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Involvement of Endoplasmic Reticulum in Paclitaxel-Induced Apoptosis

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Abstract The participation of the mitochondrial pathway in paclitaxel-induced apoptosis has been well documented. After addition of paclitaxel to U937 cells, however, we observed an early expression of five endoplasmic reticulum (ER) stress response genes that preceded the release of cytochrome *c* from the mitochondria and the cleavage of the caspases. Involvement of the ER was supported by the following evidence. Paclitaxel treatment not only activated calpain and caspase-4, but also induced a gradual increase in the cytosolic Ca^{2+} concentration at 3–6 h. Paclitaxel-induced apoptosis can be inhibited by the calpain inhibitor calpeptin and IP_3 receptor inhibitors. Either buffering of the cytosolic Ca^{2+} or inhibition of mitochondrial calcium uptake reduced BiP expression. These inhibitors also reduced mitochondrial apoptotic signals, such as mitochondrion membrane potential disruption, cytochrome *c* release and eventually reduced the death of U937 cells. Paclitaxel-induced Bax/Bak translocation to the ER and Bax dimerization on the ER membrane occurred within 3 h, which led to a Ca^{2+} efflux into cytosol. Moreover, we found that cytochrome *c* translocated to the ER after releasing from mitochondria and then interacted with the IP_3 receptor at 12–15 h. This phenomenon has been known to amplify apoptotic signaling. Taken together, ER would seem to contribute to paclitaxel-induced apoptosis via both the early release of Ca^{2+} and the late amplification of mitochondria-mediated apoptotic signals. J. Cell. Biochem. 104: 1509–1523, 2008. © 2008 Wiley-Liss, Inc.

Key words: paclitaxel; ER; apoptosis; calcium; U937 cell

Apoptosis is a highly regulated death process that occurs during development, tissue homeostasis and as a defense against pathogens [Thompson, 1995]. It is well known that the apoptotic cascades can be triggered by either extrinsic receptor-mediated or intrinsic mitochondria-mediated signaling pathways. Among them, the mitochondria play a major role. These organelles releases cytochrome *c* and apoptotic inducing factor (AIF) into the cytosol and this leads to the apoptotic process [Green and Reed, 1998]. However, recent studies have suggested that the endoplasmic reticulum (ER) also participates in execution of apoptosis as another subcellular player [Rao et al., 2001; Oyadomari et al., 2002].

The ER is well known to regulate protein synthesis, protein folding, cellular responses to stress and intracellular calcium (Ca^{2+}) levels. Alterations in Ca^{2+} homeostasis or accumulation

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Abbreviations used: ER, endoplasmic reticulum; UPR, unfolded protein response; AIF, apoptotic inducing factor; IP_3R, inositol 1,4,5-trisphosphate receptor; RT-PCR, reverse transcription-polymerase chain reaction; $\Delta\psi_m$, mitochondrial membrane potential; PTP, permeability transition pore; tBid, truncated BCL-2 interacting domain; Ire1, inositol-requiring ER-to-nucleus signal kinase 1; PERK, RNA-dependent protein kinase-like ER kinase; sXBP1, spliced X-box binding proteins-1; HO-1, Heme oxygenase-1; NCS-1, neuronal calcium sensor 1; DMSO, dimethyl sulfoxide; RR, ruthenium red; BMH, bismaleimidohexane; PBST, phosphate buffered saline with Tweens; FITC, fluorescein isothiocyanate.

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of misfolded proteins in ER can cause ER stress and ultimately lead to apoptosis [Ermak and Davies, 2002]. Increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$) occur at both early and late stages of the apoptotic pathway [Tombal et al., 1999; Masuda et al., 2007]. ER mediates apoptosis via several different mechanisms. Among these, exchange of calcium between the mitochondria and the ER plays a major role. Stimulus generating inositol 1,4,5-trisphosphate (IP_3) causes Ca^{2+} release from the ER and the released Ca^{2+} is rapidly taken up by the closely juxtaposed mitochondria. The calcium that is taken up decreases the mitochondrial membrane potential and releases cytochrome *c*, which consequently activates various apoptotic pathways [Oakes et al., 2005]. Another mechanism has been suggested whereby the ER itself is capable of initiating cell death signals. Caspase-12, localizing on the cytosolic face of the ER, is able to induce apoptosis in response to prolonged ER stress in mice [Nakagawa and Yuan, 2000]. In humans, the ER-mediated killing role of caspase-12 has been found to be replaced by caspase-4 and caspase-4 has recently been identified as a mediator of ER stress [Fischer et al., 2002; Hitomi et al., 2004].

One more possible mechanism of ER-mediated apoptosis is associated with endogenous Bcl-2 family members including Bcl-2, Bcl-xL, Bax, Bak and Bik [Ng et al., 1997; Wei et al., 2001; Germain et al., 2002]. These proteins are key players in apoptosis. Overexpression of Bax and Bak leads to Ca^{2+} efflux from the ER. The process, by which an influx of Ca^{2+} into the mitochondria increases cytochrome *c* release and leads to cell death, can be inhibited by Bcl-2 [Ng et al., 1997]. Bax/Bak double deficient mouse embryonic fibroblasts are resistant to both mitochondrial-mediated apoptosis and ER stress stimuli [Scorrano et al., 2003]. These investigations suggest that there is crosstalk between the ER and the mitochondria and that this is involved in the regulation of cell death.

Paclitaxel is currently used in the treatment of patients with various malignant diseases and leukemia [Munker et al., 1998; Crown et al., 2004; Lee et al., 2006]. This drug binds microtubules, stabilizes microtubule dynamics and arrests the cell at the mitotic phase [Jordan and Wilson, 1998]. It has been proposed that the mitotic arrest trigger the mitotic spindle checkpoint, which somehow induces a mitochondrial permeability transition, a release of prodeath molecules into the cytosol and eventually a caspase-dependent apoptosis of neoplastic cells [Jordan et al., 1996; Wang et al., 2000]. In spite of extensive investigation, how this microtubule polymerizing agent initiates apoptosis remains incompletely understood. Whether ER stress contributes to the cytotoxicity of paclitaxel against tumor cells is also unclear. Using leukemic cells, the apoptotic phenomena can be observed in more detail under submicromolar concentration of paclitaxel [Lieu et al., 1997; Wan et al., 2004; Bozko et al., 2005]. For example, we have shown that paclitaxel-induced apoptosis in leukemia cells can be triggered at different cell cycle stages through different mechanisms [Liao and Lieu, 2005]. In the present study, we investigated the role of ER stress in paclitaxel-induced apoptosis by employing the leukemic cell line U937. We demonstrated that, in addition to the mitochondrial death pathway, paclitaxel can exert its cytotoxicity via induction of ER stress. This provides further insights into the mechanisms of how paclitaxel induces cell death.

MATERIALS AND METHODS

Cell Line and Reagents

Human histocytic lymphoma U937 cells were maintained in PRMI 1640 medium (GIBCO Laboratory, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 100 units/ml of penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES and 2 mM L-glutamine (complete medium) at 37°C and 5% CO_2 atmosphere. Exponentially growing cells were used for all experiments. The Western-blotting detection kit was obtained from Amersham International (Buckinghamshire, UK). The mitochondria marker (Clone MTC02) was purchased from Lab Vision (Fremont, CA). All other chemicals were purchased from Sigma Chemical Co. Paclitaxel and caspase inhibitors were dissolved in dimethyl sulfoxide (DMSO) to make a 10 mM stock, which was than diluted to desired concentration with complete medium. DMSO concentrations were kept under 0.5% in all experiments.

