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Simultaneous mitochondrial Ca^{2+} overload and proteasomal inhibition are responsible for the induction of paraptosis in malignant breast cancer cells

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ABSTRACT

In this study, we investigated the role of Ca^{2+} in curcumin-induced paraptosis, a cell death mode that is accompanied by dilation of mitochondria and the endoplasmic reticulum (ER). Curcumin induced mitochondrial Ca^{2+} overload selectively in the malignant breast cancer cells, but not in the normal breast cell, contributing to the dilation of mitochondria/ER and subsequent paraptotic cell death. In addition, we found that simultaneous inhibition of the mitochondrial Na^+/Ca^{2+} exchanger (mNCX) and proteasomes can trigger a sustained mitochondrial Ca^{2+} overload and effectively induce paraptosis in malignant breast cancer cells.

1. Introduction

Chemotherapy improves disease-free survival in patients with breast cancer, but does not cure patients with advanced or metastatic disease. Intrinsic resistance to chemotherapy due to dysregulation of apoptosis is a significant cause of treatment failure in advanced breast cancer [1]. Thus, strategies to induce non-apoptotic cell death may provide an alternative and effective therapeutic option in malignant breast cancers that show resistance to various pro-apoptotic therapeutics.

Paraptosis is a cell death mode that is accompanied by dilation of mitochondria and the endoplasmic reticulum (ER) without the characteristic apoptotic features of pyknosis, DNA fragmentation or caspase activation [2]. Paraptosis requires new protein synthesis [3], and recent reports have shown that it can be inhibited by the expression of AIP-1/Alix [3,4]. However, the underlying mechanisms of paraptosis, particularly the signals responsible for triggering dilation of mitochondria and the ER, have not yet been fully determined.

Curcumin (diferuloylmethane), a major active component of turmeric, has shown promising abilities as a cancer chemoprevention and chemotherapeutic agent *in vitro* and *in vivo* [5–7]. Furthermore, curcumin has been demonstrated selective killing of various cancer cell types, while sparing normal cells [8–10]. While much of the research into the cancer-killing effects of curcumin has predominantly focused on its ability to induce apoptosis [11,12], it also reportedly induces non-apoptotic cell death modes, such as mitotic catastrophe [13,14] or autophagic cell death [15], in several types of cancer cells. Recently, we showed that curcumin also induces paraptosis selectively in malignant breast cancer cells [10]. Since both mitochondria and the ER are major reservoirs for intracellular Ca^{2+} [16], we investigated the role of Ca^{2+} in curcumin-induced paraptosis. Here, we report that uniporter-mediated Ca^{2+} influxes into the mitochondria act as an initial and critical signal for curcumin-induced paraptosis, triggering dilation of both mitochondria and the ER. In addition, we found that simultaneous inhibition of the mitochondrial Na^+/Ca^{2+} exchanger (mNCX) and proteasomes can trigger a sustained mitochondrial Ca^{2+} overload and effectively induce paraptosis in malignant breast cancer cells. Our findings suggest novel insights into the molecular basis of paraptosis, as well as an alternative therapeutic option for killing conventional-therapy-resistant malignant breast cancer cells via the induction of paraptosis.

2. Materials and methods

2.1. Chemicals and antibodies

Curcumin, lactacystin, MG132, N-acetylcysteine (NAC), cycloheximide (CHX), ethylene glycol tetraacetic acid (EGTA), 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), ruthenium red, RNase A, propidium iodide and crystal violet were purchased from Sigma (St. Louis,

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MO). Rhod-2-AM, Fluo-3-AM, MitoSOX-Red, MitoTracker-Green (MTG), MitoTracker-Red (MTR), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), calcein acetoxymethyl ester (calcein-AM) and ethidium homodimer (EthD-1) were purchased from Molecular Probes (Carlsbad, CA). Mn(III)tetrakis (4-benzoic acid) porphyrin Chloride (MnTBAP), 7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3*H*)-one (CGP-37157), Ru360, 2-Aminoethoxydiphenyl borate (2-APB), PD98059, U0126, and SP600125 were obtained from Calbiochem (San Diego, CA). Dantrolene was obtained from Alexis Biochemicals (San Diego, CA). The following antibodies were used: monoclonal anti-β-actin (Sigma); anti-ubiquitin and CHOP (GADD153) (Santa Cruz Biotechnologies, Santa Cruz, CA); anti-phospho-ERK1/2, total ERK1/2, phospho-JNK, total JNK, and AIP-1/Alix (Cell Signaling, Beverly, MA); HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG (Molecular Probes).

