The Mitochondrial Permeability Transition Regulates Cytochrome c Release for Apoptosis during Endoplasmic Reticulum Stress by Remodeling the Cristae Junction*

Dawei Zhang†, Chao Lu†, Matthew Whiteman†, Britton Chance†, and Jeffrey S. Armstrong††

From the †Department of Biochemistry, National University of Singapore, Singapore 117597, Singapore, the ‡Institute of Bioengineering and Nanotechnology, Singapore 138669, Singapore, ¶Peninsula Medical School, Universities of Exeter and Plymouth, Exeter EX1 2LU, United Kingdom, and the ††Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The role of the mitochondrial permeability transition (MPT) in apoptosis and necrosis is controversial. Here we show that the MPT regulates the release of cytochrome c for apoptosis during endoplasmic reticulum (ER) stress by remodeling the cristae junction (CJ). CEM cells, HCT116 colon cancer cells, and murine embryo fibroblast cells were treated with the ER stressor thapsigargin (THG), which led to cyclophilin D-dependent mitochondrial release of the profusion GTPase optic atrophy 1 (OPA1), which controls CJ integrity, and cytochrome c, leading to apoptosis. Interferon RNA knockdown of Bax blocked OPA1 and cytochrome c release after THG treatment but did not prevent the MPT, showing that Bax was essential for the release of cytochrome c by MPT. In isolated mitochondria, MPT led to OPA1 and cytochrome c release independently of voltage-dependent anion channel and the outer membrane, indicating that the MPT is an inner membrane phenomenon. Last, the MPT was regulated by the electron transport chain but not mitochondrial reactive oxygen species, since THG-induced cell death was not blocked by antioxidants and did not occur in cells lacking mitochondrial DNA. Our results show that the MPT regulates CJ remodeling for cytochrome c-dependent apoptosis induced by ER stress and that mitochondrial electron transport is indispensable for this process.

The endoplasmic reticulum (ER) regulates protein synthesis and intracellular calcium (Ca\(^{2+}\)) homeostasis (1). Excessive ER stress triggers apoptosis through a variety of mechanisms, including activation of the unfolded protein response (UPR), alterations in Ca\(^{2+}\) levels, and activation of Bcl-2 family proteins (1–3). The SERCA-ATPase inhibitor thapsigargin (THG) has been used to study ER stress-induced apoptosis implicating Bax (4, 5), the UPR (5), and the mitochondrial permeability transition (MPT) (6) as key factors involved. However, the relationship between these events and the mechanism regulating apoptosis by the MPT remains unclear.

The role of the MPT in cell death is controversial due, in part, to the current lack of knowledge of its molecular structure and its regulation (7, 8). For example, although recent genetic studies with cyclophilin D (Cyp-D) knock-out mice have shown that the MPT primarily regulates necrosis and not apoptosis (9, 10), many earlier studies showed that Bax interacted with components of the MPT, including voltage-dependent anion channel (VDAC) (11) and adenine nucleotide translocator (ANT) (12) to regulate apoptosis, and we recently showed that Bax and the MPT regulated the mitochondrial release of cytochrome c for apoptosis (6). Mechanistically, early studies suggested that the MPT caused cytochrome c release by inducing nonspecific mitochondrial swelling and organelle rupture (13–16). However, in contrast to this idea, other reports have shown that cytochrome c can be released from mitochondria without significant swelling or outer membrane rupture (17, 18). Recently, it has been shown that the mitochondrial cytochrome c release mechanism is highly regulated and involves a number of discrete steps (19, 20), including structural remodeling of the mitochondrial cristae junctions (CJ) and outer membrane permeabilization (20). The MPT was implicated in remodeling of the CJ, because it was blocked by the MPT inhibitor cyclosporine A (CsA) (20); however, the relationship between CJ remodeling and the MPT is currently unknown.