Immunofluorescence

After treatment with or without paclitaxel, U937 cells were cytospun onto a slide then fixed and permeabilized by 2% paraformaldehyde for 15 min at room temperature. Next, the slides

were washed and incubated with 0.1% BSA for 30 min at room temperature. Cells were then washed with PBS and incubated with rabbit anti-AIF or anti-cytochrome *c* monoclonal antibody (1:20) for 30 min at 37°C. The first antibody was then removed, goat anti-rabbit IgG antibody conjugated to FITC (1:200) in 0.1% normal BSA was added and the slides incubated for 30 min at room temperature. Fluorescent images were acquired through a fluorescence microscope. Images showing diffused staining were identified as AIF or as having cytochrome *c* translocation.

Western Blotting

Cells were incubated in the presence or absence of paclitaxel for various periods. After treatment, 2×10^6 cells were washed with PBS for three times and lysed in 100 μl of sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.002% bromophenol blue and 10% glycerol). The samples were boiled for 5 min and then centrifuged at 12,000 rpm for 3 min. Protein extracts were subjected to electrophoresis on an SDS–polyacrylamide gel and then transferred onto nitrocellulose membrane. The blots were blocked for 1 h in PBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 3% nonfat dried milk and then probed overnight with an appropriate dilution of the primary antibody. Reactions were detected with a suitable secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence kit (Amersham).

Reverse Transcription (RT)-PCR

Total RNA was prepared from U937 cells by lysing the cells in Trizol reagent (Invitrogen, Carlsbad, CA). RNA quality was evaluated by measuring the absorbance reading at 260 and 280 nm and by electrophoresis on agarose gels. Aliquot of RNA was subjected to reverse transcription using a random hexamer primer and MMLV reverse transcriptase (Fermentas, Lithuania) to synthesize cDNA. The reaction was carried out for 1 h at 42°C, followed by inactivation for 10 min at 95°C and cooling to 4°C. The cDNA was then amplified by 20 cycles of PCR using Taq polymerase (Fermantas). PCR products were subjected to electrophoresis through 1% agarose gels. The ethidium bromide-stained gels were then scanned and band density was calculated. The following sets of primers were used for the PCR amplifications: for BiP, 5′-ATCACGCCGTCCTATGTCGC-3′ and 5′-TCT-CCCCCTCCCTCTTATCC-3′; for CHOP, 5′-AGT-CATTGCCTTTCTCTTCG-3′ and 5′-GGTGCA-GATTCACCA TTCGG-3′; for sXBP-1, 5′-GG-AGTTAAGACAGCGCTTGG-3′ and 5′-ACTG-GTCCAAGTTGTCCAG-3′; for GADD34, 5′-TC-CTGGGAGTATCGTTCAGG-3′ and 5′-CAGG-GAGGACACTCAGCTTC; for ATF4, 5′-TC-AAACCTCATGGGTTCTCC-3′ and 5′-GTGTC-ATCCAACGTGGTCAG-3′ and for G3PDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCC-AC CACCCTGTTGCTGTA-3′.

Intracellular Calcium Measurement

After treatment with paclitaxel (100 μM) for various periods, Fluo-3 AM (5 μM) was added to the U937 cells and the cells incubated for another 45 min. The cells were then harvested, washed twice with DPBS (PBS without calcium) and resuspended in 1 ml of DPBS. The mixture was immediately analyzed by FACS or cytospun onto the slides and observed using a fluorescence microscope.

Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$)

A reduction in mitochondrial membrane potential following drug treatment was monitored by flow cytometry. The $\Delta\psi_m$ was estimated by staining 1×10^6 cells with 40 μM $DiOC_6$ (Molecular Probes), a cationic lipophilic dye, for 15 min at 37°C in the dark and then the cells were subjected to flow cytometry (excitation 488 nm; emission 525 nm; recorded in FL-1). The fluorescence of $DiOC_6$ is oxidation-dependent and correlates with $\Delta\psi_m$. Cells without treatment were processed as the control. Mitochondrial damage was recognized by the presence of cells displaying "low" uptake of $DiOC_6$.

Subcellular Fraction Preparation

After treatment with paclitaxel, U937 cells were harvested and washed once with cold PBS. The washed pellet was subjected to one freeze–thaw cycle in liquid nitrogen and then was resuspended in 1 ml buffer A 250 mM sucrose, 10 mM Tris–HCl at pH 7.5, 1 mM EGTA, 1mM phenyl methylsulphonyl fluoride (PMSF) and protease inhibitor cocktail (Calbiochem, Darmstadt, Germany). Samples were transferred to Eppendorf tubes and centrifuged at 1,000*g* for 15 min at 4°C to eliminate nuclei and unbroken cells. The resulting supernatant was further

centrifuged at 10,000*g* for 15 min at 4°C, to yield a 10,000*g* pellet (mitochondria fraction). The 10,000g supernatant was then again centrifuged at 100,000*g* for 1 h at 4°C. The 100,000*g* supernatant was collected as the cytosolic fraction and the pellet (ER fraction) was resuspended in 40–70 μl of buffer A.

Immunoprecipitation

After various treatments, 5×10^6 cells were washed twice with cold PBS, resuspended in lysis buffer (14.5 mM KCl, 5 mM MgCl, 10 mM HEPES pH7.2, 1 μM EGTA, 0.2% NP-40, 0.2 μM PMSF, 0.1% aprotinin, 0.7 μg/ml pepstatin and 1 μg/ml leupeptin) and incubated for 30 min. Lysed samples were centrifuged at 15,000*g* for 10 min to remove nuclei and cellular debris. Lysates were precleared with 50% protein-G Sepharose (v/v) for 3 min and centrifuged at 400*g* for 2 min. The antibody specific to the various different proteins was added for 90 min and the immunocomplex was captured by addition of 50% protein-G Sepharose for 60 min. After centrifugation, the precipitate was resuspended in SDS–PAGE sample buffer and subsequently resolved by 10% SDS–PAGE.

RESULTS

Paclitaxel Induces the Mitochondrial Apoptotic Pathway

Activation of the mitochondrial death pathway can be identified by releasing of mitochondrial cytochrome *c* and AIF and also by cleavage of caspases. To confirm that our paclitaxel treatment protocol caused release of cytochrome *c* and AIF, we detected cytochrome *c* and AIF using an immunofluorescence method. After treatment with 100 μM paclitaxel for 12 h, the U937 cells showed fragmented nucleus and a diffuse staining for cytochrome *c* and AIF in cytosol was found as shown in Figure 1a. The diffused pattern indicated that these molecules were released from mitochondria to cytosol. In contrast, a punctuated mitochondria-located fluorescence signal was observed in untreated cells. In the kinetic study, about 17% of the cells exhibited the diffused pattern after 12 h of paclitaxel treatment and this increased to about 40% after 18 h of treatment. Figure 1b shows that the translocation of AIF and cytochrome *c* took place to the same extent over this time period. After released from the mitochondria, cytochrome *c* can bind to dATP and apoptotic protease-activating factor-1 (Apaf-1) and activate caspase-9 and caspse-3. Whether paclitaxel can induce cleavage of caspase-3, caspase-9, caspase-8, PARP and Apaf-1 was further examined. Figure 1c shows that the caspase-3 proenzyme (32 kDa) was cleaved and the product (17 kDa) appeared after 9–12 h of drug treatment. The initiator caspase-9 was also activated in 6–9 h after paclitaxel treatment and this could be recognized by loss of its pro-enzyme form. The caspase-3 target PARP was cleaved to its 89 kDa product in about 12 h. Cleavage of caspase-8, a crucial mediator of death receptor signaling, was also observed in 12 h. In contrast, paclitaxel has little effect on Apaf-1 expression. Taken together, our results indicated that under our treatment protocol paclitaxel activated profound mitochondria-dependent death events after 6–9 h.

