et al., 2017; Ali et al., 2017). GRP78, a well-characterized glucose-regulated protein, also known as BiP (immunoglobulin heavy-chain-binding protein), has been recognized as a fundamental regulator of UPR. Despite the general localization of GRP78 to the ER, recent studies have pointed out its other functions beyond the ER, which may be critical for cell viability and therapeutic targeting (Ni et al., 2011; Ghafar et al., 2017). An increasing number of studies indicate that ERS activates the UPR; however, a high degree of ER damage may trigger apoptotic pathways (Bravo et al., 2011). Among all of ER partners, mitochondria maybe the most remarkable modulator of cellular metabolism and survival (Bravo et al., 2012). From the energetic point of view, the need for metabolic substrates is enhanced to allow adaptation to different stress conditions (Liu, 2005). On the basis of these premises, mitochondria might participate in the cellular adaptive response to ERS, possibly by determining cellular function after activation of the UPR (Bravo et al., 2011). Trichostatin A (TSA), a broad spectrum histone deacetylase inhibitor (HDAC), showed potent anticaner activities (Usami et al., 2008; Lu et al., 2013). Our previous experiments successfully confirmed that TSA ameliorated ERS-induced injury by ER-associated pathways (Yu et al., 2012). However, whether mitochondrial repair is involved in this process remains to be determined. To address this issue, we first tested the effect of TSA on TG-induced cell apoptosis. Subsequently, the protective effects of TSA and the underlying mechanisms related to the modulation of mitochondria and
GRP78 were explored. Our data suggest that TSA could protect cells from ERS-induced injury by mitochondrial pathways.

2. Materials and methods

2.1. Regents

TSA was purchased from TCI (Shanghai) Development Co., Ltd. Polyclonal rabbit antibody against GRP78 was purchased from Abcam (Cambridge, MA). Polyclonal rabbit antibodies calnexin was purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal mouse antibodies PDI and COXIV and Hsp60 were purchased from Cell Signaling Technology (Danvers, MA, USA). Glyceraldelye 3-phosphate dehydrogenase (GAPDH) antibody was from the Proteintech (Chicago, IL, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L), goat anti-mouse IgG (H + L) secondary antibodies and Cell Counting kit-8 (CCK8) were from Beyotime Institute of Biotechnology. Enhanced chemiluminescence (ECL) detection reagents were from Thermo Scientific (Waltham, MA). Other chemicals were available as analytical reagent.

2.2. Cell culture

H9c2 was purchased from ATCC and were cultured in 10% FBS-containing DMEM and 10 units/ml penicillin. Cells were main-
tained in an incubator at 37 °C and 5% CO2. Cells were incubated with/without the TSA (50 nM) for 1 h and then incubated with/without TG (3 μM) for 8 h.

2.3. Mitochondrial membrane potential (Δψm)

To examine mitochondrial function, cells were stained with the JC-1 (Molecular probes, Life Technologies, USA). In brief, treated H9c2 cells were incubated with JC-1 dye (10 μM) for 20 min and washed twice with dyeing buffer (1×). JC-1 is a lipophilic cationic fluorochrome, which can selectively enter into mitochondria and reversibly change its color from green to red. The fluorescence was measured by the wave lengths of 490 nm excitation and 530 nm and the fluorescence was observed by fluorescence microscope and Flow cytometry.

2.4. Flow cytometry assay

Incubated cells that treated with TG for 8 h or pre-treatment with TSA for 2 h and with TG for another 8 h, were collected by trypsinization, washed with PBS, stained with the AnexinV-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions, and then analyzed using FACS Calibur (BD Biosciences, CA, USA).

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Fig. 1. H9c2 cells pre-treated with TSA for 1 h following with/without TG incubation for another 8 h. TSA strongly reduced cells apoptosis rate as detected by Annexin V-FITC/PI staining (A), and improved mitochondrial function analyzed by JC-1 staining (B) and FACS analysis (C) (n = 3).
2.5. Western blot

Treated cells were lysed by radio immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, USA) containing the 1% protease inhibitor cocktail (Sigma-Aldrich, USA) and 2% phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, USA). Qproteome Mitochondria Isolation Kit was purchased from Qiagen, then lysed with RIPA buffer and separated by SDS-PAGE. 30 µg proteins were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% skimmed dry milk for 2 h at room temperature and washed three times in tris-buffered saline with Tween (TBST), and then the primary antibody GRP78, Calnexin, and GAPDH were incubated overnight at 4 °C. HRP-conjugated secondary antibodies (1:1000) were added and incubated for 2 h (R.T). Immunoblots were incubated with ECL and visualized by Tanon Chemiluminescent Imaging System (Tanon Science & Technology). The results were then quantified using Quantity One software from Bio-Rad Laboratories.

