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Molecules and Cells

Overexpression of Ornithine Decarboxylase Suppresses Thapsigargin-Induced Apoptosis

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Ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis, has paradoxical roles in apoptosis. Our published papers show overexpression of ODC prevents the apoptosis induced by many cytotoxic drugs. Thapsigargin (TG) is an inhibitor of the sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+} ATPase (SERCA) pumps and causes ER stress-induced apoptosis. We used ODC overexpressing cell lines to examine whether overexpression of ODC inhibits TG-induced apoptosis. Our results indicated overexpression of ODC attenuated TG-induced apoptosis. Overexpression of ODC blocked procaspse-4 cleavage and phosphorylation of protein kinase-like ER-resident kinase (PERK), triggered by TG. It also attenuated the increase in CAAT/enhancer binding protein homologous protein (CHOP). Cells with overexpressed ODC had greater Bcl-2 expression. Overexpression of ODC preserved the expression of Bcl-2, inhibited the increase in Bak and stabilized mitochondrial membrane potential without the influences of TG. Cytochrome *c* release and downstream caspase activation were blocked. That is, overexpression of ODC inhibits the mitochondria-mediated apoptotic pathway, induced by TG. Finally, overexpression of ODC maintains the protein and mRNA expression of SERCA. In conclusion, overexpression of ODC suppresses TG-induced apoptosis by blocking caspase-4 activation and PERK phosphorylation, attenuating CHOP expression and inhibiting the mitochondria-mediated apoptotic pathway.

INTRODUCTION

Ornithine decarboxylase (ODC, EC 4.1.1.17) is the first and rate-limiting enzyme of polyamine biosynthesis (Tabor and Tabor, 1984). It decarboxylates L-ornithine to synthesize putrescine. Polyamines, including putrescine, spermidine and spermine, are aliphatic cations and are essential for life. Polyamines and ODC have multiple biological functions which regulate embryonic development, cell cycle, proliferation and apoptosis (Thomas and Thomas, 2001). In apoptosis, polyamines and ODC have paradoxical roles. Apoptosis is induced by two major pathways, the extrinsic (death receptor-mediated) pathway and the intrinsic pathway, which is mediated through mitochondria or the endoplasmic reticulum (ER) (Gupta et al., 2006). Our published papers show overexpression of ODC prevents apoptosis induced by both the extrinsic pathway (tumor necrosis factor-α) (Liu et al., 2005) and intrinsic pathway (methotrexate, curcumin, etoposide, paclitaxel and cisplatin) (Hsu et al., 2008; Huang et al., 2005; Liao et al., 2008). Overexpression of ODC reduces the accumulation of reactive oxygen species, maintains the mitochondrial membrane potential ($\Delta\Psi_m$) and then blocks the mitochondria-mediated death pathway, triggered by these drugs.

The ER is the principal site for protein synthesis, folding and proper targeting. It is the major site for intracellular calcium storage and is involved in calcium signaling and maintenance of intracellular calcium homeostasis (Rao et al., 2001). Many cellular stress conditions, such as hypoxia, nutrient deprivation, alterations in glycosylation status, and disturbances of calcium flux, result in accumulation and aggregation of unfolded and/or misfolded proteins in the ER lumen and cause so-called ER stress (Harding et al., 2002). With ER stress, accumulation of unfolded proteins in the ER triggers evolutionarily conserved responses, termed the unfolded protein responses (UPR). Glucose-regulated protein 78 (GRP78), an ER chaperone, becomes occupied by unfolded proteins and releases transmembrane ER-stress sensor proteins. Released transmembrane sensor proteins aggregate and launch the UPR. In mammals, protein kinase-like ER-resident kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) are three critical transmembrane ER sensor proteins, providing a bridge that connects the ER and cytosol (Xu et al., 2005). Initially, the UPR helps adapt to the changing environment, and reestablish normal ER functions. However, it can eventually

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trigger cell death if ER stress is severe or prolonged, that is, ER stress-induced apoptosis (Xu et al., 2005). ER stress induces apoptosis through various different signals, including the activation of transcription factors, such as C/EBP homology protein (CHOP) (Reddy et al., 2003; Xu et al., 2005), ER-resident caspases (Breckenridge et al., 2003) and proteins from the Bax/Bcl-2 family (Scorrano et al., 2003; White et al., 2005).

