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Paclitaxel attenuates Bcl-2 resistance to apoptosis in breast cancer cells through an endoplasmic reticulum-mediated calcium release in a dosage dependent manner

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ABSTRACT

To address the controversy regarding efficacy of paclitaxel in the presence of the anti-apoptotic protein Bcl-2, we investigated calcium stored in the endoplasmic reticulum as a potential factor. Our results showed that the ER calcium store is a common target for both paclitaxel and Bcl-2 protein. Paclitaxel directly associates with the endoplasmic reticulum to stimulate the release of calcium into the cytosol, contributing to the induction of apoptosis. However, Bcl-2 expression suppresses the cell's pro-apoptotic response of endoplasmic reticulum calcium release, thus inhibiting susceptibility of cancer cells to undergo apoptosis. Depending upon dosage, a paclitaxel-induced stimulatory effect can overcome the Bcl-2-mediated inhibitory effect on endoplasmic reticulum calcium release, thus attenuating the resistance of Bcl-2 to apoptosis. Our finding is the first to demonstrate that endoplasmic reticulum calcium plays a key role in the efficacy of paclitaxel in the presence of Bcl-2, thus providing insight into the complex but crucial paclitaxel–calcium–Bcl-2 relationship, which may impact breast cancer treatment.

1. Introduction

Many chemotherapy agents induce cancer cell death primarily through apoptosis [1–3]. Therefore, expression of anti-apoptotic factors can convey resistance to chemotherapy agents [4]. Specifically, the anti-apoptotic protein Bcl-2, initially discovered in human follicular lymphoma, may resist apoptosis induced by paclitaxel, a major chemotherapy agent for breast cancer treatment [5,6]. However, a controversy regarding the resistance of Bcl-2 and efficacy of paclitaxel remains unsettled. Some studies suggest that Bcl-2 protects cells from paclitaxel-induced apoptosis [7,8], while other studies showed that Bcl-2 expression is correlated with susceptibility to paclitaxel and can be used as a good predictive marker in chemotherapy [9–11]. In addition, paclitaxel-induced apoptosis has been reported to be independent of Bcl-2 expression [12]. To clarify this controversy, key factors determining the relationship between paclitaxel action and Bcl-2 resistance need to be clarified. Previous studies proposed estrogen receptor or multiple-drug resistance receptors as candidates [13,14]. This study is the first to focus on direct involvement of ER calcium as a key factor, based on the following rationale.

First, ER calcium store may be involved in the relationship between paclitaxel action and Bcl-2 resistance due to its importance as a gateway in apoptosis [15–18]. Physiologically, ER calcium homeostasis is maintained by the dynamic balance between calcium release (through ER calcium channels) and calcium refilling (through ER calcium pumps) [19]. Additionally, normal ER calcium release is required to maintain the function of downstream organelles such as mitochondria [19]. However, increased ER calcium release can sensitize the mitochondrial-apoptosis pathway, or activate calcium-dependent enzymes to induce or regulate apoptosis [20–23]. Therefore, ER calcium mobilization can determine whether the cell undergoes apoptosis depending on cell type and inducer type [15].

In addition, the involvement of the ER calcium store in Bcl-2 resistance is further supported by ER calcium–Bcl-2 coupling, evidenced by the co-localization of Bcl-2 and calcium in the ER. Thus, Bcl-2 may be able to inhibit apoptosis by regulating ER calcium homeostasis [5,23–26]. Some studies [27,28] proposed that Bcl-2 may inhibit ER Ca^{2+} release by regulating membrane conductance

Abbreviations: ATCC, American tissue culture center; ANOVA, the analysis of variance; Bcl-2, B cell lymphoma 2 protein; CCE, capacitative calcium entry; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; FRET, fluorescence resonance energy transfer; HRP, horseradish peroxidase; HSD, honestly significant difference; M468, MDA-MB-468 breast cancer cell line; PI, propidium iodide; ROS, reactive oxygen species; RT, room temperature; SD, standard deviation; SERCA, sarco(Endo)plasmic reticulum calcium ATPase; TG, thapsigargin.

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or Ca^{2+} channels/pumps. Specifically, one recent study by Rong et al., identified that Bcl-2 physically interacts with the ER calcium channel, inositol 1,4,5-trisphosphate receptor (IP3R) to inhibit pro-apoptotic ER calcium release [29]. Although this ER calcium–Bcl-2 coupling has not been fully understood, it strongly suggests Bcl-2-mediated resistance to apoptosis.