Here we show, using isolated mitochondria and leukemic CEM cells, that the MPT induces mitochondrial structural remodeling of the cristae membrane by releasing OPA1 from the CJ to facilitate mitochondrial release of cytochrome c for apoptosis. In cells, the MPT failed to release cytochrome c from mitochondria in the absence of Bax, indicating that the MPT does not rupture the organelle’s outer membrane for cytochrome c release. In isolated mitochondria, the MPT caused OPA1 and cytochrome c release independently of the mitochondrial outer membrane and the putative component
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VDAC. Last, we show that the MPT is dependent on a functional electron transport chain (ETC) but does not depend on mitochondrial ROS production and therefore is not redox-regulated.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—All chemicals, including THG, ruthenium 360 (Ru360), staurosporine (STS), CsA, BAPTA-AM, N-acetylcysteine (NAC), and the manganese-superoxide dismutase antioxidant Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) were purchased from Sigma. Bongkrekic acid (BGK) was purchased from Calbiochem. Ionomycin (Iono) and tunicamycin (TM) were purchased from AG scientific (San Diego, CA). Fluorescent probes, including tetramethylrhodamine methyl ester (TMRM), Fluo-3-AM, Rhod2-AM, propidium iodide, and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) were obtained from Molecular Probes, Inc. (Eugene, OR). CEM cells were passaged daily to maintain them in log phase growth and kept at a nominal concentration of 2.5–5 × 10^5/ml. Cell viability studies were performed by the trypan blue exclusion method. CEM parental cells were treated with ethidium bromide in the presence of 110 μg/ml pyruvate and 50 g/ml uridine to generate cells lacking mitochondrial DNA (ρ^0) as previously described (21). ρ^0 status was determined by monitoring cells for KCN-sensitive oxygen consumption and cytochrome oxidase II subunit protein expression by Western blot until oxygen consumption was reduced 90%. Murine embry fibroblast (MEF) wild type cells and Bax/Bak double knock-out cells were generously given by Dr. L. Scorrano (Venezian Institute of Molecular Medicine, Venice, Italy). HCT116 colon cancer Bax^+/− and Bax^−/− cells were generously given by Dr. V. Yu (Institute of Molecular and Cellular Biology, Singapore). MEF cells and HCT116 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 μg/ml penicillin, 100 units/ml streptomycin, 1% glutamate, and 10% fetal bovine serum.

Aptosis Assays—THG and STS were used at a concentration of 1 μM and 500 nm, respectively, unless otherwise stated. Cellular apoptosis was determined by performing FACS cell cycle analysis of propidium iodide (20 μg/ml)-stained CEM cells. The percentage of cells in the sub-G₁ zone was taken to indicate the percentage of cells undergoing apoptosis. DAPI (3 μM) staining was also monitored by fluorescence microscopy to visually estimate cells containing apoptotic nuclei. Caspase-3 activity was determined using the EnzChek® caspase-3 fluorescence assay kit according to the manufacturer’s instructions (Molecular Probes, Inc., Eugene, OR), using a Molecular Probes fluorometer model GEMINI XS (excitation at 342 nm and emission at 441 nm).

Determination of Cytosolic and Mitochondrial Ca^{2+} Levels—Cytosolic Ca^{2+} levels were determined by FACS analysis using Fluo-3-AM (1 μM) (log mode in fluorescein isothiocyanate (FITC) setting) with results displayed as FACS analysis histograms as described in Ref. 6. Mitochondrial Ca^{2+} levels were determined by FACS analysis using Rhod2-AM (250 nM) (log mode in phycoerythrin (PE) setting) with results displayed as color density dot plots as described in Ref. 6. For the spectro-photometric determination of mitochondrial Ca^{2+}, cells were loaded with 1 μM Rhod2-AM in a buffer (100 mM KCl, 10 mM MOPS, pH 7.2) for 60 min and transferred to primary culture for an additional 18 h to eliminate the residual cytosolic fraction of the Rhod2 dye. Mitochondrial Ca^{2+} was determined by measuring fluorescence at 552 and 581 nm. For the single cell mitochondrial Ca^{2+} assay, laser-scanning confocal microscopy (LSCM) was used as described previously (6). Mitochondrial localization of the Rhod2 probe was performed by simultaneously loading cells with 100 nM MitoTracker green (a mitochondria-selective dye that is concentrated by active mitochondria) and 300 nM DAPI. Fluorescence images of Rhod2, MitoTracker green, and DAPI were obtained at 552/490/358 nm excitation and 581/516/461 nm emission, respectively.