Paclitaxel Administration Causes ER Stress in U937 Cells

To examine whether ER stress is involved in paclitaxel-induced cellular death, we detected a number of marker substances that always express in ER-induced apoptosis and unfolded protein response (UPR). These markers include activation of the protease caspase-4 and calpain, activation of the transcription of stress response genes such as GRP78/BiP (glucose-regulate protein/immunoglobulin heavy chain binding protein), CHOP/GADD153 (c/EBP homology protein/growth arrest and DNA damage), the ATF4 (activating transcription factor 4), GADD34 and sXBP-1 (spliced X-box binding protins-1) and phosphorylation of JNK (c-jun N-terminal kinase).

Human caspase-4 has been suggested as the counterpart of murine caspase-12, which can be activated by ER stress. We thus examined whether caspase-4 was activated in U937 cells after paclitaxel treatment. As shown in Figure 2a, paclitaxel diminished the amount of inactive caspase-4 and increased the cleaved product. In addition, the paclitaxel-induced calpain cleavage was also found at 9 h.

The UPR pathway induces expression of the ER chaperone BiP and the transcription factor CHOP. We thus measured both mRNA and protein expression of *BiP* and *CHOP* after paclitaxel treatment. As shown in Figure 2b, both the *BiP* and *CHOP* mRNA were increased at 1–3 h after paclitaxel treatment, which is

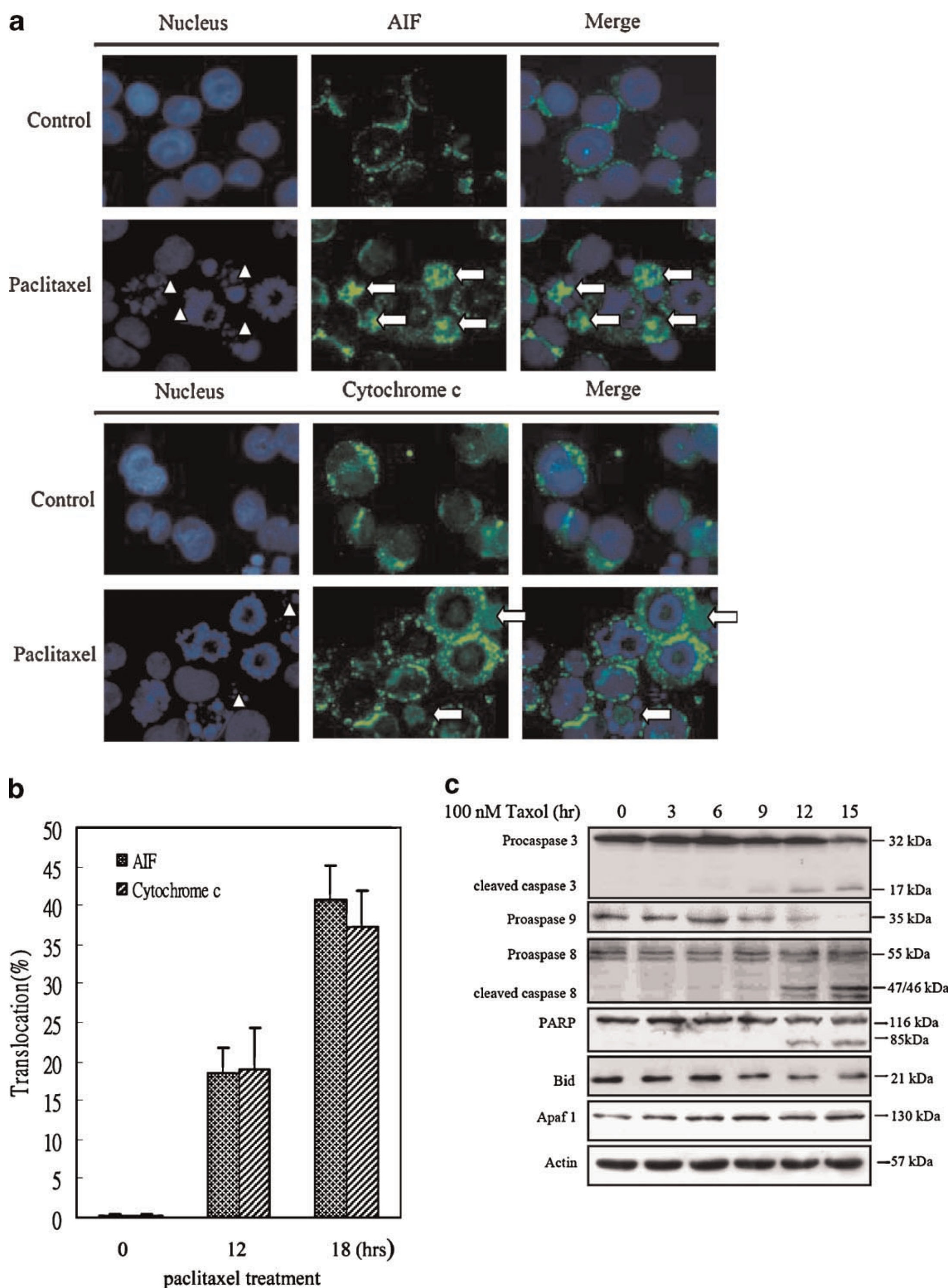

Fig. 1. The paclitaxel-induced mitochondria-mediated apoptotic pathway. **a**: Cells were treated with 100 μM paclitaxel for 12 h. After treatment, the cells were immunostained with an antiserum specific for AIF or cytochrome *c*. Cell nuclei were counterstained with Hoechst 33342 (blue) and observed by fluorescence microscopy (40× magnification). Arrowheads identify apoptotic nucleus. Arrows indicate translocation of cytochrome *c* or AIF. **b**: Kinetics of paclitaxel-induced AIF and cytochrome *c* translocation in U937 cells. Data are means ± SD from three independent experiments. **c**: U937 cells were exposed to 100 μM paclitaxel for the indicated time. At the end of treatment, the treated cells were subjected to SDS–PAGE and the presence of caspase-3, caspase-9, caspase-8, PARP, Bid and Apaf 1 detected. The loading control was actin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