Finally, not only does Bcl-2 regulate ER calcium release, a previous study from our laboratory showed that the ER calcium store is also involved in paclitaxel-induced apoptosis. Our results, using clinically-relevant doses, showed that paclitaxel at higher doses ($\sim 10^{-6}$ M) directly attacked the ER inducing significant ER calcium release, resulting in an ER calcium-dependent apoptosis Ref. In contrast, lower dose ($\sim 10^{-7}$ M)-induced apoptosis was independent of ER calcium release [30]. Our finding that paclitaxel can induce ER-mediated apoptosis is also supported by another study focusing on paclitaxel-induced activation of ER stress proteins [31]. Therefore, paclitaxel may attack the ER through multiple mechanisms.

Taken together, these data suggest strong involvement of the ER calcium store in the relationship between paclitaxel action and Bcl-2 resistance, since both paclitaxel and Bcl-2 can mediate apoptosis-related ER calcium changes albeit in opposite ways. To our knowledge, our research is the first study that has focused on the role of calcium, especially the internal ER calcium store, to elucidate this relationship. Our study is composed three parts: (1) test the efficacy of paclitaxel in the presence of Bcl-2. (2) Test whether Bcl-2 presence can change the cell's ability to release pro-apoptotic calcium from the ER. (3) Test whether paclitaxel still has direct stimulatory effects on pro-apoptotic ER calcium release in the presence of Bcl-2 protein.

2. Materials and methods

2.1. Cell agents

Paclitaxel was dissolved in dimethyl sulfoxide (DMSO) solution and then diluted to the desired concentration with medium. Two concentrations of paclitaxel, 2.5 μM ($\sim 10^{-6}$ M) and 0.2 μM ($\sim 10^{-7}$ M) were tested. These are the estimated plasma levels in patients after using a common dosage 135 mg/m^2 for a 3 h and 24 infusion schedules, respectively, according to the drug manufacturer [32]. DMSO concentrations were kept below 0.5% in all experiments. Paclitaxel, DMSO, and the ER calcium pump inhibitor thapsigargin (TG) were purchased from Sigma (St. Louis, MO).

2.2. Stable transfections

Breast carcinoma cell line MDA-MB-468 (M468), which is Bcl-2, estrogen receptor, progesterone receptor and HER-2 receptor negative, was obtained from the American Type Culture Collection (ATCC, Manassas, VA) [33]. The Bcl-2 expression plasmid containing the human Bcl-2 cDNA in a pCMV-Tag 2B (Stratagene) backbone and its vehicle control vector, were kindly provided by Dr. Clark Distelhorst (Case Western Reserve University) [34]. According to the manufactures's instruction, plasmid vectors were transfected into M468 cells using SuperFect™ (QIAGEN), followed by selection with the neomycin selection antibiotic G418 (USB, Cleveland, OH). M468 control cells were cultured as previously described [30,33]. M468 Bcl-2 transfectant cells were maintained in medium containing 100–200 μl/mg of G418, with all other culture conditions the same as that of M468 control cells. No significant difference can be observed between wild M468 and vector control cells in calcium and apoptosis tests.

2.3. Immunoblotting

Major procedures and agents were same as previously described [30]. β-Tubulin expression was used as an internal control for equal protein loading. A mouse anti-human Bcl-2 monoclonal antibody (Sigma, St. Louis, MO) were used to detect protein levels of Bcl-2, and a Rabbit anti-human IP3R polyclonal IgG antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was used to detect ER calcium channel IP3R levels. A horseradish peroxidase (HRP) secondary goat anti-rabbit antibody (Calbiochem, San Diego, CA) was used to visualize signals. 25 μg of total cellular protein lysate from Bcl-2 stable transfected cells were applied. The expected Bcl-2 band was ~26 kD in size. Anti-IP3R antibody was raised against amino acids 2402–2701 mapped at the C terminus of human IP3R, and the resulting band is ~115 kD in size.

2.4. Immunocytochemistry

Cells were fixed in pure methanol for 10 min at −20 °C, and incubated in 1× PBS with 0.1% Tween 20 solution for 10 min at room temperature (*RT*). The same anti-human Bcl-2 antibody as above was used to detect the location of transfected Bcl-2 protein. A secondary goat anti-mouse rhodamine antibody (Molecular probes, Eugene, OR) was used to visualize the signals. Rhodamine red fluorescence imaging was accomplished under 40X using an Olympus IX 71-based deconvolving epifluorescence microscope.