Thapsigargin (TG) is a sesquiterpene lactone and is a natural product, originally isolated from the umbelliferous plant *Thapsia garganica*. It is one of the pharmacologic agents which can induce ER stress experimentally. Thapsigargin disrupts ER Ca^{2+} stores through inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps. SERCA pump inhibition by TG results in depletion of ER Ca^{2+} and elevation of cytoplasmic calcium. Depletion of ER Ca^{2+} can activate the UPR, and if sustained, can cause ER stress-induced apoptosis (Denmeade and Isaacs, 2005). There is no published paper directly examining the relationship of ODC and ER stress-induced apoptosis. Polyamine depletion by alpha-difluoromethylornithine (DFMO) causes swelling and involution of ER (Parkkinen et al., 1997) and prevents a GABA-agonist mediated increase in the cytoplasmic density of rough ER in cultured rat cerebellar granule cells (Abraham et al., 1993). Rough ER seems to be one of the targets where polyamines affect cell proliferation and differentiation. Here, our aim is to examine whether overexpression of ODC inhibits TG-induced apoptosis.

MATERIALS AND METHODS

Cell culture and chemicals

HL-60 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at a temperature of 37°C under humidified air and 5% CO_2. Thapsigargin, acridine orange, and rhodamine 123 were purchased from Sigma.

Cell viability

Cells were counted using the trypan blue exclusion assay. The extent of cell viability was calculated and the viable cell numbers from experiment groups were compared with those in the untreated control group.

Human *odc* gene sub-cloning and cell transfection

Parental HL-60 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS for 3 h, and the harvested cells were then gently rinsed in PBS. Purification of mRNA was carried out according to the supplier's instructions (MDBio, Taiwan) and the cDNA was synthesized by reverse transcriptase (RT) (Promega, USA). Polymerase chain reaction (PCR) amplification of the coding region of the human *odc* cDNA was performed with our primers, which were designed based on the human odc sequence. The PCR product was sub-cloned into a eukaryotic expression vector, pCMV-Tag (Stratagene, USA), and then sequenced. The plasmid for ODC expression was constructed by inserting the *Bam*HI–*Eco*RI 1415 bp coding region fragment. Parental HL-60 cells were transfected with WT-ODC (overexpressing ODC) and m-ODC (frame-shift mutant causing a nonsense stop codon) plasmids according to calcium phosphate-mediated transfections. Stably transfected cells were selected with the antibiotic G418 (400 μg/ml). Three weeks later, isolated G418-resistant clones were individually analyzed for expression of ODC. The ODC expression for individual clones was examined by RT-PCR, immunoblotting, and enzyme activity assays (Liu et al., 2005).

Apoptotic sub-G1 analysis

The 1×10^6 cells were cultured in 35-mm petri dishes and incubated for 24 h. Cells were treated with TG for the specified dose, then harvested, washed with PBS, resuspended in 0.2 ml of PBS and fixed in 0.8 ml of ice-cold 100% ethanol at -20°C overnight. The cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/ml RNase A) and incubated at 37°C for 30 min. Then, 1 ml of PI solution (50 μg/ml) was added and the mixture was allowed to stand on ice for 30 min. The nuclei were analyzed in a FACSCAN laser flow cytometer (Becton Dickenson, USA).

DNA fragmentation assay

Cells were harvested and lysed overnight in a digestion buffer (0.5% sarkosyl, 0.5 mg/ml proteinase K, 50 mM Tris-HCl, pH 8.0 and 10 mM EDTA) at 55°C. Subsequently, cells were treated with 0.5 μg/ml RNase A for 2 h. The genomic DNA was extracted by phenol/chloroform/isoamyl alcohol extraction and analyzed by gel electrophoresis using 2% agarose.

Analysis of the mitochondrial membrane potential ($\Delta\Psi_m$)

The $\Delta\Psi_m$ was monitored by fluorescence of rhodamine 123 (Liu et al., 2005). Cells were incubated with 10 μM rhodamine 123 for 10 min. Finally, cells were detached and the fluorescence was measured in a flow cytometer. In each study, 10,000 events (cells) were counted. Data were acquired and analyzed using WinMDI software.

Mitochondrial calcium determination

Cells were resuspended in 10% FBS RPMI 1640 without phenol red containing 10 μM Rhod2-AM for 30 min. Then, cells were washed two times with RPMI 1640 without phenol red and resuspended in the same medium. The cells were dispatched in 96-well white plates at 1×10^5 cells per well and the volume of each well was adjusted to 200 μl with 10% FBS RPMI 1640 without phenol red. The variations of fluorescence of the Rhod2-AM dye (λ_{exc} = 540 nm λ_{em} = 595 nm) were measured in a Perkin-Elmer HTS 7000 Plus Bio Array Reader. TG was added after stabilization of the fluorescence baseline. The fluorescence was then recorded during 30 min.