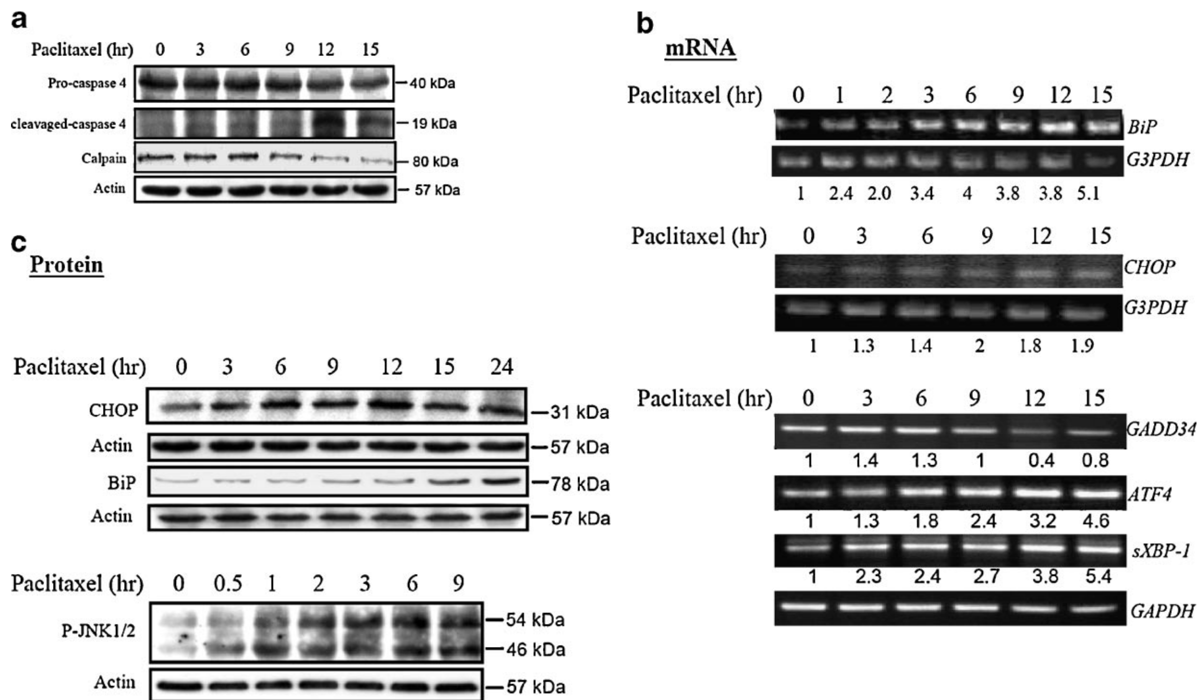

Fig. 2. Paclitaxel treatment induced ER stress in U937 cells. **a**: U937 cells were treated with 100 μM paclitaxel for indicated times and then the caspase-4 and calpain were detected by immunoblot analysis. Actin was used as a loading control. **b**: Paclitaxel-induced expressions of *BiP, CHOP, GADD34, ATF4* and *sXBP-1* mRNA levels for cells after the same treatment as in (a). *G3PDH* was used as an internal control. The density of bands was quantified using ImageQuant software. The cDNA levels of *BiP, CHOP, GADD34, ATF4 and sXBP-1* were normalized against *G3PDH* and are shown under each band. **c**: Western analysis showed increases in CHOP, BiP and phospho-JNK. All of the experiments were conducted three times and a representative result is shown.

much earlier than the release of cytochrome *c*. The increases of CHOP and BiP protein were observed at 6 and 12 h, respectively, as shown in Figure 2c. In addition, activation of three sensor proteins, namely inositol-requiring ER-to-nucleus signal kinase 1 (Ire1), activating transcription factor 6 (ATF6) and RNA-dependent protein kinase-like ER kinase (PERK), was detected via monitoring of their downstream molecules. Activation of ATF6 induced expression of XBP1 mRNA and the activated Ire1 spliced a 26 nucleotide-fragment out of the XBP1 mRNA and formed the sXBP1 (frameshift spliced product of XBP1) product. In Figure 2b, the sXBP1 mRNA can be seen to increase over the first 3 h of paclitaxel exposure. This indicates that both ATF6 and Ire1 were activated at 3 h. Another UPR pathway, namely activation of the PERK pathway, can be identified by the presence of its target molecules, the protein GADD34 and the transcription factor ATF4. The mRNA of both molecules increased during our treatment protocol.

The JNK cascade is also known to be activated in ER stress-induced apoptosis. In Figure 2c, JNK was found to be phosphorylated at 3 h of treatment and the phosphorylation increased with time. All of these results confirm that paclitaxel induces ER stress after 1–3 h of treatment.

Buffering of Cytosolic Ca^{2+} in Paclitaxel-Treated U937 Cells Reduces Apoptotic Cell Death

Uncontrolled cytosolic Ca^{2+} overload is a common cause of cell death in several pathological conditions and calcium dependent calpain has been found activated after treatment with paclitaxel. We analyzed the intracellular calcium to determine whether paclitaxel caused cytosolic Ca^{2+} overload. U937 cells were treated with 100 μM paclitaxel for 3 and 6 h and then loaded with the Ca^{2+}-sensitive dye Fluo-3 AM to measure cytosolic Ca^{2+} levels. Fluorescence was detected by flow cytometry or fluorescence microscopy as shown in Figure 3a. Compared to untreated cells, treatment with paclitaxel

resulted in a time-dependent increase in cytosolic Ca^{2+}. Figure 3b shows that the increase was independent of the extracellular Ca^{2+} since EGTA treatment had no blocking effect. To determine whether calcium plays an essential role in the progression of paclitaxel-induced cell death, we employed two calcium inhibiting agents. BAPTA-AM reduces the intracellular Ca^{2+} concentration and ruthenium red (RR) inhibits mitochondrial Ca^{2+} uptake. It is evident that BAPTA-AM reduced the amount of paclitaxel-induced BiP protein in Figure 3c. This indicates that cytosolic calcium overload is imperative to paclitaxel-induced ER response. In Figure 3d, we observe that both inhibitors significantly reduced the level of apoptosis. It suggests that an increase in the calcium concentration of either the cytosol or mitochondria is essential to the death process induced by paclitaxel.

Reduction of the Mitochondrial Calcium Uptake Inhibits Paclitaxel-Induced Apoptotic Events

It has been demonstrated that mitochondrial calcium overload activates the permeability transition pore (PTP), which leads to transient depolarization of the mitochondria and this causes the release of pro-apoptosis proteins. We assessed these events in cells pretreated with either BAPTA-AM or RR and then treated with 100 μM paclitaxel for 12 h. As shown in Figure 4a, about 16% of cells showed a decrease in $\Delta\psi_m$ after paclitaxel treatment for 12 h. About 20% cells showed cytochrome *c* and AIF releases over the same period as seen in Figure 4b. Pretreatment with either BAPTA-AM or RR significantly reverses these apoptotic events.

We next examined the paclitaxel-induced mitochondrial downstream events after inhibition of calcium uptake. The Western blot in Figure 4c indicates that the cleavage of procaspase-9 was reduced after pretreatment with RR and BAPTA-AM. Similar results were observed for the activation of caspase-3. These results indicate the calcium signals are essential to the mitochondria-mediated pathway induced by paclitaxel.

Inhibition of Calpain Activity Reduces Paclitaxel-Induced Cell Apoptosis

It was noted that calpain was activated during paclitaxel-induced apoptosis as is shown in Figure 2a. We then treated U937 cells with paclitaxel for 12 and 15 h in the presence of calpeptin, an inhibitor of calpain. Calpeptin inhibited 60% of the apoptosis at 15 h as shown in Figure 4d. This demonstrates that the calpain is a major mediator in the apoptosis induced by paclitaxel.

Paclitaxel Induces Accumulation of Bax and Bak in ER

We next studied how the ER is involved in the paclitaxel-induced apoptosis. It is possible that paclitaxel induces calcium efflux from the ER. It has been reported that when ER stress occurs, Bak and Bax are localized to the ER membrane, undergo conformational changes and this permits Ca^{2+} efflux into cytosol [Boehning et al., 2003; Scorrano et al., 2003]. We examined whether these two proteins were translocated to the ER during paclitaxel-induced apoptosis. U937 cells were treated with paclitaxel for 3, 6 and 9 h. ER fractions were isolated and the Bcl-2 family proteins were determined by Western blot analysis. As shown in Figure 5a, the amounts of Bax and Bak at the ER increased after 3–6 h of treatment. The increased Bax/Bak might not be a result of new protein synthesis because we have previously demonstrated that paclitaxel does not increase amount of Bax and Bak proteins after 24 h of our treatment protocol [Liao and Lieu, 2005]. This suggests that paclitaxel induces Bax/Bak translocation to the ER. At the same time, paclitaxel also induced a phosphorylation shift of Bcl-2 and increased the amount of truncated BH3-only protein Bid (tBid) at ER membrane. This suggests that paclitaxel also reduce Bcl-2 activity at the ER by post-translational modification.