2.5. ER calcium measurements

To detect free calcium in the endoplasmic reticulum (ER) lumen of cultured cells, an ER-targeted calcium indicator, D1ER cameleon (kind gift of Dr. Roger Tsien, University of California, San Diego) was transfected into the cells using lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were imaged 48 h after transfection using a time-lapsed FRET fluorescence imaging technique. Images were collected every 15 s for 5 min at *RT* and analyzed using SimplePCI software (Compix Inc., Sewickley, PA). For each D1ER expressed cell, the FRET535/CFP480 emission ratio proportional to the $[calcium]_{ER}$ was fixed arbitrarily as 1 at the start of the acquisition. Calibration, background and crosstalk were corrected according to the protocol developed by Dr. Tsien's laboratory [35,36].

2.6. Cytosolic calcium measurements

Cytosolic calcium changes were measured using the calcium dye Fluo4-AM, which was loaded into cells and calibrated using a modified procedure adapted from the manufacturer (Molecular Probes, Eugene, Oregon). Fluo4 imaging was accomplished using the above time-lapsed fluorescence imaging technique. Mean Fluo4 green intensity for 10–15 cells at each time point, measured by SimplePCI software, was fixed arbitrarily as 1 at the start of the acquisition to generate the intensity plot over time.

2.7. Apoptosis measurements

Changes in apoptosis levels of cultured cells, under different treatments, were measured by double labeling using an annexin V-fluorescein isothiocyanate (FITC, green) and propidium iodide (PI, red) apoptosis kit (Biovision, Mountain View, CA) according to the manufacturer's instructions. An Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL) was used to count the populations of living (no staining), early apoptotic (FITC green only), late apoptotic (both FITC green and PI red) and necrotic cells (PI red only) based on their fluorescent properties.

2.8. Statistical analysis

Data from this study was generated from three independent experiments. Apoptosis levels measured were represented as mean ± standard deviation (SD) in the figures. To show the calcium changes over time clearly, calcium curves were represented as mean values in the figures, and peak levels were shown as mean ± SD in the additional figures for comparison. Two-sided independent Student's *T*-test and one-way ANOVA (the analysis of variance) were used to compare two means or more than two means of one variable, respectively. If one-way ANOVA demonstrated unequal means, the Tukey–HSD (honestly significant difference) test was used to find which mean was different by conducting multiple pair-wise comparisons. The two-way ANOVA test was further performed to determine whether an interaction between two independent variables exists. For all analyses, differences with $p < 0.05$ were considered statistically significant and indicated with. Differences with $p < 0.01$ and $p < 0.001$ were indicated with ** and ***, respectively.

3. Results and discussion

The purpose of this study was to investigate whether ER calcium plays a key role in defining the relationship between paclitaxel's action and Bcl-2's resistance in breast cancer cells. After stable Bcl-2 transfectants were established from an endogenous Bcl-2 negative breast cancer cell line M468 (Fig. 1A and B), then the efficacy of paclitaxel in the presence of Bcl-2 protein was examined.

3.1. Paclitaxel can overcome the resistance of Bcl-2 to apoptosis depending on dosage

As shown in Fig. 1(C and D), while Bcl-2+ cells became more resistant to paclitaxel at the lower dose ($\sim 10^{-7}$ M) than control M468 cells ($p < 0.05$), they did not show any significant resistance to paclitaxel at the higher dose ($\sim 10^{-6}$ M). Unlike the dose-dependent change, the presence of Bcl-2 has no effect in a time-dependent manner for either dose since the apoptosis level in Bcl-2+ cells increased in a pattern similar to control M468 cells (two-way ANOVA showed $p = 0.21$ for low dose paclitaxel-induced apoptosis in Fig. 1(C), and $p = 0.99$ for higher dose paclitaxel-induced apoptosis in Fig. 1(D).