FACS Analysis for Determination of Δψ_m, and Reactive Oxygen Species (ROS) Production—Δψ_m was determined by monitoring the fluorescence of the cationic potentiometric dye TMRM as described previously (6). Briefly, cells were loaded with TMRM (250 nm) for 15 min, and red fluorescence was determined by FACS analysis using the PE setting. Representative results were shown as two-dimensional color density plots of TMRM-stained cells showing the percentage of cells with intact Δψ_m (TMRM fluorescence in top right quadrant). For semiquantitative determination of Δψ_m, TMRM (40 nm) fluorescence was monitored in parental CEM ρ^+ cells and CEM ρ^0 cells before and after treatment with the detergent Nonidet P-40 using a modified Nernst equation, Δψ_m = −60 mV × log(TMRM fluorescence of control)/(TMRM fluorescence intensity of Nonidet P-40 treatment) (22). ROS assays were determined using dichlorofluorescin diacetate and dihydroethidium. Representative results of at least five independent experiments are shown. In each analysis, 10,000 events were recorded.

Transmission Electron Microscopy—CEM cells in the logarithmic proliferation phase were treated with THG (1 μM) with or without the inhibitors as described in figure legends 1, 2, 3, and 5. Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 1 h, washed with 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated with graded series of ethanol, and embedded in LX112. Thin sections were prepared and stained with uranyl acetate. Specimens were examined on a JEOL 1000X electron microscope operating at 80 kV (6).

Subcellular Fractionation and Western Blotting—Subcellular fractionation was performed as described previously in Ref. 6. Protein assay was performed by Dc protein assay (Bio-Rad). Proteins were separated by electrophoresis on 14% SDS-polyacrylamide gels. Bax determination was performed using anti-Bax conformation-dependent antibody (N20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (6). Bip/Grp78 was detected by rabbit polyclonal antibody (Stressgen Biotechnologies); CHOP/GADD153 was detected by rabbit polyclonal antibody (Santa Cruz Biotechnology). Cyp-D was determined using rabbit polyclonal antibody (Santa Cruz Biotechnology). Bak was determined by goat polyclonal antibody (Santa Cruz Biotechnology). Bel-2 was determined by mouse monoclonal antibody (Santa Cruz Biotechnology). Mouse monoclonal anti-OPA1, anti-cytochrome c, anti-β-actin, and anti-manganese-superoxide dismutase antibodies were from BD Biosciences, Pharmin-
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gen. Chemiluminescence detection was performed using an ECL detection kit according to the manufacturer’s instructions (Pierce).

Mitochondrial Isolation and Determination of Large Amplitude Swelling—Rat liver mitochondria were isolated by conventional differential centrifugation from the livers of male adult Sprague-Dawley rats as described previously (6). Isolated rat liver mitochondria were incubated in isolation buffer (200 mM sucrose, 20 μM EGTA, 5 mM succinate, 2 μM rotenone, 1 μg/ml oligomycin, 20 mM Tris, 20 mM HEPES, and 1 mM KH2PO4, pH 7.2). EGTA (1 mM) was used to prevent MPT. For the MPT experiments, EGTA was omitted from the buffers, and 200 μM Ca2+/mg of protein was used to induce large amplitude swelling, which was measured by recording absorbance measurements at 540 nm every 10 s for a total of 8 min (6, 19). At the end of the incubation period, mitochondrial suspensions were centrifuged at 10,000 × g for 5 min, and the resulting supernatants were used for Western blot analysis for OPA1 and cytochrome c. Digitonin (DIG) (100 pmol/mg protein)-treated mitochondria was incubated on ice for 10 min and centrifuged at 10,000 × g for 10 min at 4 °C, and the resulting supernatants were Western blotted for the indicated protein (20). Proteinase K digestion of mitochondrial outer membrane proteins, including VDAC, was used at a concentration of 10 μg/ml. Oxygen consumption was measured with a Clark oxygen electrode (Oxygraph model 5300; Yellow Springs Instrument Co., Yellow Springs, OH) as described previously (6). Mitochondria were energized with 5 mM succinate, and respiratory control ratios were calculated after the addition of 200 μM ADP.