Immunoblotting

To extract mitochondrial proteins, cells were washed once with PBS, and then resuspended with mitochondrial buffer (25 mM Tris, pH 6.8, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 250 mM sucrose). To purify the total proteins, cells were harvested and lysed in cold lysis buffer (10% v/v glycerol, 1% v/v Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris, pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin), homogenized, centrifuged, and then the supernatant was boiled in loading buffer with an aliquot corresponding to 50 μg of protein. Samples were then separated by SDS-PAGE and transferred to PVDF membranes. After blotting, the PVDF membranes were incubated with primary antibodies for 6 h and with the secondary antibody labeled with horseradish-peroxidase for 1 h. The antigen-antibody complexes were visualized by enhanced chemiluminescence.

RT-PCR

RNA was isolated from cells by Trizol (MDBio) according to the manufacturer's instructions. Synthesis of cDNA was performed using mRNA templates, reverse transcriptase (RT) and 500 ng

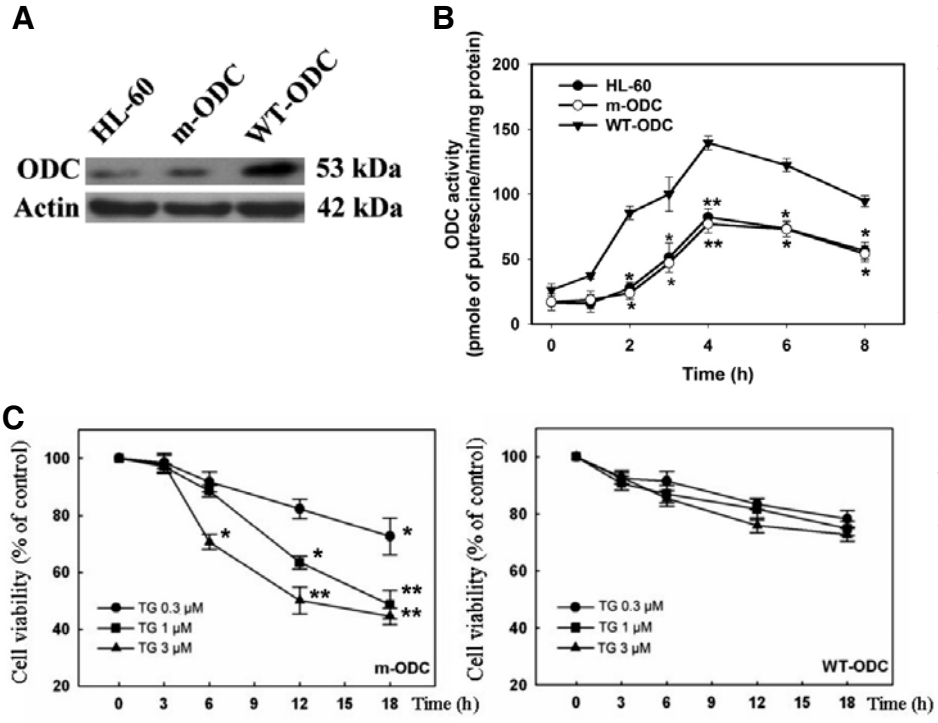


Fig. 1. Overexpression of ODC attenuates the cell death, caused by TG. After stimulating by 10% FBS on parental HL-60, m-ODC and WT-ODC cells, protein expression was detected by immunoblotting with anti-ODC antibody (A) and enzymatic activity was examined (B). After being stimulated by 10% FBS for 3 h, m-ODC and WT-ODC cells (stimulated m-ODC and WT-ODC cells) were treated with TG at the indicated concentrations for 18 h. Cell viabilities were counted by light microscopy at the indicated time points (C). Data are representative of three experiments. Bars represent SD from three independent experiments. **P* < 0.05 and ***P* < 0.01 as compared with WT-ODC.

of random primers. The reaction mixture was incubated for 90 min at 42°C. For PCR assay, cDNA was added to 40 μl mixture buffer containing 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20 (v/v), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM forward and reverse primers, and 1 U Taq DNA polymerase (MDBio). PCR was set on the condition of 2 min at 94°C, 25-35 cycles (30 s, 94°C; 30 s, 56-60°C; 90 s, 72°C) and 10 min at 72°C by a Mastercycler (Eppendorf), and products were analyzed on a 2% agarose gel. The following primer pairs were used: β-actin (309 bps) 5′-AGCGGGAAATCGTGCGTG-3′ and 5′-CAGGGTACATGGTGGTGC-3′; SERCA (312 bps) 5′-GGGAAGTTGTCTGTATTTTCC-3′ and 5′-GGAAATGACTCA GCTGGTAG-3′.