This result indicates that paclitaxel has the potential to overcome Bcl-2's resistance to apoptosis at a higher dosage, which raises two questions: (1) how does Bcl-2 protect cancer cells from apoptosis induced by paclitaxel at the lower dosage? (2) How can paclitaxel at the higher dosage overcome the anti-apoptotic action of Bcl-2 to induce significant apoptosis? To answer these questions, we initially tested whether Bcl-2 had an effect on the cell's ability

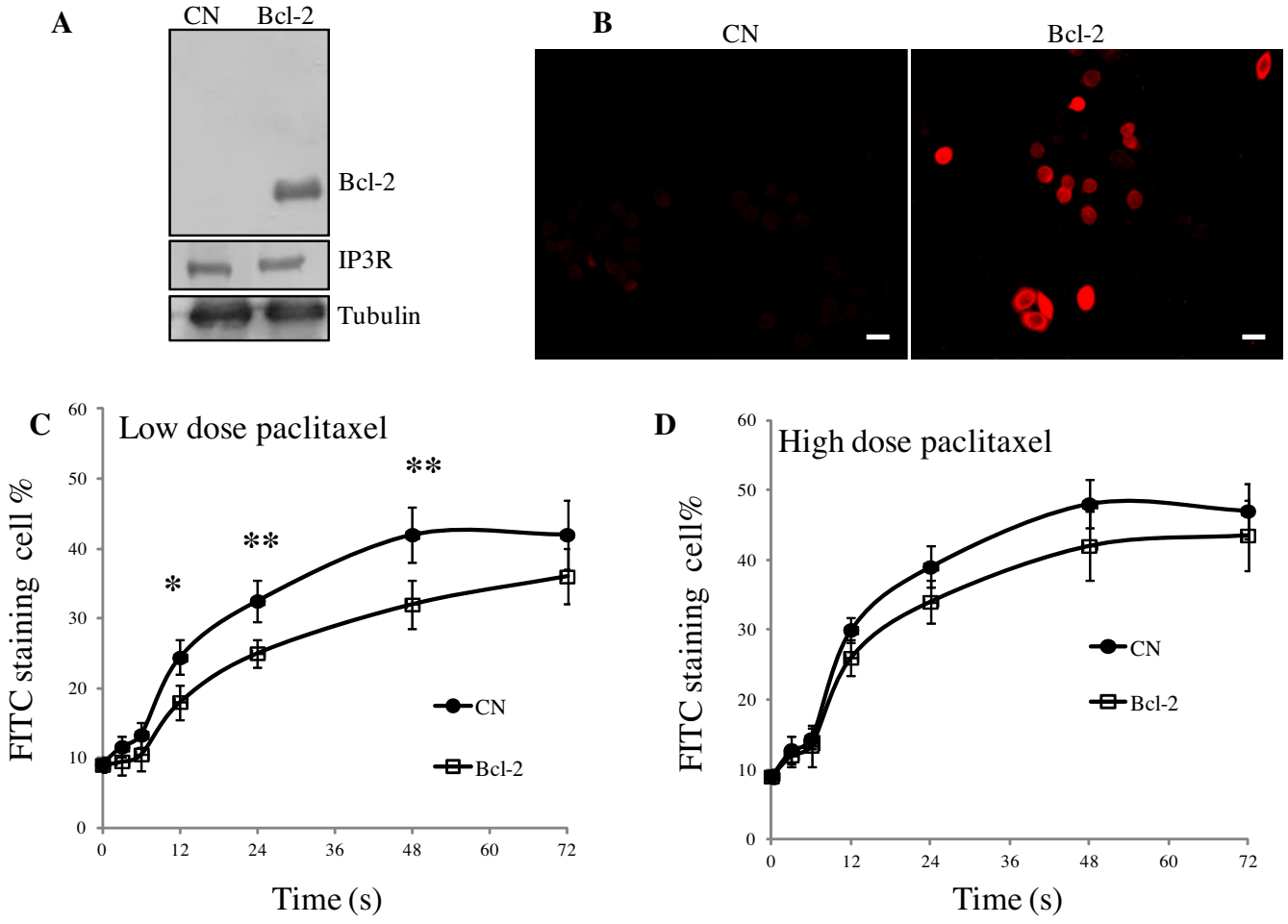

Fig. 1. The efficacy of paclitaxel in the presence of Bcl-2. (A) Western blot of Bcl-2 and IP3R expression in Bcl-2 transfected cells. (B) Immunocytochemistry analysis of Bcl-2 expression in transfected cells. Left panel = control cells, and right panel = Bcl-2 cells. Bar scale = 50 μm. (C) Low dose ($\sim 10^{-7}$ M) paclitaxel-induced apoptosis. (D) High dose ($\sim 10^{-6}$ M) paclitaxel-induced apoptosis. Two different doses of paclitaxel were applied for six different exposure times (3, 6, 12, 24, 48, and 72 h) in M468 control and Bcl-2+ cells. Total apoptosis was calculated as a percentage of annexin V-FITC stained cells in the population.

to release ER calcium into the cytosol and then examined the effects of paclitaxel on Bcl-2 associated calcium regulation.