Bax and Cyp-D Gene Silencing with Small Interference RNAs (siRNAs)—Gene silencing with siRNAs (sense and antisense strands) were purchased from 1st Base. The sense strand sequences were: control (5'-UUCUCCGAACGUCCGACGU(dTdT)-3'), Bax (5'-GGUGCGCCGAAACGUGACGA(dTdT)-3'), and Cyp-D (5'-CCUCGUAAAUUGUCCGCUA(dTdT)-3'). Cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions for 48 h as described in Ref. 6.

Statistical Analysis—Data were expressed as the S.E. of three or more separate experiments performed in duplicate. Analysis of variance was used for significance testing (p < 0.05).

RESULTS

MPT Activation Induces the Co-release of OPA1 and Cytochrome c from Isolated Mitochondria and from Mitochondria in Situ—Since structural remodeling of the CJ is sensitive to CsA (20) and is accompanied by the mitochondrial release of the OPA1 protein (23), which allows for the complete release of cytochrome c from mitochondria (24), an initial aim for this study was to determine the relationship between the MPT and structural remodeling of the CJ for cytochrome c release from mitochondria. We determined the release of OPA1 and cytochrome c from rat liver mitochondria after Ca2+ treatment to induce the MPT. Fig. 1A shows a representative example of Ca2+-induced mitochondrial swelling profile of rat liver mitochondria. Mitochondrial swelling was inhibited by MPT inhibitors CsA, BGK, and Ru360. The amount of OPA1 and cytochrome c released into the incubation buffer after MPT was determined. Results indicated that both proteins were co-released from mitochondria in an MPT-dependent manner, since their release was blocked by CsA, BGK, and Ru360, inhibitors of the MPT (Fig. 1B). We next determined whether the MPT in cells would cause the co-release of both OPA1 and cytochrome c. CEM cells were treated with THG with or without CsA, BGK, and Ru360 and mitochondrial Ca2+ levels, Δψm (as an indicator of MPT activation), and cytosolic OPA1 and cytochrome c were determined. THG treatment increased mitochondrial Ca2+ levels (blocked by Ru360 but not CsA or BGK), reduced the percentage of cells displaying Δψm and caused loss of mitochondrial cristae structure blocked by MPT inhibitors (Fig. 1C). THG treatment also caused OPA1 and cytochrome c release from mitochondria of CEM cells blocked by MPT inhibitors (Fig. 1D). Overall, these results indicated that the Ca2+-induced MPT caused co-release of OPA1 and cytochrome c from isolated mitochondria and mitochondria in situ, suggesting that the MPT controls remodeling of the CJ for cytochrome c release.