Statistical analysis

Data shown represent the mean ± standard deviation (S.D.). Statistical analyses for detecting significant differences between the control and experimental groups were carried out using the Student's *t*-test.

RESULTS

Overexpression of ODC attenuates TG-induced apoptosis

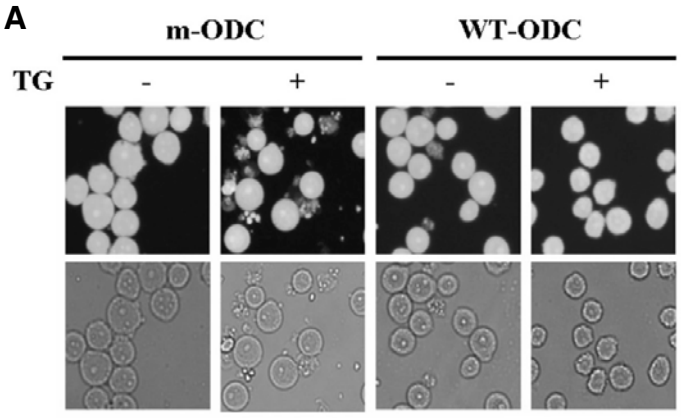
We produced an ODC overexpressing cell line (WT-ODC cells) and vector- control cell line (m-ODC cells). After being stimulated by 10% FBS for 3 h, WT-ODC cells show a 2 fold increase in ODC protein expression and enzyme activity, compared with m-ODC cells and HL-60 cells (Figs. 1A and 1B). TG decreased cell viability of stimulated m-ODC cells (that is, being stimulated by 10% FBS for 3 h) in a dose-dependent manner. However, stimulated WT-ODC cells resisted TG-induced cell death at the concentration of 1 μM and 3 μM (Fig. 1C). After being incubated with 1 μM TG for 18 h, stimulated m-ODC cells showed typical apoptotic morphologic characteristics, including chromatin condensation, membrane blebbing and shrinkage and apoptotic body formation, and DNA fragmentation, but stimulated WT-ODC cells didn't (Figs. 2A and 2B). In a dose dependent manner, TG induced greater increase of the sub-G1 ratio of stimulated m-ODC cells than stimulated WT-ODC cells (Fig. 2C). Taken together, overexpression of ODC attenuates TG-induced apoptosis.

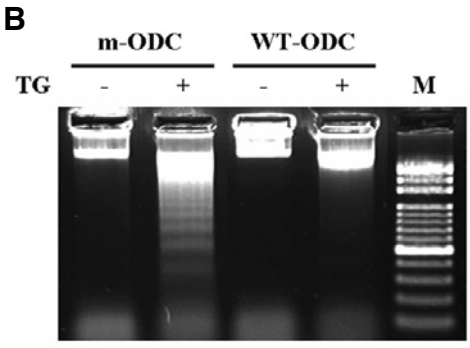
Overexpression of ODC blocks procaspase-4 cleavage and PERK phosphorylation, and attenuates the increase in CHOP

TG is a well-known ER stressor. To examine the influences of ODC on ER stress-induced apoptosis, triggered by TG, we chose several ER-specific molecules. At first, we assessed the protein expression of ODC. After being incubated by 1 μM TG for 18 h, stimulated m-ODC and WT-ODC cells were harvested for the experiments of immunoblotting. As shown in Fig. 3, TG didn't change the protein expression of ODC. Stimulated WT-ODC cells had more protein expression of ODC than stimulated m-ODC cells (Fig. 3). Next, we examined the phosphorylation of PERK and the expression of procaspase-4, GRP78 and CHOP. TG induced procaspase-4 cleavage and PERK phosphorylation in stimulated m-ODC cells, but not in stimulated WT-ODC. TG increased GRP78 and CHOP in both stimulated m-ODC and WT-ODC cells (Fig. 3). However, stimulated WT-ODC cells had less protein expression of CHOP than stimulated m-ODC cells did upon the treatment of TG (Fig. 3).