2.6. Immunohistochemistry

Treated cells were fixed with 4% paraformaldehyde for 30 min and washed three times in PBS. Blocking solutions (1%TritonX-100 and sheep serum in PBS) were added to fixed cells and incubated for 30 min at 37 °C. Primary antibody against GRP78 was incubated overnight at 4 °C. After washed with PBS again, the appropriate secondary antibody was added and incubated for 1 h in the dark at room temperature. The nuclei were stained by Hochest33342 for 20 min, RT. After being washed with PBS, immunofluorescence was examined by epifluorescence microscope or laser-scanning confocal microscopy.

2.7. Statistical analyses

Data are shown as mean ± standard error of the mean (SEM). A One-way variance analysis (ANOVA) followed by Dunn’s test was used to detect statistical significance. P value less than 0.05 was considered statistically significant. Data were analyzed with GraphPad Prism v5.0.

3. Results

3.1. Protective effect of TSA on TG-induced cell apoptosis and mitochondrial damage

To study the protective effect of TSA on TG-induced apoptosis, H9c2 cultures were subjected to TG treatment and Annexin V-FITC/PI staining for apoptosis detection by flow cytometry. The results showed that at a 3 µM concentration of TG for 8 h, the cell apoptosis rate was 22.58%. The apoptosis rate of the cell group that was pre-treated with TSA for 2 h decreased to 16.24% (Fig. 1A). In conclusion, cells pre-treated with TSA were more resistant to TG-induced apoptosis than those in the TG group.

The cell has evolved a sophisticated communication system between the ER and other intracellular organelles, especially the physical interaction with mitochondria. As the power-plants of the cell, mitochondria generate ATP by utilizing the proton electrochemical gradient potential, or the electrochemical proton motive force (Δp), which is generated by the serial reduction of electrons through the respiratory electron transport chain (ETC) (Perry et al., 2011). The ER Ca2+ signals could modulate mitochondrial bioenergetics, thus influencing cellular metabolism and survival (Bravo et al., 2012).

We first treated H9c2 cells with TSA for 1 h and then incubated them with TG (3 µM) for another 8 h. Next, we used JC-1 fluorescent dye to test mitochondrial membrane potential measured by fluorescence microscopy and flow cytometry. The results showed that TG, reduced mitochondrial activity in H9c2 cells ad compared to control group; however, a significant elevation in mitochondrial activity was detected in the cells after treatment with TSA (Fig. 1B and C).

3.2. Effects of TSA on subcellular localization of GRP78 under ERS

The expression of GRP78 was measured by immunostaining and western blotting. The results revealed that TSA exposure signifi-
cantly elevated GRP78 levels in both the TSA-only control group and the TG-pretreatment group. Compared to the non-TSA control, the TSA-only group had strikingly increased levels of GRP78, while the expression of GRP78 was further increased by TSA in the presence of TG ($P < 0.01$; Fig. 2A and B).

GRP78 is regarded as a major ER chaperone, facilitating protein folding and assembly (Ni et al., 2011). Research has shown that GRP78 also exists outside the ER (Reddy et al., 2003), which prompted us to examine the changes in intracellular distribution of GRP78 in response to ERS, specifically within the mitochondria.

Fig. 3. Effects of TSA on subcellular localization of GRP78 under ERS. By immunofluorescent assay (A) GRP78 (green) localization in ER were visualized via its co-staining by calnexin (red). Merged pictures indicate partial colocalization of GRP78 and calnexin (yellow); (B) GRP78 (green) localization in the mitochondrion were visualized via its co-staining by MitoTracker (red). Merged pictures indicate GRP78 in the mitochondrion (yellow). $n = 3$. Scale bar 20 $\mu$m.
The low expression levels of GRP78 in normal cells were noted. Then, the co-localization of GRP78 with the ER marker protein calnexin was visualized. When the cells were exposed to TG, a higher expression of GRP78 and an increased co-localization with the ER were observed (Fig. 3). We also found that GRP78 was ubiquitously expressed in non-treated cells. Under ERS induced by TG, GRP78 accumulated in the perinucleus region (Fig. 3A). In a parallel experiment, co-staining with the mitochondria-selective marker MitoTracker showed that the diffusive expression of GRP78 in cytoplasm became more concentrated and partially co-localized with the MitoTracker under ERS (Fig. 3B).