Cyp-D Regulates the Release of OPA1 and Cytochrome c during THG-dependent Apoptosis—Since pharmacological inhibitors are nonspecific, siRNA was used to knock down the MPT component Cyp-D to confirm the role of the MPT in the release of OPA1 and cytochrome c from mitochondria induced by THG. STS was used as a control, since genetic studies have demonstrated that STS-induced apoptosis is not Cyp-D-regulated (9, 10). Cyp-D siRNA effectively reduced Cyp-D expression ~90% compared with scramble control siRNA or untreated cells (Fig. 2A, top left) and blocked the mitochondrial release of OPA1 and cytochrome c in response to THG (Fig. 2A, top right); however, as expected, Cyp-D knockdown did not block OPA1 and cytochrome c release in response to STS (Fig. 2A, bottom). Δψm and mitochondrial cristae ultrastructure were determined in parental CEM and Cyp-D knockdown cells before and after treatment with THG and STS. Cyp-D knockdown blocked loss of Δψm and mitochondrial cristae ultrastructure after treatment with THG but not STS (Fig. 2, B and C). Cyp-D knockdown also prevented caspase-3 processing and the increase in fraction of apoptotic cells after THG treatment but not STS treatment (Fig. 2D). The indispensable role of Cyp-D in the co-release of OPA1 and cytochrome c was not stimulus- or cell type-specific, since Cyp-D knockdown blocked cytochrome c and OPA1 release caused by other ER stress inducers, including Iono and TM (Fig. 2E, top) and by THG in other cell types, including MEF cells and HCT116 cells (Fig. 2F, middle and bottom).

The MPT and Bax Are Required for the Release of OPA1 and Cytochrome c from Mitochondria—The mechanism of cytochrome c release from mitochondria by the MPT has been suggested to involve matrix swelling and organelle rupture (13–16). However, Bax is also known to play a role in apoptosis by the matrix swelling model, we silenced Bax expression and then induced the MPT by treating Bax knockdown CEM cells with THG. Bax siRNA effectively reduced Bax expression.
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~90% compared with scramble control siRNA or untreated cells but did not significantly change the levels of Bak and Bcl-2 (Fig. 3A). In Bax knockdown cells, THG treatment caused CsA-sensitive loss of Δψm and loss of normal mitochondrial cristae ultrastructure, indicating that THG treatment induced the MPT in Bax knockdown cells (Fig. 3B). However, after Bax knockdown, THG treatment did not release mitochondrial OPA1 and cytochrome c compared with parental CEM cells (Fig. 3C), indicating that the MPT did not induce mitochondrial rupture and cytochrome c release but required Bax for this function. Bax knockdown also prevented caspase-3 processing and the increase in fraction of apoptotic cells after THG treatment (Fig. 3D). Cytochrome c and OPA1 release induced by Iono and TM was blocked by Bax knockdown (Fig. 3E, top), and we did not observe the co-release induced by THG in HCT116 Bax−/− cells and MEF Bax/Bak DKO cells (Fig. 3E, middle and bottom), suggesting that the requirement of MPT and Bax for the co-release of OPA1 and cytochrome c is a universal mechanism.