3.2. Bcl-2 inhibits ER calcium release, which contributes to its apoptosis resistance

To detect Bcl-2-induced ER calcium release into the cytosol, thapsigargin (TG) was added to an extracellular calcium free medium. TG is a highly specific ER Ca^{2+} pump (SERCA) inhibitor, which irreversibly blocks calcium entry into the ER. Without the impact of the extracellular calcium pool and ER calcium refilling, the detectable changes in ER free calcium level induced by TG originated from ER calcium release into the cytosol, resulting in an increase in the cytosolic calcium level. The calcium changes in the ER and subsequently in the cytosol, were detected using D1ER and Fluo4 indicators, respectively. Results showed that cell's ability to release ER calcium into the cytosol is suppressed in the presence of Bcl-2, as described below.

First, in response to TG, the decrease in the ER free calcium level, determined by FRET/CFP ratio, was significantly lower in Bcl-2+ cells than in control cells (Fig. 2A and B $p < 0.001$). Since the calcium release from the ER into the cytosol was suppressed in Bcl-2+ cells, it was assumed that the amplitude of subsequent cytosolic calcium elevation was also decreased. This was confirmed through analysis of the cytosolic calcium indicator Fluo4. As shown in Fig. 2(C) and (D), the amplitude of the subsequent cytosolic calcium increase was lowered significantly in Bcl-2+ cells to only 65% of that observed in control M468 cells ($p < 0.05$). Taken together, the result confirmed that Bcl-2 expressing breast cancer cells' capability to release calcium from the ER in response to inducers is greatly reduced.

As discussed in the introduction, ER calcium release determines the cell's ability to undergo apoptosis through activating the downstream apoptotic pathways such as mitochondria sensitization and ER-associated enzyme activation. Therefore, the inhibited ER calcium release and subsequent decreased cytosolic calcium elevation in the Bcl-2+ cells have an inhibitory effect on induction of apoptosis, which is consistent with Bcl-2's resistance to the apoptosis inducers. However, if Bcl-2 can protect cells by lowering the ER sensitivity to apoptotic inducers, why is Bcl-2 not resistant to paclitaxel at higher doses? Thus, the next step was to determine whether paclitaxel could affect Bcl-2-mediated anti-apoptotic changes in ER calcium release.

3.3. Dependent upon dosage, paclitaxel directly stimulates pro-apoptotic ER calcium release in the presence of Bcl-2 and thus overcomes Bcl-2's inhibition on basal ER calcium release

Although the expression of Bcl-2 suppresses the cell's ability to release calcium from the ER, these results, as illustrated in Fig. 3(A) and (B), showed that paclitaxel at a high dose ($\sim 10^{-6}$ M) can still induce a significant ER calcium release, resulting in a rapid de-