Overexpression of ODC inhibits mitochondrial apoptotic pathway, induced by TG

After being incubated with 1 μM TG for 18 h, stimulated m-ODC cells expressed less Bcl-2 and more Bak, compared with the untreated control group (Fig. 4). TG reduced the protein amount of procaspase-9 and procaspase-3, that is, induced the cleavage or activation of procaspase-9 and 3 (Fig. 4). Under the same circumstances, stimulated WT-ODC cells expressed more Bcl-2 than their counterparts of stimulated ODC cells, both in the treated and untreated groups. Cells with overexpres-





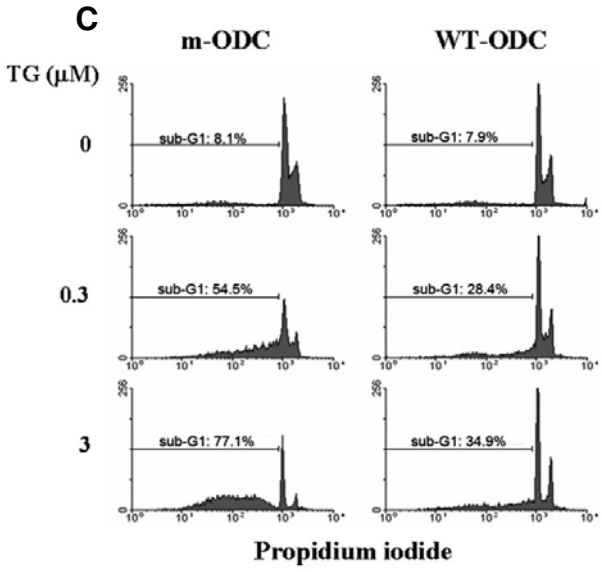


Fig. 2. Overexpression of ODC suppresses TG-induced apoptosis. Stimulated m-ODC and WT-ODC cells were treated with 1 μM TG for 18 h. Cells were observed by fluorescence-microscope (A), and harvested for the experiments of DNA fragmentation assay (B) and flow cytometry with PI fluorescence to determine sub G1 peak ratio (C). The arrows indicate the apoptotic body. Data are representative of at least three experiments.

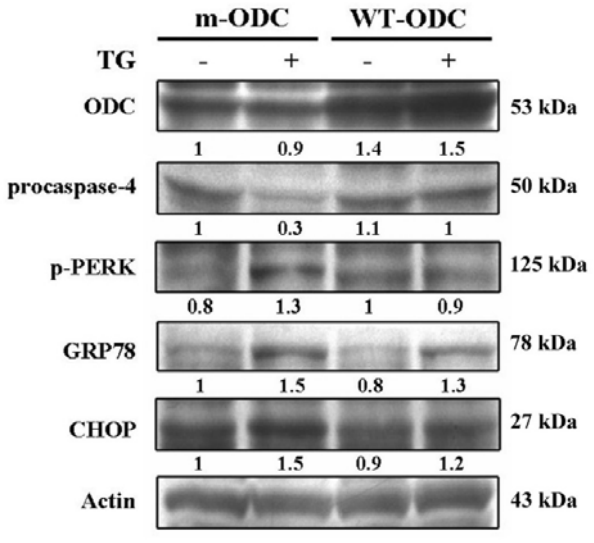


Fig. 3. The effects of ODC on SERCA, procaspase-4, p-PERK, GRP78 and CHOP. Stimulated m-ODC and WT-ODC cells were treated with 1 μM TG for 18 h. Total protein was extracted for immunoblotting with antibodies of ODC, procaspase-4, p-PERK, GRP78, CHOP and actin. Data are representative of at least three experiments.

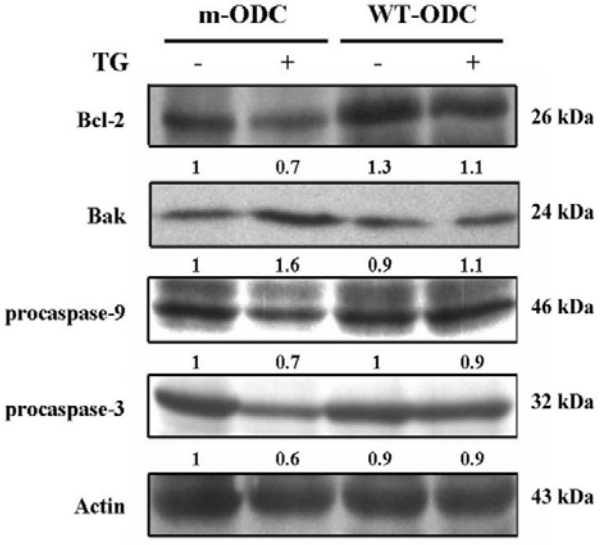


Fig. 4. The effects of ODC on Bcl-2, Bak, procaspase-9 and 3. Stimulated m-ODC and WT-ODC cells were treated with 1 μM TG for 18 h. Total protein were extracted for immunoblotting with antibodies of Bcl-2, Bak, procaspase-9 and 3, and actin. Data are representative of at least three experiments.