OPA1 and Cytochrome c Release from Isolated Mitochondria Occurs Independently of VDAC and the Mitochondrial Outer Membrane—Since our model suggested that the MPT regulated the structure of the cristae and C (i.e. the mitochondrial inner membrane), we considered that outer membrane VDAC might not be a necessary component of the MPT, as previously suggested (25). Mitochondria were first treated with proteinase K to remove the VDAC by digestion (26) and then treated with Ca2+ to induce MPT. Mitochondrial swelling profiles before and after proteinase K treatment were monitored (Fig. 4A). We observed no significant difference either between the mitochondrial swelling profiles or the quantity of OPA1 and cytochrome c released into the incubation buffer before or after proteinase K treatment (Fig. 4, A and B). Mitochondria were next preincubated with DIG at concentrations previously found to remove the outer membrane (6, 20) and treated with Ca2+ to induce MPT. Mitochondrial swelling profiles before and after DIG treatment were monitored (Fig. 4C). We observed no significant difference either between the mitochondrial swelling profiles or the quantity of OPA1 and cytochrome c released into the incubation buffer before or after DIG treatment (Fig. 4, C and D, bottom). The effects of DIG on the extent of mitochondrial coupling were determined by monitoring mitochondrial respiratory control ratios, as previously described (6, 20), and were not found to be significantly different before or after DIG treatment at the concentration of DIG used (100 pmol/mg protein) (respiratory control ratio before DIG treatment = 6.2 compared with 5.6 after DIG treatment) (Fig. 4D, top). Overall, the results indicated that the major fraction of cytochrome c is stored in mitochondrial cristae together with other ETC components, as previously found (6, 19, 20), and its release is independent of VDAC and the outer membrane.
FIGURE 2. THG-induced loss of ΔΨm, mitochondrial ultrastructure, and caspase-3-dependent apoptosis is regulated by Cyp-D. A, CEM cells were treated with either incubation medium, control scramble siRNA, or siRNA Cyp-D for 48 h, and cell lysates were separated by SDS-PAGE and Western blotted for Cyp-D as described under “Experimental Procedures” (top left). Parental CEM cells and Cyp-D knockdown cells were treated with THG or STS for the times indicated, and OPA1 and cytochrome c (Cyt c) release were determined as described in the legend to Fig. 1 (top right and bottom). B, ΔΨm was determined in CEM parental and Cyp-D knockdown cells before and after treatment with THG and STS for the times indicated as described in the legend to Fig. 1. Values shown indicate the percentage of cells with intact ΔΨm. C, transmission electron microscopy was determined in CEM parental and Cyp-D knockdown cells before (NT) and after treatment with THG for 36 h as described in the legend to Fig. 1. D, CEM parental and Cyp-D knockdown cells were treated with THG for 36 h or STS for 12 h, and caspase-3 activity and cell cycle analysis was performed as described under “Experimental Procedures.” The bar graphs show caspase-3 activity (left) and the percentage of cells in the sub-G1 fraction (right) determined (mean ± S.E., n = 3). E, parental CEM cells and Cyp-D knockdown cells were treated with Iono (2 μM) or TM (10 μg/ml) for 24 h, and OPA1 and cytochrome c release were determined as described in the legend to Fig. 1 (top). Wild type HCT116 and MEF cells before and after Cyp-D knockdown were treated with THG for the times indicated, and OPA1 and cytochrome c release were determined as described in the legend to Fig. 1 (middle and bottom). WT, wild type.
The ETC Is Indispensable for THG-induced MPT—To investigate the role of the ETC in THG-dependent cell death, we created CEM cells lacking mitochondrial DNA ($\rho^0$) as previously described (21). CEM cells were assigned $\rho^0$ status when KCN-sensitive oxygen consumption was reduced by $\sim 90\%$ (data not shown), and cytochrome oxidase II expression was reduced by $\sim 90\%$ compared with parental CEM ($\rho^+$) cells (Fig. 5A, top left). $\rho^+$ and $\rho^0$ cells were treated with THG, and $\Delta\psi_{m}$, mitochondrial ultrastructure, and OPA1 and cytochrome c release were determined. Cells were also treated with STS for control purposes. $\rho^0$ cells resisted loss of $\psi_{m}$ and mitochondrial cristae ultrastructure compared with $\rho^+$ cells in response to THG (Fig. 5B), and mitochondrial release of OPA1 and cytochrome c, caspase-3 processing, and induction of apoptosis were significantly blocked compared with $\rho^+$ cells (Fig. 5A, top right), C, and D). In contrast, $\rho^0$ cells did not prevent STS-induced cell death when the same parameters were analyzed (Fig. 5, A (bottom), B, C, and D).