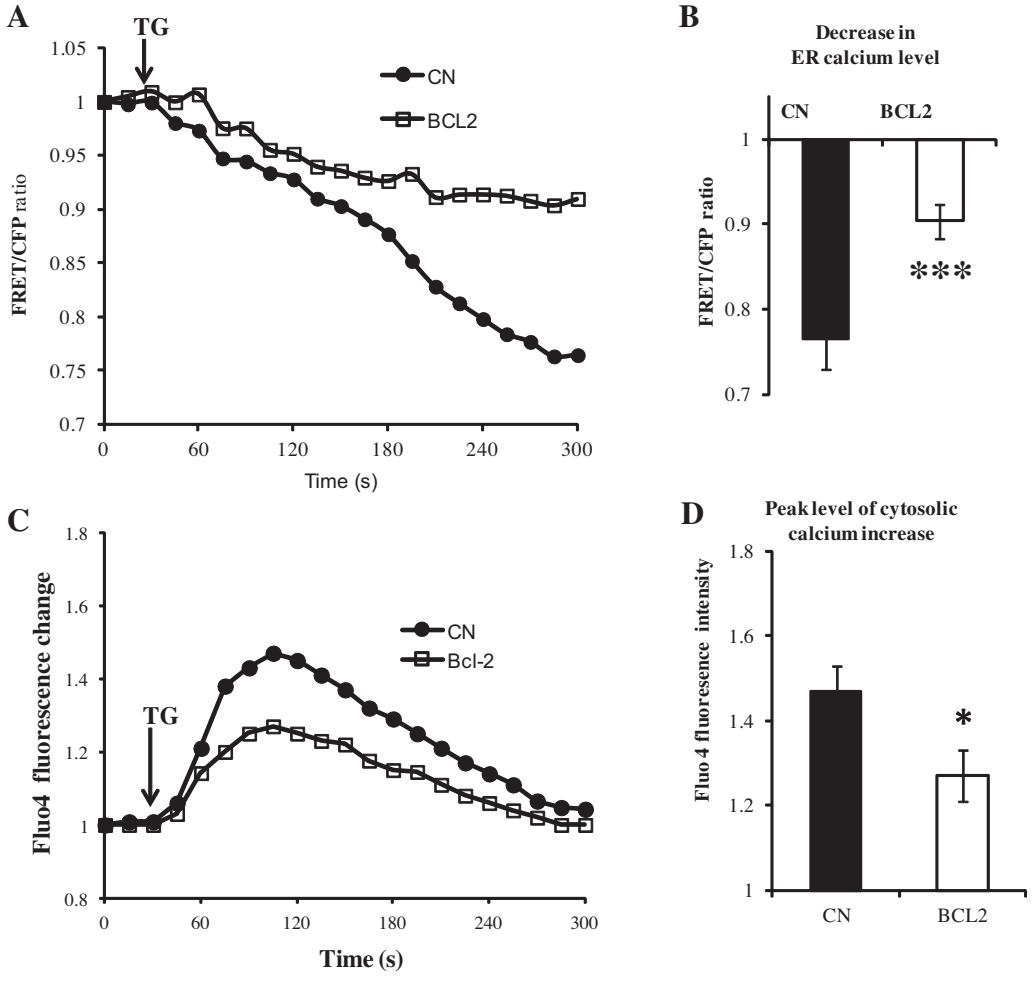

Fig. 2. The effects of Bcl-2 expression on ER calcium release into the cytosol. The arrows indicate the time points of TG (2 μM) treatment in the absence of external calcium (A) time–response curve of free ER calcium levels, (FRET/CFP ratio using D1ER cameleon). (B) The decrease in ER free calcium levels after TG addition. (C) Time–response curve of cytosolic calcium levels, (intensity of cytosolic calcium indicator Fluo 4). (D) Peak levels of cytosolic calcium elevation after TG addition.