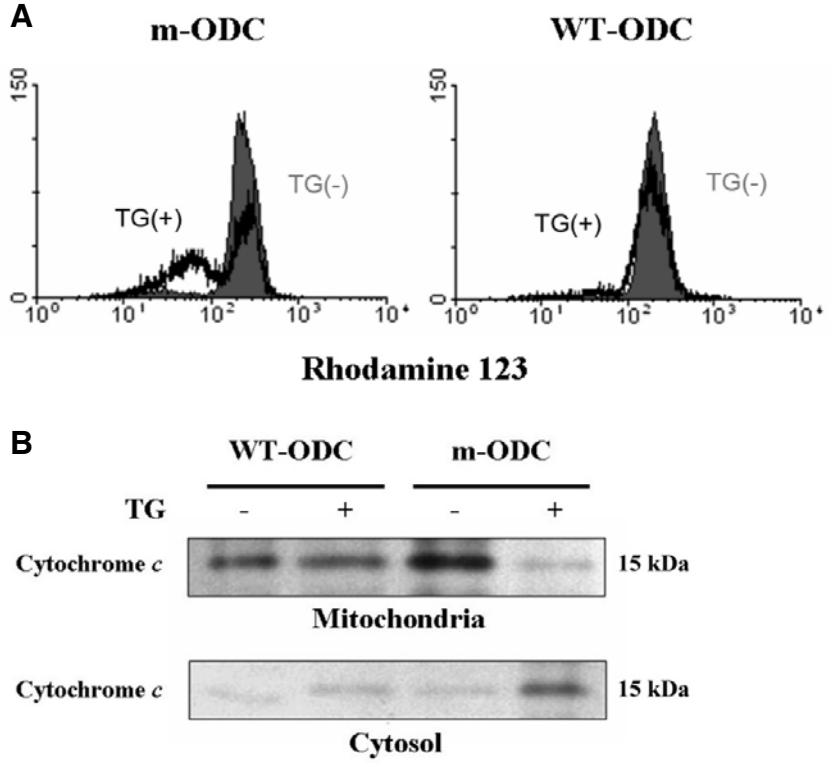


Fig. 5. Overexpression of ODC inhibits mitochondrial membrane potential loss and cytochrome *c* release, induced by TG. Stimulated m-ODC and WT-ODC cells were treated with 1 μM TG for 18 h. Intracellular $\Delta\psi_m$ were detected by flow cytometry measuring the fluorescence of rhodamine 123 (18 h after) (A). Mitochondrial and cytosol protein were separated for immunoblotting with anti-cytochrome *c* antibody (B). Data are representative of at least three experiments.

sed ODC maintained the same expression of Bcl-2, Bak, procaspase-9 and procaspase-3 without the influences of TG (Fig. 4). TG induced $\Delta\Psi_m$ loss (Fig. 5A) and cytochrome *c* release from mitochondria to cytosol in stimulated m-ODC cells, but not in stimulated WT-ODC cells (Fig. 5B). Compatible with previous studies, TG decreases anti-apoptotic protein Bcl-2, increases pro-apoptotic protein Bak, and triggers mitochondrial dysfunction and releasing cytochrome *c*, and then activates caspase cascades, including caspase-9 and 3. Overexpression of ODC inhibits the intrinsic pathway of apoptosis, induced by TG.

Overexpression of ODC maintains the expression of SERCA and reduces mitochondrial calcium accumulation

We examined the effects of TG and ODC on the expression of SERCA in stimulated m-ODC and WT-ODC cells. After being treated by 1 μM TG, stimulated m-ODC cells expressed more SERCA protein at 6 h and less at 18 h than untreated control group. Stimulated WT-ODC cells expressed more SERCA protein then m-ODC cells and kept the protein expression of SERCA from the influence of TG (Fig. 6A). We further examined the transcription level of SERCA. After being incubated with 1 μM TG for 18 h, stimulated m-ODC cells expressed less mRNA of SERCA than untreated control group, but stimulated WT-ODC cells had the same mRNA expression (Fig. 6B). Furthermore, the mitochondrial calcium accumulation was down-regulated in WT-ODC cells to compare with m-ODC cells (Fig. 6C).

DISCUSSION

ER-stress triggers specific signaling pathways to protect the cell, including reduction of newly synthesized proteins, increased translocation and degradation of misfolded proteins and increased protein folding capacity within ER (Rao et al., 2001). If sustained, ER-stress will induce apoptosis. TG is a specific blocker of SERCA. The interaction of TG with the SERCA isoforms is rapid, stoichiometric, and essentially irreversible (Lytton et al., 1991). Through inhibition of SERCA pumps, thapsigargin depletes ER Ca^{2+}, induces ER-stress and apoptosis. In our experiments, stimulated WT-ODC cells significantly attenuated TG-induced apoptosis in the experiments of cell viability, DNA fragmentation assay and apoptotic sub-G1 analysis. We further explored the influences of ODC on ER stress-induced apoptosis, triggered by TG. Among all the reported death pathways of ER stress-induced apoptosis, PERK-CHOP pathway, Bcl-2 family proteins, and capspase-4 were examined.