2.2. Cell culture

The MDA-MB 435S and MDA-MB 231 human breast cancer cell lines, HCT116 human colon cancer cell line, and the MCF-10A non-tumorigenic human mammary epithelial cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB 435S, MDA-MB 231, and HCT116 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies, Grand Island). Normal human mammary epithelial cells (HMEC) were purchased from Clonetics Corp. and maintained in Mammary Epithelial Growth Medium (MEGM, Clonetics Corp., San Diego, CA) supplemented with bovine pituitary extract, insulin, human epidermal growth factor, hydrocortisone, and antibiotics (Clonetics Corp.). The MCF-10A cells were cultured in the same MEGM additionally supplemented with 100 ng/mL cholera toxin (Calbiochem). Cell culture passage number less than five was used in the present study. Curcumin (>94% purity, Sigma) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 40 mM and stored in a dark colored bottle at −20 °C. This stock solution was diluted to the required concentration when need.

2.3. Measurement of cellular viability

Cell viability was assessed by double labeling of cells with 2 μM calcein-AM and 4 μM EthD-1. The calcein-positive live cells and EthD-1-positive dead cells were visualized using fluorescence microscope (Axiovert 200 M, Zeiss) equipped with Zeiss filter set #10 (excitation band pass, 450–490 nm; emission band pass, 515–565 nm) and #20 (excitation band pass, 546 nm; emission band pass, 575–640 nm) and counted.

2.4. Clonogenic assay

To determine long-term anti-cancer effects, cells were treated with the indicated concentrations of curcumin for 6 h, rinsed with fresh medium, allowed to grow for 14 days to form colonies, and then stained with 0.5% crystal violet.

2.5. Analysis of DNA content

MDA-MB 435S cells (2×10^5 cells) or HCT116 cells (4×10^5 cells) were plated in 60-mm dishes and treated with 10 μM z-VAD-fmk and/or 40 μM curcumin for 24 h. After treatment, cells were collected by trypsinization, fixed in 70% ethanol for 30 min on ice, washed with ice-cold PBS, resuspended in 1 ml PBS containing 100 μg/ml RNase A and 1 mg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed using FACScan flow cytometer (BD Biosciences, CA). The data was analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

2.6. Establishment of the stable cell lines expressing the fluorescence specifically in mitochondria or ER

To establish the stable cell lines expressing the fluorescence specifically in mitochondria or the ER, MDA-MB 435S cells were transfected with the pEYFP-Mito or pEYFP-ER vector (Clontech Laboratories, Mountain View, CA). Stable cell lines overexpressing pEYFP-Mito or pEYFP-ER (YFP-Mito or YFP-ER) were selected with fresh medium containing 500 μg/mL G418 (Calbiochem). Images of mitochondria or ER were obtained from the fluorescence microscopy using a Zeiss filter set #10.

2.7. Measurement of mitochondrial superoxide production

To measure mitochondrial superoxide production, cells were loaded with 2.5 μM MitoSOX-Red for 20 min in the dark, washed with Hank's Buffered Salt Solution (HBSS) containing Ca^{2+} and Mg^{2+}, and further processed for flow cytometry. In addition, following staining of cells with MitoSOX-Red, the production of mitochondrial superoxide was confirmed by fluorescence microscopy using a #20 filter set.