ER stress induces conformational changes and oligomerization of the proapoptotic proteins Bax and Bak on the ER membrane. This may lead to a lesion of the ER membrane integrity and cause Ca^{2+} efflux out of the ER [Scorrano et al., 2003]. Therefore, we examined whether these Bax or Bak proteins form dimers on the ER membrane. After paclitaxel treatment, a sulfhydryl-reactive cross-linker bismaleimidohexane (BMH) was used to cross-link the oligomerized proteins. The ER fraction was collected and examined for Bax oligomerization. We found a slow mobility band, which seems to be associated with the Bax-containing protein complex (~43 kDa), and this appeared at 3 h treatment. This is probably the Bax dimer form since its molecular weight is about twice of the

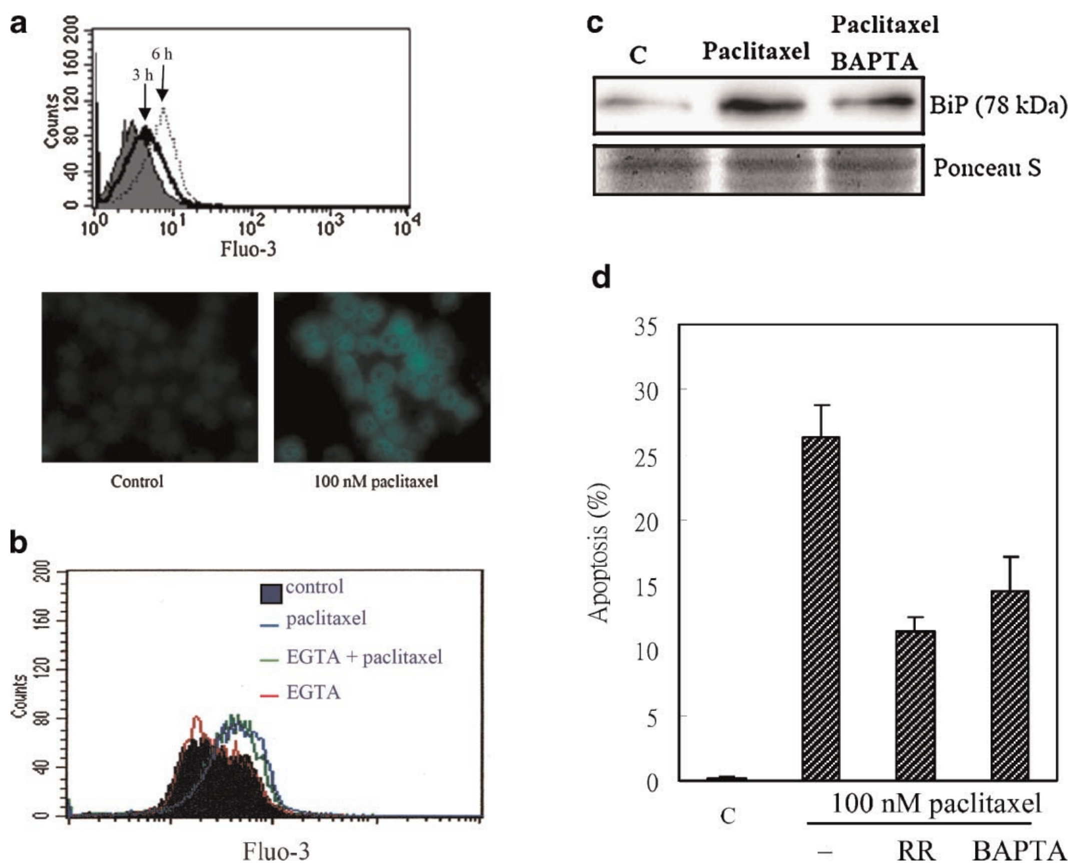

Fig. 3. Paclitaxel-induced an increase in cytosolic calcium concentration in U937 cells. **a**: U937 cells were treated with 100 μM paclitaxel for 3 and 6 h. After treatments, the cells were incubated with Fluo-3AM and subjected to flow cytometry. The solid line represents 3 h of treatment and the dotted line indicates 6 h of treatment. The shadow peak represents untreated control cells. Lower panel shows the fluorescence in treated and untreated cells detected by immuno-fluorescence. **b**: The calcium levels of U937 under the paclitaxel treatment for 6 h in the presence and absence of EGTA. **c**: Cells were pretreated with 2 μM BAPTA-AM for 30 min and incubated with 100 μM paclitaxel for 12 h. The BiP protein was determined by immunoblotting equal protein loading was confirmed by staining the membranes with Ponceau S. **d**: U937 cells were pretreated with 50 μM RR or 2 μM BAPTA-AM for 30 min and then treated with 100 μM paclitaxel for 15 h. The apoptotic cells were determined by morphological analysis. At least 200 cells were analyzed for each treated group. Error bars indicate ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

monomer Bax protein in Figure 5b. However, we cannot rule out the existence of a heterodimer of Bax with other protein(s).

To further confirm whether the dimer form of Bax increased in ER after paclitaxel treatment, we employed a monoclonal antibody 6A7; this has been demonstrated to show specificity against activated multimer forms of Bax [Hsu and Youle, 1997]. As shown in Figure 5c, after treatment with paclitaxel, the active form of Bax that was present in the ER fraction significantly increased after 3–9 h. This supports the hypothesis that paclitaxel induces Bax dimerization, which can cause efflux of Ca^{2+} out of the ER.

Cytochrome *c* Binds to Inositol (1,4,5) Trisphosphate Receptors on the ER During Paclitaxel-Induced Apoptosis

There is a known crosstalk between the ER and mitochondria whereby cytochrome *c* released from mitochondria may induce Ca^{2+} efflux from ER through the IP_3 receptor (IP_3R). This generates a positive feedback loop, whereby Ca^{2+} causes the mitochondrial permeability transition and stimulates further cytochrome *c* release. To examine whether this cytochrome *c*-mediated crosstalk also plays a role in paclitaxel-induced apoptosis, we detected the cytochrome *c* translocation process