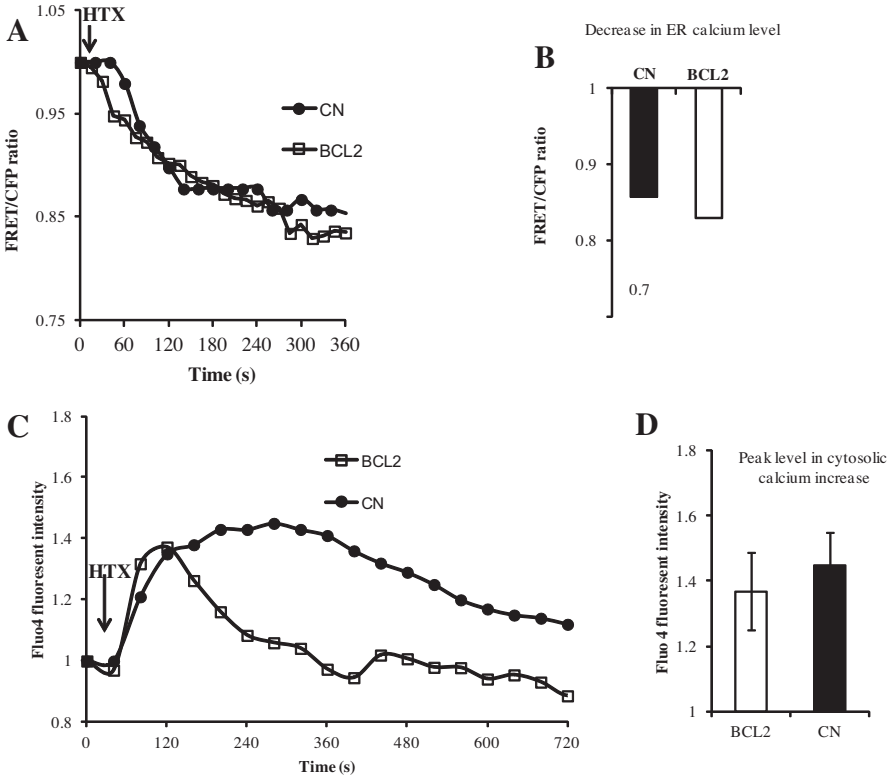

Fig. 3. The short-term effect of high dose paclitaxel on ER calcium release in the presence of Bcl-2 (A and C): time–response curves of free ER calcium levels, determined by FRET/CFP ratio in control and BCL2+ cells. (B) The decrease in ER free calcium levels upon addition of paclitaxel. (C) Time–response curve of cytosolic calcium levels, (determined by intensity of cytosolic calcium indicator Fluo 4), reflects release of calcium from the ER to the cytosol after exposure to paclitaxel. (D) Peak levels of ER calcium release upon addition of paclitaxel.

crease in the ER calcium levels, similar to control cells. No significant difference in the amplitude of these paclitaxel-induced ER calcium changes was observed between Bcl-2+ cells and control cells, indicating that paclitaxel's direct effect on ER calcium stores can overcome Bcl-2's inhibition of ER calcium release. Paclitaxel-induced ER calcium release also results in a cytosolic calcium elevation, without significant difference in amplitude between Bcl-2+ (40%) and control cells (50%) (Fig. 3C and D). In contrast, a low dose of paclitaxel ($\sim 10^{-7}$ M) only induced small, slow changes in both control and Bcl-2+ cells, and was not significant from either regular medium or DMSO vehicle controls (data is not shown). This dose-dependent paclitaxel-induced stimulation of ER calcium explains why paclitaxel at a high dose can remove Bcl-2's inhibition, while a lower dose cannot.

In addition to the short-term effects, the capability of Bcl-2+ cells to release ER calcium into the cytosol after long-term paclitaxel treatment was also tested using the TG method. As shown in Fig. 4(A) and (C), remarkably, treatment of high dose paclitaxel over hours restores the cells' ability to release ER calcium into the cytosol. This is indicated by the gradual increase in amplitude of TG-induced ER calcium release ($p < 0.05$ for 6 h treatment, and $p < 0.01$ for 12 h treatment). In contrast, the lower dose of paclitaxel cannot counteract the intensity of the inhibitory effect of Bcl-2 on the ER calcium release as high dose paclitaxel did (Fig. 4B). The amplitude of TG-induced ER calcium release was not increased until 12 h treatment (Fig. 4D), at which time significant apoptosis was already induced (Fig. 1C). Interestingly, low dose paclitaxel can moderately prolong the duration of cytosolic calcium recovery time. During this attenuated recovery time, cytosolic calcium levels are above baseline (20% after 6 h and 35% after 12 h after reaching amplitude (Fig. 4C, $p < 0.01$). This keeps released ER calcium in the cytosol for a longer period, potentially increasing the susceptibility of the cells to apoptosis. However, this change is not markedly effective since Bcl-2+ cells were still significantly resistant to paclitaxel treatment at lower doses.

Based on this study, the relationship between paclitaxel and Bcl-2 on ER calcium regulation can be summarized as follows: expression of Bcl-2 can suppress the cell's ability to release ER calcium. This protects the cell from releasing too much ER calcium and thus inhibits the induction of subsequent apoptosis. Meanwhile, the ER calcium store also serves as an important direct target for paclitaxel at higher dosage, and Bcl-2 expression has no effect on this dose-dependent action of paclitaxel. Therefore, paclitaxel at the higher dose is capable of releasing significant ER calcium to overcome Bcl-2's inhibitory effect and thus attenuate Bcl-2 resistance.

Our results showed that the ER calcium store is a key common target for paclitaxel and Bcl-2, since both can directly change ER calcium release independent of each other. This direct ER calcium involvement, linking the actions of palitaxel and Bcl-2 together, helps elucidate the underlying mechanisms of the paclitaxel–Bcl-2 relationship in a novel way, outside of the previously described, indirect ER calcium regulation through the phosphorylation of Bcl-2 by paclitaxel [37–40]. Furthermore, the dosage of paclitaxel is crucial for inducing significant ER calcium release to overcome Bcl-2-mediated apoptotic resistance. Therefore, our findings not only help clarify the controversy regarding the efficacy of paclitaxel in the presence of Bcl-2 expression, but may also assist oncologists in optimizing the usage of paclitaxel in breast cancer treatment. More research is needed to fully understand the complex relationship between paclitaxel, calcium and Bcl-2.