Ca$^{2+}$ Signaling, Bax Activation, and Induction of the UPR Is Conserved in CEM $\rho^0$ Cells—Three independent experiments showed that mitochondrial Ca$^{2+}$ signaling in $\rho^0$ cells was not significantly different from that in $\rho^+$ cells. THG-induced Rhod2 fluorescence was determined by LSCM in single cells and by spectrofluorimetry in cell populations. The THG-dependent Rhod2 fluorescence increase was similar in magnitude in $\rho^+$ and $\rho^0$ cells, and both signals were inhibited by Ru360 (Fig. 6, A and B). Rhod2 fluorescence was also determined by FACS analysis, producing similar results (data not shown). Next, we semiquantitatively estimated the $\Delta\psi_{m}$ of $\rho^0$ cells to determine whether the value obtained was consistent with electrophoretic uptake of Ca$^{2+}$, since the $\Delta\psi_{m}$ of $\rho^0$ cells is known to be lower than $\rho^+$ cells (27). $\Delta\psi_{m}$ of $\rho^0$ cells was estimated to be $\sim 20\text{ mV}$.
that THG treatment induced a similar level of ER stress in both \( \rho^+ \) and \( \rho^0 \) cells, which was independent of Ca\(^{2+} \). Since ER-cytosolic-mitochondrial Ca\(^{2+} \) signaling, Bax activation, and the UPR were similar in both \( \rho^+ \) and \( \rho^0 \) cells, the results indicated that MPT activation depended on a functional ETC.

**Mitochondrial ROS Do Not Regulate the MPT and Apoptosis during THG-mediated ER Stress**—Since ROS are important regulators of the MPT (29, 30), we considered that ROS produced by the ETC might be the trigger for MPT activation, which would explain the resistance to MPT in \( \rho^0 \) cells, because they do not produce mitochondrial ROS (31). To investigate this, \( \rho^+ \) cells were incubated with the antioxidants NAC and MnTBAP and treated with THG. Since STS-induced apoptosis is known to be regulated by ROS (32), cells were also treated with STS with or without the antioxidants NAC and MnTBAP for control purposes. \( \rho^0 \) cells were used to determine whether ROS were of mitochondrial origin (31). Cells were treated with either THG or STS with or without NAC or MnTBAP, and ROS production was determined by FACS analysis by monitoring the fluorescence of ROS-sensitive dyes dichlorofluorescein and hydroethidine (31). Although ROS production was observed after THG treatment in \( \rho^+ \) cells, no ROS increase was found to occur in \( \rho^0 \) cells, indicating that the source of the ROS was the ETC (Fig. 7A, top). Antioxidant pretreatment with NAC and MnTBAP blocked THG-induced ROS increase (Fig. 7A, top) but did not prevent either mitochondrial release of OPA1 and cytochrome \( c \), loss of \( \Delta \psi_m \), caspase-3 activation, or loss of cell viability (Fig. 7, B–E). In contrast, ROS production was observed in both \( \rho^+ \) and \( \rho^0 \) cells after STS treatment, indicating that the source of the ROS was not the ETC (Fig. 7A, bottom). Also, although antioxidant pretreatment with NAC and MnTBAP blocked STS-induced ROS increase (Fig. 7A, bottom), it also effectively prevented the mitochondrial release of OPA1 and cytochrome \( c \), loss of \( \Delta \psi_m \), caspase-3 activation, and loss of cell viability (Fig. 7, B–E). Taken together, the results show that the MPT was not triggered by mitochondrial ROS and that THG-dependent apoptosis was not redox-regulated.

**DISCUSSION**

In this study, we investigated the relationship between the MPT and remodeling of the mitochondrial CJ for cytochrome \( c \) release and apoptosis. Three lines of evidence indicated that the MPT regulated CJ remodeling and cytochrome \( c \) release from mitochondria and that death signaling was transduced by the mitochondrial matrix peptidyl-prolyl isomerase Cyp-D (33). First, the inhibition of mitochondrial Ca\(^{2+} \) uptake with the ruthenium analogue Ru360 prevented OPA1 and cytochrome \( c \) release from isolated mitochondria and mitochondria of CEM cells, which blocks apoptosis (6). Second, CsA, which blocks the interaction of Cyp-D with the ANT (34), also prevented OPA1 and cytochrome \( c \) release from isolated mitochondria and mitochondria of CEM cells, which blocks apoptosis (6). Third, siRNA knockdown of Cyp-D also blocked OPA1 and cytochrome \( c \) release from mitochondria of CEM cells and prevented loss of \( \Delta \psi_m \) and caspase-3-dependent apoptosis. These results are in agreement with the ideas that the MPT regulates the structural reorganization of mitochondria, in