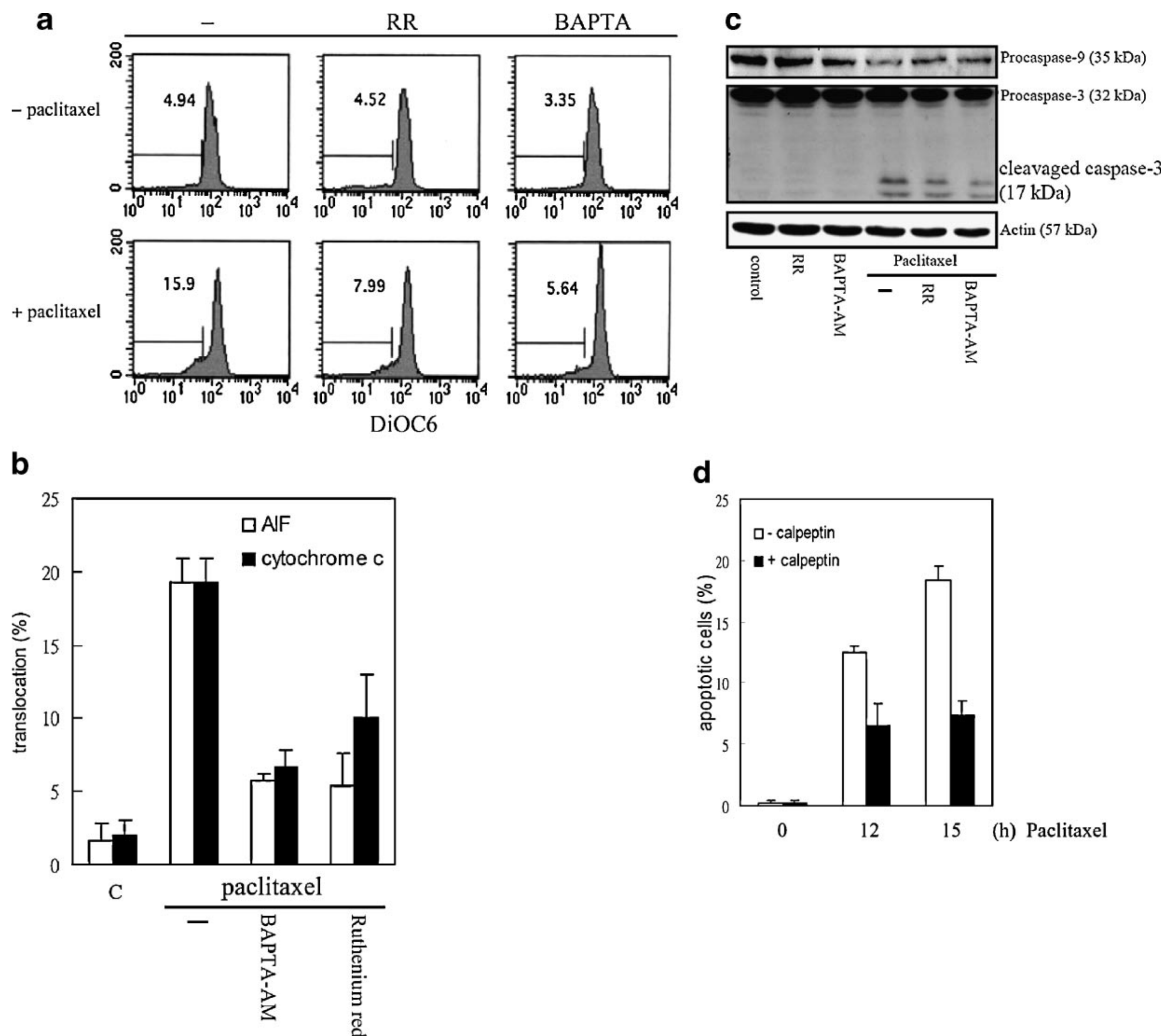

Fig. 4. RR and BAPTA-AM inhibited paclitaxel-induced mitochondrial events. Cells were pretreated with 50 μM RR or 2 μM BAPTA-AM for 30 min and then incubated with 100 μM paclitaxel for 12 h. The effects of these two inhibitors on (**a**) disruption of MMP, (**b**) release of AIF and cytochrome *c* from mitochondria and (**c**) cleavage of both procaspase-9 and -3 were determined. **d**: After same treatment as in (a–c) was carried out, the cells were harvested at the indicated time. The percentage of apoptotic cells was determined by morphological examination. Each histogram indicates mean ± SD of three examinations of at least 200 cells.

in treated U937 cells. The mitochondria, the ER membrane and the cyotsolic fraction were prepared by ultracentrifugation. Figure 6a shows a decrease in the cytochrome *c* level in the mitochondrial fraction that coincides with an increase in the ER fraction after 12–18 h of paclitaxel treatment. The emergence of cytochrome *c* in cytosolic fraction was also increased at 15 and 18 h. To verify the purity of each fraction and ensure equal protein loading in each lane, specific markers for each subcellular fraction were employed and are shown in Figure 6a. The results showed that the mitochondrial marker was expressed exclusively in the mitochondrial fraction. The protein p38, a cytosolic marker, was detected only in the cytosolic fraction and was not present in either the mitochondrial or ER fractions. However, Heme oxygenase-1 (HO-1), an ER marker, was detected in both the ER and mitochondria fractions. Thus, the mitochondrial fraction could not be completely separated from the ER fraction, but the ER and cytosol fractions were free of contamination as shown in Figure 6a. The results clearly demonstrate the translocation of cytochrome *c* from mitochondria to the

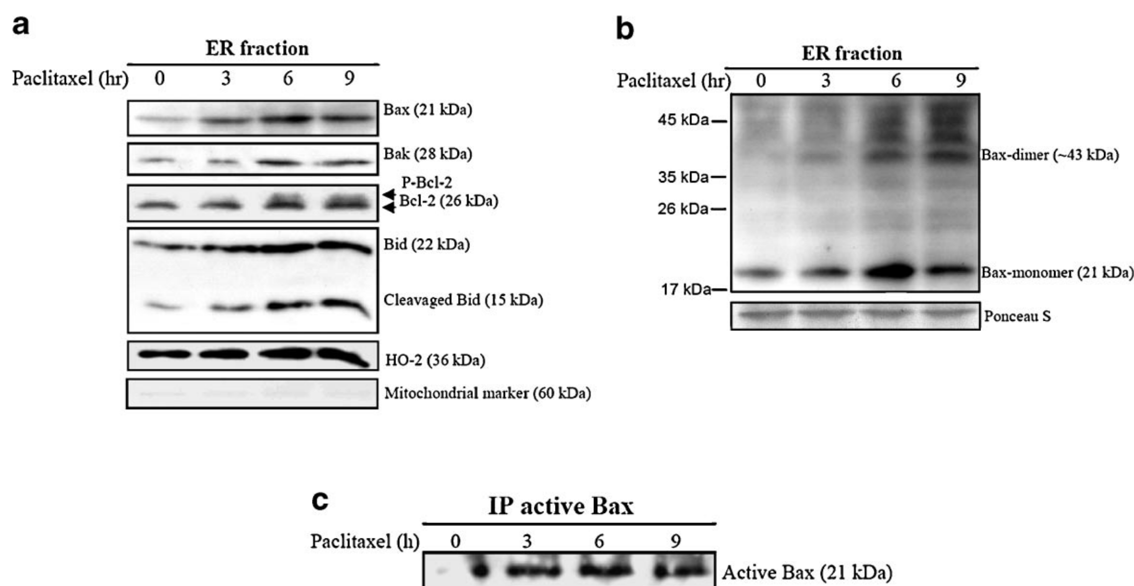

Fig. 5. Paclitaxel induces Bax accumulation and dimerization in the ER. **a**: U937 cells were treated with 100 μM paclitaxel for 3, 6 and 9 h. After treatment, subcellular fractionation was performed to obtain the ER enriched fraction. The Bax, Bak, Bcl-2 and Bid proteins at ER fraction were determined by immunoblotting. The purity of each fraction was verified by a mitochondrial marker and HO-2, which is an ER marker. **b**: U937 cells were treated with 100 μM paclitaxel for 3, 6 and 9 h. Cells were resuspended in hypotonic buffer A to disrupt the cell membrane. Five micrometer BMH reagent was added to cross-link the oligomerized proteins. Cell lysates were subjected to subcellular fractionation to obtain ER enriched fractions. Immunoblotting was performed to detect Bax-containing oligomers. Equal protein loading was confirmed by staining membranes with Ponceau S. **c**: The ER fraction of U937 cells was collected after treatment of paclitaxel. Protein (500 μg) was immunoprecipitated with an active Bax multimer-specific antibody 6A7 under non-denaturing condition. The precipitates were then analyzed by immunoblot (IB) using an antibody against total Bax.

ER in response to paclitaxel treatment in 15–18 h. AIF redistributed to the nucleus was also observed.