Upon ER stress, PERK undergoes oligomerization and transautophosphorylation (Bertolotti et al., 2000). The phosphorylated PERK activates eukaryotic translation initiation factor-2 alpha, resulting in decreased translation initiation and protein synthesis (Harding et al., 1999; 2000; Liu et al., 2000; Scheuner et al., 2001; Shi et al., 1998). A recent report showed sustained PERK signaling impairs cell proliferation and promotes apoptosis (Lin et al., 2009). Our data indicate overexpression of ODC blocks the phosphorylation of PERK and attenuates the expression of CHOP. CHOP is a transcription factor induced by both the PERK and ATF6 pathways upon ER stress (Ma et al., 2002; Ron and Habener, 1992; Yoshida et al., 2000). CHOP activates the transcription of growth arrest and DNA damage gene 34 (Novoa et al., 2001), endoplasmic reticulum oxidoreductin 1 (Marciniak et al., 2004), death receptor 5 (Yamaguchi and Wang, 2004), and carbonic anhydrase VI (Sok et al., 1999; Wang et al., 1998), which are responsible for apoptosis. In addition, Bcl-2 is down-regulated by CHOP, leading to enhanced oxidant injury and apoptosis (McCullough et al., 2001). Overexpression of ODC suppresses the PERK-CHOP death pathway of ER-stress.

Our published papers have shown overexpression of ODC prevents apoptosis induced by several cytotoxic drugs, including tumor necrosis factor-α, methotrexate, curcumin, etoposide,

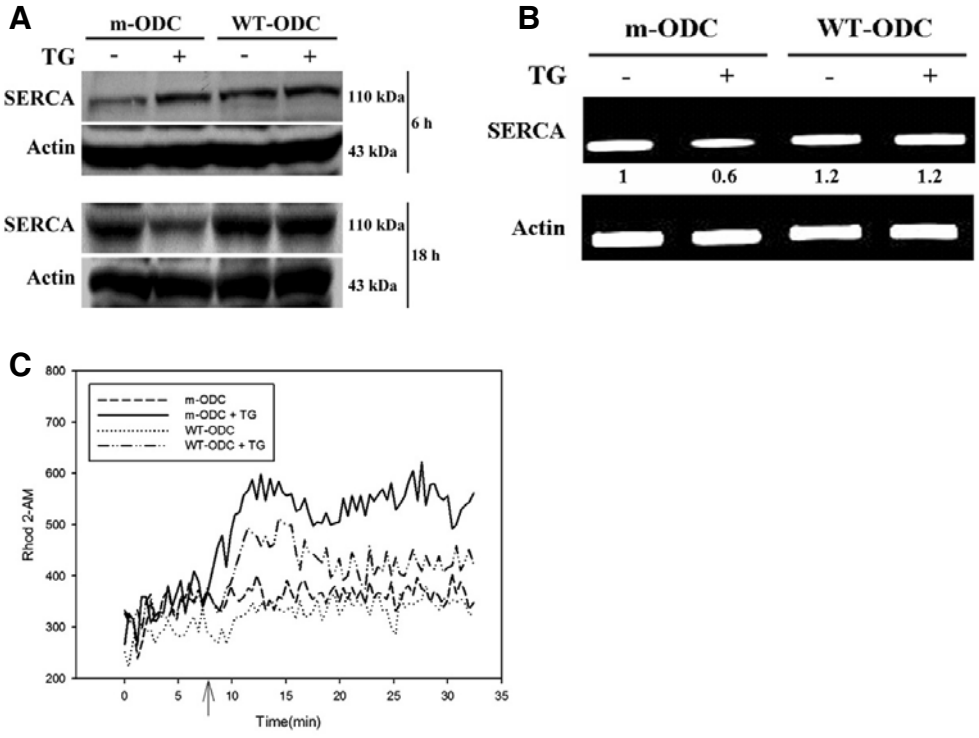


Fig. 6. The effects of TG and ODC on the expression of SERCA and mitochondrial accumulation of calcium. Stimulated m-ODC and WT-ODC cells were treated with 1 μM TG for 6 h and 18 h. SERCA and actin protein (A) and mRNA (B) expressions were detected by immunoblotting with anti-SERCA antibody and anti-actin antibody, and RT-PCR. Actin protein and mRNA expression were used as internal controls. The levels of SERCA mRNA relative to actin were determined by densitometry analysis. m-ODC and WT-ODC cells were stimulated by TG and labeled with Rhod 2-AM to visualize the mitochondrial accumulation of calcium (C). Data are representative of at least three experiments.