2.8. Measurement of cytosolic and mitochondrial Ca^{2+} levels

To measure cytosolic Ca^{2+} levels, treated cells were incubated with 2.5 μM Fluo-3-AM at 37°Cfor 20 min, washed with HBSS (without Ca^{2+} or Mg^{2+}), and analyzed immediately by flow cytometry. To measure mitochondrial Ca^{2+} levels, treated cells were incubated with 2.5 μM Rhod-2-AM at 4 °C for 30 min, washed with HBSS (without Ca^{2+} or Mg^{2+}), further incubated with HBSS at 37 °C for 20 min, and then analyzed by flow cytometry. To confirm the mitochondrial localization of the Rhod-2 probe, cells were loaded with 2.5 μM Rhod-2-AM in HBSS (without Ca^{2+} or Mg^{2+}) for 30 min at 4 °C. The cells were then washed with HBSS, loaded with 100 nM MitoTracker-Green for 20 min in HBSS, and visualized by the fluorescence microscopy using Zeiss filter sets #10 and #20.

2.9. Western blotting

Cells were washed in PBS and lysed in boiling sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (62.5 mM Tris [pH 6.8], 1% SDS, 10% glycerol, and 5% β-mercaptoethanol). The lysates were boiled for 5 min, separated by SDS–PAGE, and transferred to an Immobilon membrane (Millipore, Bredford, MA, USA). After blocking nonspecific binding sites for 1 h using 5% skim milk, membranes were incubated for 2 h with specific Abs. Membranes were then washed three times with TBST and incubated further for 1 h with horseradish peroxidase-conjugated anti-rabbit, -mouse or -goat antibody. Visualization of protein bands was accomplished using ECL (Amersham Life Science, Buckinghamshire, UK).

2.10. Transmission electron microscopy

Cells were prefixed in Karnovsky's solution (1% paraformaldehyde, 2% glutaraldehyde, 2 mM calcium chloride, 0.1 M cacodylate buffer, pH 7.4) for 2 h and washed with cacodylate buffer. Post-fixing was carried out in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 h. After dehydration with 50–100% alcohol, the cells were embedded in Poly/Bed 812 resin (Pelco, Redding, CA, USA), polymerized, and observed under electron microscope (EM 902A, Zeiss, Oberkohen, Germany).

2.11. Statistical analysis

All data were presented as mean ± S.D. (standard deviation) from at least three separate experiments. Student's *t* test was applied to evaluate the differences between treated and control groups with cell viability. Data from multiple groups were analyzed by one-way ANOVA, followed by Bonferroni multiple comparison test. For all the tests, the level of significance was values of $P < 0.05$.

2.12. Isobologram analysis

To determine the effect of combination of CGP-37157 and bortezomib on MDA-MB 435S or MDA-MB 231 cells, dose-dependent effects were determined for each compound and for one compound with fixed concentrations of another. The interaction of CGP-37157 and bortezomib was quantified by determining the combination index (CI), in accordance with the following classic isobologram [17]. The equation for the isobologram is shown as $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$, where $(Dx)_1$ and $(Dx)_2$ indicate the individual dose of CGP-37157 and bortezomib required to produce an effect, and $(D)_1$ and $(D)_2$ are the doses of CGP-37157 and bortezomib, respectively, in combination that produce the same effect. From this analysis, the combined effects of the two drugs can be summarized as follows: CI < 1 indicates synergism; CI = 1 indicates summation (additive and zero interaction); and CI > 1 indicates antagonism.

3. Results

3.1. Curcumin induces paraptosis selectively in malignant breast cancer cells

To investigate the effect of curcumin on the viability of various breast cells, we treated these cells with different doses of curcumin and measured the cell viability using calcein-AM and EthD-1. Curcumin was more cytotoxic to two malignant breast cancer cell lines (MDA-MB 435S and MDA-MB 231), compared to a normal breast cell line (MCF-10A) or human mammary epithelial cells (HMEC) (Fig. 1A). To further examine the antitumor activities of curcumin, clonogenic assays were performed and we observed that the clonogenicities of MDA-MB 435S and MDA-MB 231 cells were dose-dependently reduced by curcumin treatment (Fig. 1B). Since curcumin is well-known to induce apoptosis in many cancer cells, including HCT116 colon cancer cells [18], we used flow cytometry to compare the changes in DNA content between HCT116 and