Cytochrome *c* translocation to the ER is known to be associated with the IP_3R. We precipitated type 1 IP_3R with a specific antibody. Figure 6b shows that amount of cytochrome *c* co-precipitated with IP_3R increased in the ER fraction after paclitaxel treatment. This suggests that, after 15–18 h of treatment, cytochrome *c* releases from the mitochondria and then binds to IP_3R at the ER. To confirm that the increase in cytochrome *c* at the ER was not a result of new protein synthesis, we monitored cytochrome *c* expression especially at 12–15 h by Western blotting. Figure 6c shows that the cytochrome *c* levels remain constant during paclitaxel treatment. Although IP_3R could be cleaved by caspase 3, its levels were found to have increased in response to paclitaxel treatment for 15 h. In addition, the increase in cytochrome *c* in the ER was not inhibited by pretreatment with the protein synthesis inhibitor cycloheximide (please refer to Supplement 1). Our data provides direct evidence that cytochrome *c* is released from the mitochondria and then binds to IP_3R in the ER during the late stage of paclitaxel-induced apoptosis.

Inhibitors of IP_3R Reversed Disruption of the Mitochondria Membrane Potential and Cytochrome *c* Release as well as Attenuating Paclitaxel-Induced Apoptosis

To confirm that the ER is essential for mitochondria-mediated apoptotic events, we employed two IP_3R inhibitors, TMB-8 and 2-APB, to attenuate the ER response. We found that both inhibitors were able to reduce by about 45% the paclitaxel-induced apoptosis at its highest concentration (Fig. 7a). In addition, the decrease in MMP was also reversed, respectively from 16% to 3% and from 19% to 6% by TMB-8 and 2-APB, respectively (Fig. 7b). Pretreatment with either TMB-8 or 2-APB significantly reduced cytochrome *c* release from the mitochondria (Fig. 7c). These results indicated that the ER plays an important role in the mitochondria responses as well as the process of apoptosis.

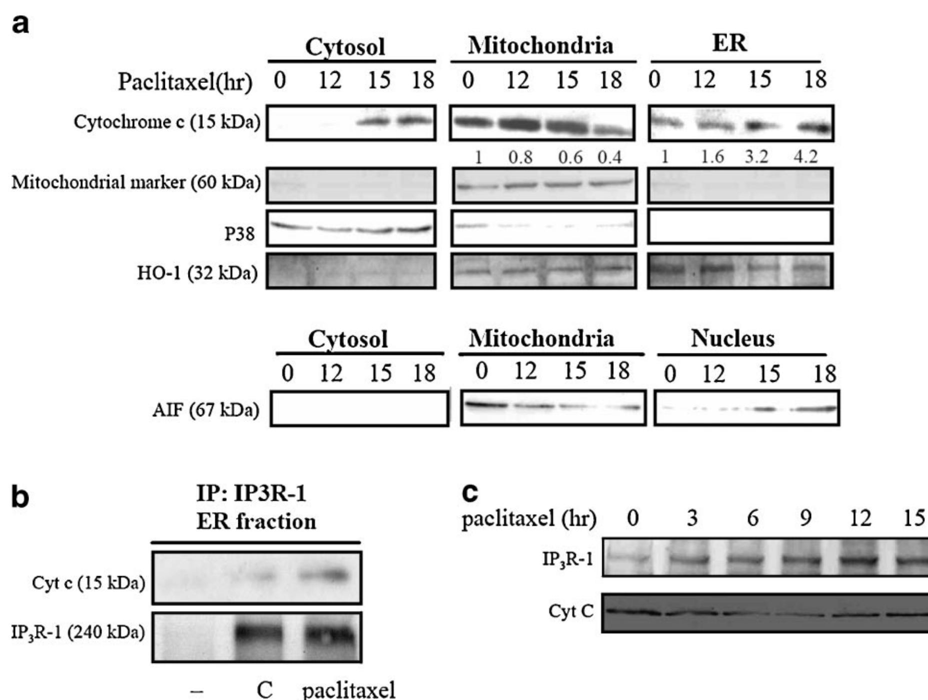

Fig. 6. Cytochrome *c* translocates to the ER and binds to IP_3R during paclitaxel-induced apoptosis. **a**: U937 cells were treated with 100 μM paclitaxel for the indicated time and then the amounts of cytochrome *c* in cytosol, mitochondria and ER fractions were determined. The mitochondrial marker, HO-1 (ER) and p38 (cytosol) were used to verify the purity of each fraction and were also loading controls. The numbers beneath the cytochrome *c* panel are the normalized values of the treatments relative to each specific marker. **b**: ER fractions from paclitaxel-treated U937 cells were immunoprecipitated with antibody against IP_3R-1. The immunocomplex was then immunoblotted with anti-cytochrome *c* and IP_3R-1 antibodies. **c**: Amounts of IP_3R-1 and cytochrome *c* in U937 cells in response to paclitaxel detected by Western blotting.

DISCUSSION

Paclitaxel is known to stabilize microtubules and cause G2/M cell cycle blockade, mitochondria damage and p53-independent apoptosis. Previous studies of the molecular mechanism involved in paclitaxel-induced apoptosis mostly have focused on the mitochondria-mediated signaling pathways. Our study, rather, presents evidence of an ER response during this type of apoptosis.

In the present study, all the ER-related events can be observed within a period of 3–6 h after paclitaxel treatment, whereas the mitochondria-related apoptosis events, such as caspase activation and cytochrome *c* translocation, occurs at 6–9 h after treatment. This suggests that paclitaxel induces excessive ER stress at an early stage prior to the mitochondria response.

Calcium plays a key role in the paclitaxel-induced apoptosis. Paclitaxel can increase cytosolic Ca^{2+} at as early as 3 h. The ER response and the paclitaxel-induced cell death can be reduced by RR or BAPTA-AM. These two inhibitors can also reduce the $\Delta\psi_m$ collapse, apoptogenic factor release and caspase activation induced by paclitaxel.

This opens up the question as to what mechanism medicate the cytosolic Ca^{2+} increase during paclitaxel treatment. It has been suggested that apoptotic agents induce cytochrome *c* releasing from mitochondria. The released cytochrome *c* then binds to IP_3R and controls Ca^{2+} movement [Boehning et al., 2003]. IP_3R can be cleaved by caspase-3 and the cleaved IP_3R became an active channel that continuously leaks Ca^{2+} into the cytosol [Nakayama et al., 2004]. Although both the binding of cytochrome *c* to IP_3R and the activation of caspase 3 were observed in our experiments, they do not seem to be responsible for the early release of Ca^{2+}. This is because the activation of caspase-3 was detected at 9 h (Fig. 1c) and the translocation of cytochrome *c* was only evident at 12 h (Fig. 6a). Therefore, the interaction of IP_3R with cytochrome *c* might function as an apoptotic signal amplifier at