paclitaxel and cisplatin, though blocking the mitochondria-mediated pathway. Again, the present data demonstrate overexpression of ODC enhances the expression of Bcl-2 and decreases Bak. It stabilizes the $\Delta\Psi_m$, prevents cytochrome *c* release and the activation of caspase-9 and 3. Overexpression of ODC inhibits the mitochondria-mediated pathway of TG-induced apoptosis.

In the murine system, caspase-12 is localized to the ER membrane, and activated by ER stress (Tan et al., 2006). Then, caspase-12 activates caspase-9, which in turn activates caspase-3, leading to cell death (Morishima et al., 2002). Overexpression of Bcl-2 inhibits the activation of caspase-12 and apoptosis during ER stress (Contreras et al., 2003). Aside from being present on the membrane of mitochondria, Bax (Bcl2-associated X protein) and Bak (Bcl-2 homologous antagonist/killer) are also present in the ER membrane. During ER stress, Bax and Bak oligomerize and activate caspase-12 (Hetz et al., 2006; Zong et al., 2001). Caspase-12 can activate caspase-9, independent of cytochrome *c* and Apaf-1 released from the mitochondria (Rao et al., 2001). There is evidence indicating caspase-4 in humans may be the caspase-12 homolog that is activated in an ER-stress-specific manner (Hitomi et al., 2004). Overexpression of ODC blocks the cleavage of procaspase-4, that is, inhibits the activation of caspase-4, an apoptotic pathway without involving mitochondria.

GRP78, a well-known chaperone, belongs to the highly conserved heat shock protein 70 family chiefly residing in the ER membrane (Gething, 1999; Liu et al., 1997; Morris et al., 1997). CRP78 not only binds to unfolded protein and facilitates folding, but negatively regulates the sensors of ER stress, including PERK, ATF6 and IRE1 (Rao et al., 2001). Many studies have shown GRP78 plays an anti-apoptotic role against several kinds of stress in various types of cancer (Fu and Lee, 2006; Gazit et al., 1999; Li and Lee, 2006). Stimulated WT-ODC cells express the same amount of GRP78 protein as stimulated m-ODC cells. Overexpression of ODC seems to be selectively inhibiting death signals, and protecting survival molecules, such as Bcl-2 and GRP78.

After being treated by 1 μM TG, stimulated m-ODC cells expressed more SERCA protein at 6 h and less at 18 h than the untreated control group. Under the same conditions, stimulated WT-ODC cells expressed more SERCA protein then m-ODC cells and kept the protein and mRNA expression of SERCA from the influence of TG. With ER stress, the activated PERK kinase phosphorylates eukaryotic translation initiation factor-2 alpha (eIF2α) results in decreased translation initiation and protein synthesis (Harding et al., 1999; 2000; Liu et al., 2000; Scheuner et al., 2001; Shi et al., 1998). Overexpression of ODC maintains the expression of SERCA both transcriptionally and translationally. SERCA pump inhibition by thapsigargin leads to a depletion of ER Ca^{2+}. Additionally, overexpression of Bax or Bak causes Ca^{2+} efflux from the ER (Nutt et al., 2002a; 2002b; Scorrano et al., 2003). The release of Ca^{2+} from the ER lumen induces the eletrophoretic accumulation of Ca^{2+} influx from the cytosol in the mitochondria. Sustained Ca^{2+} accumulation in the mitochondria, induced by ER stress, triggered signs of proapoptotic mitochondrial alteration, relocalization of Bax to mitochondria and the release of cytochrome *c* and apoptosis-inducing factor from mitochondria (Deniaud et al., 2008). As discussed above, overexpression of ODC maintains the expression of

SERCA and decreases Bak expression. Thus, inhibition of sustained Ca^{2+} accumulation in cytosol and mitochondria is another possible way that ODC inhibits apoptosis.

In conclusion, overexpression of ODC suppresses TG-induced apoptosis, blocks caspase-4 activation and PERK phosphorylation, and attenuates CHOP expression. It inhibits Bak increase and maintains Bcl-2 expression, blocking the intrinsic pathway of apoptosis. In our studies, we observed ODC seemed to protect cells from ER stress-induced apoptosis in many different ways.

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