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Thapsigargin induces apoptosis when autophagy is inhibited in HepG2 cells and both processes are regulated by ROS-dependent pathway

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ABSTRACT

Thapsigargin (TG), is widely used to induce endoplasmic reticular stress. Treated with TG for a long time, cells suffer the unfolded protein response (UPR) to elude apoptosis, but may activate autophagy. However, the switch between autophagy and apoptosis is unclear. To clarify the key signal for selection of these two protective responses, we studied the correlation of autophagy and apoptosis in HepG2 cells exposed to TG with time. TG induced apoptosis in HepG2 cells was evidenced by typical cell morphological changes and the activation of caspase-12, caspase-9 and caspase-3. Meanwhile, cytochrome c was released following with the dissipation of mitochondrial membrane potential (MMP), and the ratio of Bax/Bcl-2 was increased. TG-induced autophagy was confirmed by the accumulation of MDC, GFP-LC3 staining autophagic vacuoles, and the improved expression of LC3 II and Beclin-1. Additionally, inhibited autophagy via chloroquine (CQ) markedly enhanced the apoptosis induced by TG, which was linked to the Bcl-2 family. Furthermore, TG induced the generation of reactive oxygen species (ROS), and the ROS scavenger effectively suppressed TG-induced apoptosis and autophagy. All these results proved that restraint of autophagy may enhance TG-induced apoptosis through increasing the Bax/Bcl-2 ratio and both processes were regulated by ROS.

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1. Introduction

Autophagy is a highly-conserved intracellular catabolic process which plays a critical role in the homeostatic process of degradation and recycling defective organelles, aggregated or long lived proteins (Mizushima et al., 2002). The autophagy process begins with the formation of double-membrance bound vesicles (autophagosomes), which engulf long-lived proteins and excess or damaged organelle. Then the autophagosomes fuse with lysosomes for degradation and reuse of the components (Baba et al., 1994; Strmhaug et al., 1998; Yang and Klionsky, 2010). Two major cell signaling pathways involved in the regulation of autophagy, one is the PI3K-Akt-mTOR signaling pathway, which represses autophagy, the other is Beclin1-Class III PI3K pathway, which regulates autophagosome formation (Yue et al., 2003; Zhao and Vogt, 2008). Autophagy has been accounted to one of the survival mechanisms for cancer cells, but it could also play a role in the remotion of cancer cells (Roy and Debnath, 2010). Autophagy can be induced by nutrient starvation (Komatsu et al., 2005), oxidative stress (Kiffin et al., 2006), endoplasmic reticulum (ER) stress (Yorimitsu et al., 2006) and so on. In mammalian cells, the unfolded protein response (UPR) signaling is the main pathway in the ER Stress signal transduction pathway (Patil and Walter, 2001). The UPR is mediated by Glucose-regulated protein-78/Binding immunoglobulin protein and three transmembrane ER stress sensors, namely PERK (Protein kinase R-like ER kinase), IRE-1 (Inositol-requiring kinase-1) and ATF6 (Activating transcription factor 6). When the UPR occurs, the three ER stress transducers were activated and transduce their own signals to the cytoplasm and the nucleus for cell survival (Lee, 2005; Verfaillie et al., 2010). Among them, it was indicated that activation of PERK and IRE-1 plays an important role in ER stress-induced autophagy (Kim et al., 2010; Ogata et al., 2006). Recently, it has been shown that ER stress could be led to the formation of

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autophagic vehicles with the expression of microtubule-associated protein 1 light chain3 (LC3)-II and Beclin-1 (Cheng et al., 2014; Kouroku et al., 2006). ER Stress can promote cell survival via UPR, however, if the stress is prolonged or too severe, ER Stress could induce apoptosis (Szegezdi et al., 2006).

Autophagy and apoptosis are two forms of programmed cell death (PCD), which play essential roles in regulating the balance of cell growth and cell death. And morphological differences are obvious between them. Autophagy is an intracellular contained event which does not necessarily mean cellular destruction, whilst apoptosis is ordered cellular destruction. Recent studies show that the molecular regulators of both pathways are interconnected and the same regulators can sometimes control both apoptosis and autophagy (González-Estévez and Saló, 2010; Yousefi et al., 2006). Multiple stress signals, such as reactive oxygen species (ROS) and ER Stress can mediate the process of both autophagy and apoptosis in several types of cancer cells. For instance, the Cas III-ia, a copper compound could induce autophagy and apoptosis through ROS and JNK activation in glioma cells (Trejo-Solís et al., 2012). The imbalance in ROS expression will injure cell, especially in mitochondria and ER. As for Saxifragifolin D, a treatment for solid tumor drug could induce interaction between autophagy and apoptosis by ROS-mediated ER Stress in breast cancer (Shi et al., 2013). Taken together, ROS and ER Stress play an important role in the interplay between autophagy and apoptosis. However, it is still unclear about them; more studies are needed to illustrate the interaction of ROS/ER Stress/autophagy/apoptosis for future research in cancer therapy.