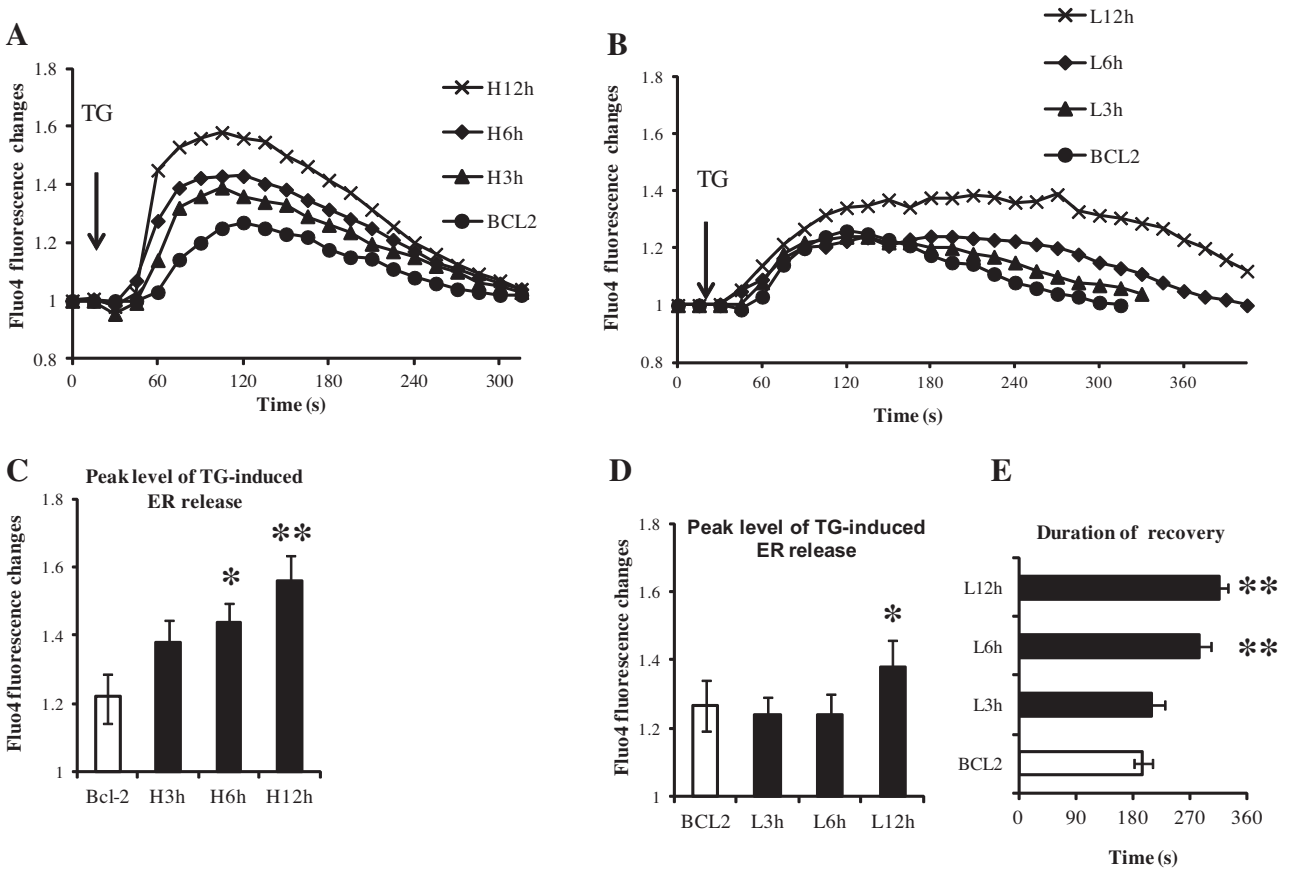

Fig. 4. The effect of long-term paclitaxel on ER calcium release into the cytosol in the presence of Bcl-2. (A and B) Time–response curve of cytosolic calcium levels upon TG addition in cells treated with high and low dose paclitaxel, respectively. After 3 h, 6 h and 12 h exposures to paclitaxel, treated Bcl-2+ cells were loaded and incubated with Fluo 4. After the incubation, cells were washed and kept in calcium free medium to remove the effect of the extracellular calcium pool. Arrows shows the addition of TG to determine the ER calcium release into the cytosol in the paclitaxel-treated sample. (C and D) Peak levels of ER calcium release. For curve (B), (E) is the duration of recovery time.

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