To further examine the localization of GRP78 in mitochondria after ERS and TSA treatment, we isolated mitochondria from the total lysate and conducted western blot analysis. We tested the purity of mitochondria using the ER marker calnexin and mitochondria marker COX IV. The expression of GRP78 was significantly enhanced in mitochondria (Fig. 4). These results confirmed the higher levels of GRP78 in mitochondria under TSA treatment.

4. Discussion

We have confirmed the protective effects of TSA on TG-induced cell apoptosis, and have clarified the underlying mechanisms related to GRP78 and its association with mitochondria. In contrast to other ERS models, our model focused on the metabolic changes that influence cellular function instead of ER-related signaling.

TSA is a histone deacetylase inhibitor (HDACi) which serves as an anti-neoplastic agent that inhibits the growth of different types of cancer (Gilardini Montani et al., 2017; Jamal et al., 2017). Our previous studies have shown the protective role of TSA on myocardial infarction, which involves ER signaling pathways. Moreover, TSA protects cardiac function against myocardial ischemia/reperfusion (I/R) injury by upregulating GRP78 and downregulating C/EBP homologous protein CHOP (Yu et al., 2012). In the present study, we demonstrated the protective effect of TSA on ERS-induced mitochondrial injury. TG, a naturally occurring sesquiterpene lactone, inhibits the ER isoform of the Ca^{2+}-ATPase with high selectivity (Thastrup et al., 1990). It elevates the free Ca^{2+} content in the cytosol in a subset of sensitive cells by acutely arresting the ER Ca^{2+} pump, followed by a rapid Ca^{2+} leak from intracellular reservoir. (Thastrup et al., 1990).

The ER is a cytosolic membranous structure interconnects the nucleus, mitochondria, as well as other intracellular organelles (Ron and Walter, 2007). It is also the major reservoir for intracellular Ca^{2+} and is considered the dominant modulator of Ca^{2+} homeostasis (Schroder and Kaufman, 2005). Its dynamic interactions with other membranous organelles, such as the late endosomes (Rocha et al., 2009), have been extensively studied. When associated with mitochondria, a specific region on the ER tethers to mitochondria via a dynamic linkage, namely the mitochondria-associated membranes (MAMs). It regulates calcium homeostasis and mitochondrial function, and has been associated with autophagy and apoptosis (Vance, 1990). The major ER-mitochondrial calcium transfer channels, IP3R and VDAC, locate in close proximity to MAM, by associating with chaperone GRP75 interconnect these two organelles (Szabadkai et al., 2006).

Studies have shown extensive physical and functional connections between ER and mitochondria (Shamsudin et al., 2017). Among them, calcium exchange is thought to be the best established mode of interorganellar communication (Giorgi et al., 2009). For example, ER-localized IP3R stimulate calcium release to mitochondria, which further tigers mitochondrial respiration and downstream ATP production. During cellular stress, ER-mitochondrial interactions facilitate the structural framework and activate signaling cascades for an appropriate cellular adaption. However, if the ER-stress continued, the mitochondria would face the risk of disintegration.

The mechanism by which mitochondrial depolarization as well as cytochrome c release from mitochondria have been implicated under various stress types. However, increasing evidence suggested other two distinct modes of actions (Marzo et al., 1998). The first mode involves the Bcl-2 family proteins, while the second mode involves a high conductive channel, namely the mitochondrial permeability transition pore (MPTP) (Feldmann et al., 2000). Under ER-stress conditions, translocation of GRP78 to mitochondria was observed. Previous studies revealed its distribution in the inter-membrane space, inner membrane, and matrix, leaving the outer mitochondrial membrane spared (Sun et al., 2006). However, GRP78 has been found in the outer mitochondrial membrane in H460 cells after similar stress (Shu et al., 2008). These observations indicate that the submitochondrial distribution of GRP78 depend highly on cell and stress type (Sindhu et al., 2017).

Our study demonstrated the metabolic changes that occur during the adaptive phase of ERS. We also clarified the role of TSA in reducing cell apoptosis; TSA recovered injured mitochondrial membrane potential and increased expression of GRP78 in the mitochondria. In conclusion, this study provides evidence that TSA exerts its protective role under TG induced ERS by modulating GRP78 localization within mitochondria.

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**Fig. 4.** TSA promotes GRP78 localization in the mitochondrion. Immunoblot of mitochondrial fraction from H9c2 cells treated with TSA (1 h) with/without TG (3 μM, 8 h) for GRP78, two independent mitochondrial marker COX IV and Hsp60 are shown. n = 3, **P < 0.05.
Conflicts of interest

The authors have declared that there is no conflict of interest.

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