Thapsigargin (TG), a highly selective inhibitor of the sarcoplasmic reticulum and sarco/endoplasmic Ca^{2+}-ATPases (SERCA), is widely used to induce ER Stress in a variety of cell types (Lytton et al., 1991; Thastrup et al., 1990). In the present study, we explored the effect of thapsigargin on human liver cancer HepG2 cells through ER Stress activation. In addition, we examined the role of ROS in TG-induced cell death. We found that TG induced mitochondrial pathway-dependent apoptosis and Beclin-1-mediated autophagy, and ROS may play an important role in these progresses. The inhibition of autophagy could accelerate the apoptosis induced by TG.

2. Materials and methods

2.1. Reagents and plasmids

Thapsigargin, monodansylcadaverin (MDC), chloroquine (CQ) and anti-DAPK1 antibody were purchased from Sigma–Aldrich (St. Louis, MO, USA). Minimum essential medium (MEM) and Fetal Bovine Serum (FBS) were purchased from Life Technologies Corporation (Grand Island, NY, USA). X-tremeGENE HP DNA Transfection Reagent was obtained from Roche (Mannheim, Germany). 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sodium dodecylsulfonate (SDS), Tween-20, trypsin and dimethyl sulfoxide (DMSO) were all purchased from AMRESCO Inc. (Solon, OH, USA). Bovine serum albumin (BSA), cell permeable pan Caspase-3 inhibitor Ac-DEVD-CHO, Rhodamine 123, PMSF, N-acetylcysteine (NAC), anti-LC3 and anti-Tubulin antibodies were purchased from Beyotime (Shanghai, China). Caspase-9 inhibitor Ac-LEHD-FMK was obtained from Beijing B&M Biotech Co, Ltd. (Beijing, China). Annexin V-FITC Apoptosis Detection kit was obtained from Keygen Biotech (Nanjing, China). Fluorescent Staining Apoptosis Detection kit and Western Luminescent Detection Kit were purchased from Vigorous (Beijing, China). The Caspase-Glo® 3/7 Assay and Caspase-Glo® 9 Assay were obtained from Promega (USA). Primary antibodies against: Caspase-9, Caspase-12 and Bax were obtained from Proteintech (Wuhan, China). Anti-Beclin1, anti-Bcl-2, anti-Caspase-3 and anti-Cytochrome (Cyt) c antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The anti-mouse and anti-rabbit antibodies were all obtained from ZSGB-BIO (Beijing, China). Fetal bovine serum was obtained from Gibco (Grand Island, USA). The GFP-LC3 plasmid was kindly supplied from Xuejun Jiang (Chinese Academy of Sciences, Beijing, China).

2.2. Cell culture and treatments

HepG2 cells were obtained from the Institute of Bio-chemistry and Cell Biology (Shanghai, China). The cells were grown in MEM culture medium containing heat inactivated 10% fetal bovine serum and 1% penicillin/streptomycin, and cultured at 37 °C in a 5% CO_2 incubator. HepG2 cells were treated with TG from a freshly prepared 5 mM stock solution in DMSO and diluted to obtain corresponding concentrations with the cell culture medium. The final DMSO concentration was less than 0.1% (v/v) for each treatment. The control cells were treated with 0.1% DMSO in minimum essential medium.

2.3. GFP-LC3 plasmid transfection

Transient transfection was performed with X-tremeGENE HP DNA Transfection Reagent following the protocol by the manufacturer. In brief, cells were seeded at 1×106 per well in 1 mL of growth medium in a 6-well plate. After 24 h, the cells were incubated with 2 μg pEGFP-LC3 plasmid and 6 μL X-tremeGENE HP DNA Transfection Reagent. Six hours later, fresh growth medium was inserted. After 48 h, cells were treated with thapsigargin (1 μM), photomicrographs of GFP-LC3 were obtained by fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

2.4. Cell viability assay

The cytotoxic effects were observed by MTT assay as previously described. Briefly, HepG2 cells (1.0×104 cells/well) were cultured in 96-well plates (100 μL culture medium per well). After 24 h, the cells were treated with different doses of TG (0, 2, 4, 8, 16 and 32 μg/mL, respectively) for 24 h or 48 h. Then the medium containing TG was removed, and cells were incubated with 100 μL fresh medium containing 10 μL MTT (5 mg/mL in PBS) for 4 h at 37 °C. After that, 200 μL DMSO was added into each well to dissolve the formazan crystals at 37 °C for 15 min in the dark. Finally, the absorbance was examined by a microplate reader at 570 nm (Molecular Devices, Sunnyvale, CA, USA). The cell viability was estimated as the percentage of the control.

2.5. Cellular morphology examination

The HepG2 cells were seeded in 6-well plates with a density of 5.0×105 cells/well, and were further exposed to TG (0, 5, 10 and 20 μg/mL) for 24 h. After incubation, cells were observed by fluorescence microscopy (Leica DMIRB, Germany) with UV excitation. For morphological examination, the apoptosis cells were detected by Fluorescent Staining Apoptosis Detection kit following the manufacturer's instructions. Briefly, the cells were washed twice with PBS, and stained with staining solution which containing 10 μL Hoechst33342 in 1 mL of growth medium at 37 °C in the dark for 30 min. MDC, an autofluorescence base that accumulates in autophagic vacuoles, is widely used as a specific marker to analyze the autophagic process. For autophagic morphological examination, the cells were washed twice with PBS and stained with 0.05 mM MDC at 37 °C in the dark for 10 min. Cells were

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