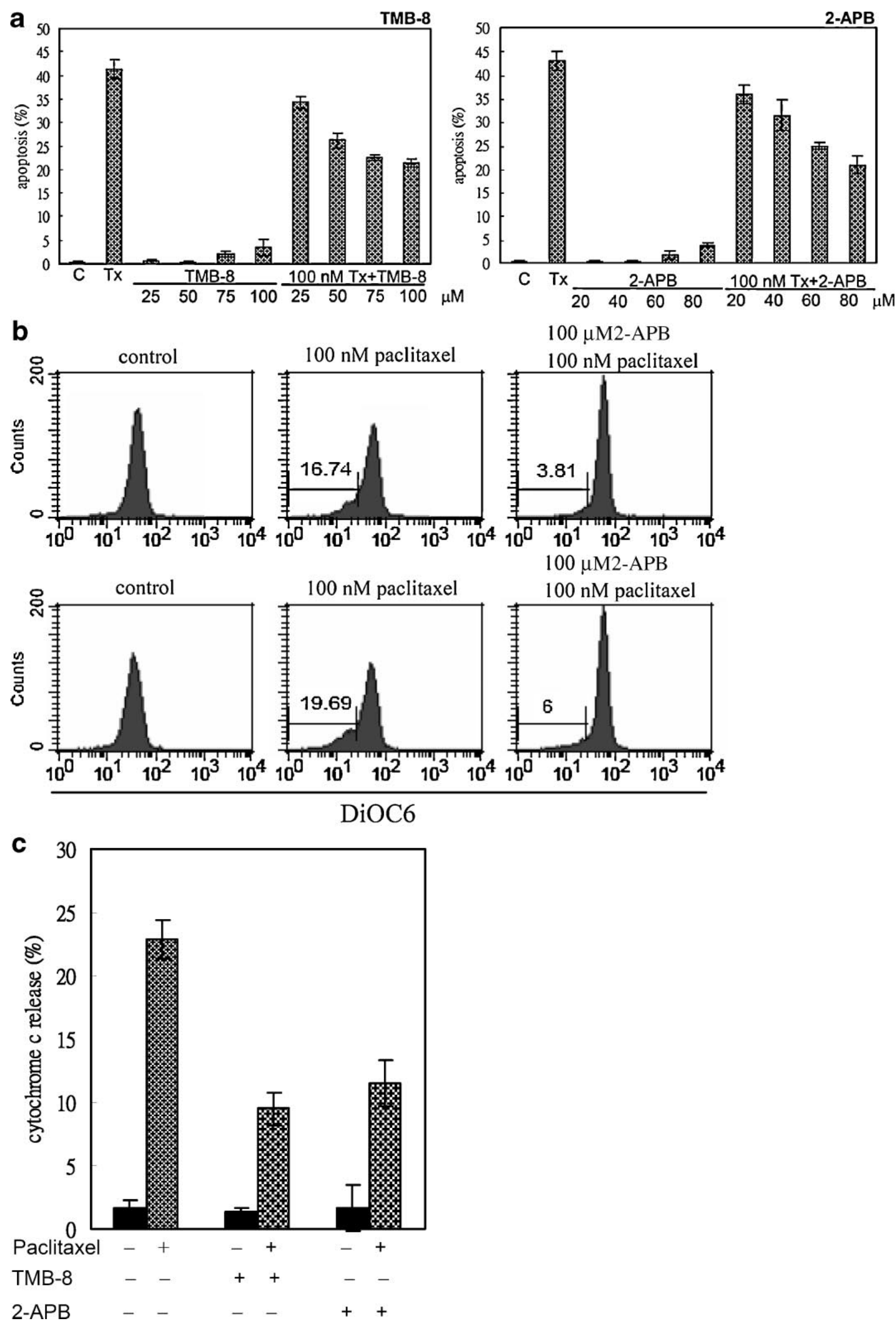

Fig. 7. Paclitaxel-induced apoptosis and mitochondria events can be inhibited by IP3R-specifc inhibitors. **a**: U937 cells were pretreated with 25–100 μM of TMB-8 and 20–80 μM of 2-APB for 30 min and then treated with 100 μM paclitaxel for 24 h. The apoptotic cells were determined by morphological analysis. At least 200 cells were analyzed for each treated group. Mean ± SD of three independent experiments is shown. **b**: One hundred micrometer TMB-8 or 80 μM 2-APB was added to cells for 30 min and then incubated with 100 μM paclitaxel for 15 h. Disruption of the MMP was determined by flow cytometry. **c**: With same treatment as in (b), cells were harvested and the cells showing a release of cytochrome *c* was determined by immunofluorescence as in Figure 1a. At least 200 cells were analyzed for each treated group. Each histogram indicates mean ± SD from three independent experiments.

about 12 h. There must be another pathway initiating the early release of Ca^{2+}. The Bcl-2 family proteins such as Bax and Bak might be responsible for such a process.

Some Bcl-2 family proteins, in addition to their mitochondrial localization, have been found at ER membrane and affect the ER Ca^{2+} store [Zong et al., 2003]. The Bax and Bak proteins, in response to apoptotic stimuli, seem to change their conformation at the ER membrane and mediate Ca^{2+} efflux into cytosol from the ER [Scorrano et al., 2003; Mathai et al., 2005]. In this study, we found the Bax and Bak proteins accumulated in the ER and formed Bax dimers after 3–6 h of treatment. These contributed to the early Ca^{2+} release in our treatment protocol.

Nevertheless, how paclitaxel induces Bax/Bak translocation to the ER causing Bax dimer formation remains unclear. Recently, Boehmerle et al. [2006] identified a novel paclitaxel binding protein neuronal Ca^{2+} sensor 1 (NCS-1) from a human brain cDNA phage display library. The NSC-1 protein is a Ca^{2+} binding protein that interacts with the IP_3R in the ER. They demonstrated that submicromolar concentration of paclitaxel-induced cytosolic calcium oscillations that depended on the ER Ca^{2+} store, IP_3R and NCS-1. This NCS-1 protein is also found in human neutrophils and HL-60 promyelocytes [Brochetta et al., 2003]. It is possible that the early Ca^{+2} increase is also involved in this pathway. Paclitaxel might directly induce calcium oscillations via IP_3R, which will lead to ER dysfunction and subsequently a release of more Ca^{2+} out of the ER. Whether NCS-1 mediates early Ca^{2+} release during paclitaxel treatment in U937 cells needs to be investigated in the future.

The mechanism of caspase-9 activation is known and results from cytochrome *c* release. Cytochrome *c*, dATP and Apaf-1 form complex in cytosol and promote caspase-9 activation. In our study, we observed cytochrome *c* release at 12 h, while the activation of caspase-9 occurred after 9 h. The earlier caspase-9 activation is probably due to a very small amount of cytochrome *c* release that was undetectable by immunoblotting under our conditions. The other possibility is that the activation of caspase-9 may result from caspase-4 activity upon ER stress. The ER-associated caspase-4 has been reported to act upstream of caspase-9 in cephalostatin-induced apoptosis and it was found that this process is independent of Apaf-1 and cytochrome *c* [Lopez-Anton et al., 2006].

Interestingly, we also observed activation of procaspase-8 after treatment of the U937 cells with paclitaxel for 12 h. Activation of caspase-8 has been found to play a crucial role in paclitaxel-induced apoptosis [Oyaizu et al., 1999]. Caspase-8 is generally considered to be an initiator caspase due to its ability associated with the cell surface death receptor. The processing of procaspase-8 has been known to precede activation of caspase-9 and caspase-3, which are downstream mediators in the mitochondrion. However, Fas-independent caspase-8 activation has been reported to occur with various chemotherapeutic agents including paclitaxel [Wesselborg et al., 1999; Newton and Strasser, 2000; Wieder et al., 2001]. Analysis of apoptosis in B-lymphoma cells has revealed that paclitaxel-induced activation of caspase-8 is independent of death receptor signaling and is mediated by caspase-3 activation [Wieder et al., 2001]. The activation of caspase-3 and caspase-8 might function as an amplification loop in mitochondria-mediated apoptosis [von Haefen et al., 2003]. These research results support the hypothesis that caspase-8 can be activated by caspase-3 in the present study.

In summary, we have observed that administration of paclitaxel to U937 cells caused activation of several different apoptotic pathways. Prior to the mitochondrial death pathway, paclitaxel can induce Bax and Bak protein translocation to the ER membrane and the formation of Bax dimers, which leads to Ca^{2+} efflux from ER and causes ER stress. The released Ca^{2+} may enter mitochondria and turn on mitochondria-mediated apoptosis. This is referred to as the first crosstalk event between the ER and the mitochondria. In addition, a second crosstalk event may exist during the later stages and this mediated through the IP_3R-cytochrome *c* interaction. Thus, the ER may contribute to paclitaxel-induced apoptosis via both an early release of Ca^{2+} and a late amplification of mitochondria-mediated apoptotic signals.

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