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**FIGURE 4.** The VDAC channel and mitochondrial outer membrane are not required for the MPT-dependent release of OPA1 and cytochrome \( c \) in isolated mitochondria. A, mitochondria were pretreated with 10 \( \mu \)g/ml proteinase K on ice for 10 min, and phenylmethylsulfonyl fluoride (1 mM) was added to stop the reaction. Mitochondria were then repelleted by centrifugation and treated with 200 \( \mu \)M Ca\(^{2+} \), and mitochondrial swelling was monitored continuously at 540 nm. Shown are representative swelling curves induced by untreated control mitochondria (trace 1), untreated control mitochondria previously incubated with proteinase K (trace 2), mitochondria incubated with proteinase K and treated with 200 \( \mu \)M Ca\(^{2+} \) (trace 3), and mitochondria treated with 200 \( \mu \)M Ca\(^{2+} \) (trace 4). B, at the end of the mitochondrial swelling experiment, samples were centrifuged, and the resulting supernatants were separated by SDS-PAGE and Western blotting for OPA1 and cytochrome \( c \) as described under “Experimental Procedures.” VDAC protein was determined in mitochondrial pellets before and after proteinase K treatment for control (CTL) purposes by SDS-PAGE and Western blotting as described under “Experimental Procedures.” C, mitochondria were incubated with 100 pmol/mg protein DIG on ice for 10 min, pelleted by centrifugation, and treated with 200 \( \mu \)M Ca\(^{2+} \). The mitochondrial swelling profile was monitored before and after DIG treatment as described in the legend to Fig. 1. Shown are representative swelling curves induced by untreated control mitochondria (trace 1), untreated control mitochondria incubated with DIG (trace 2), mitochondria incubated with DIG and treated with 200 \( \mu \)M Ca\(^{2+} \) (trace 3), and mitochondria treated with 200 \( \mu \)M Ca\(^{2+} \) (trace 4). D, top, respiratory control ratios (RCR) were determined on mitochondria before and after DIG treatment, as previously described (6). Bottom, at the end of the mitochondrial swelling experiment, samples were centrifuged, and the resulting supernatants were separated by SDS-PAGE and Western blotting for OPA1, cytochrome \( c \) (Cyt c), and VDAC as described under “Experimental Procedures.”
this case the remodeling of the CJ, in response to a Ca^{2+} challenge and regulates mitochondrial responses to cell death signals (35).

Although early ideas on how the MPT released cytochrome c for apoptosis suggested a nonspecific mechanism involving organelle swelling and rupture (13–16), this notion is controversial, since it has been found that mitochondria can release cytochrome c in the absence of swelling (17, 18). An alternative idea has shown that the cytochrome c release mechanism is highly regulated and involves discrete steps, both at the level of the mitochondrial cristae and CJ and at the mitochondrial outer membrane (19, 20). For example, studies have shown that cytochrome c needs to be solubilized, or dissociated, from other components of the ETC (19) and then diffuses into the intermembrane space following the structural remodeling of the CJ (20). This event is followed by a second major step requiring the activation and translocation to the mitochondrial outer membrane of Bcl-2 proapoptotic proteins, such as Bax (19) or Bak (20), which facilitates the release of cytochrome c to the cytosol for apoptosis (6). Although our results using isolated mitochondria confirm that in vitro the MPT leads to the release of cytochrome c, presumably by organelle rupture, as previously proposed (13–16), this does not appear to be the mechanism by which the MPT releases cytochrome c for apoptosis during ER stress, since in the absence of Bax, cytochrome c was not released from mitochondria (Fig. 3).

**FIGURE 5.** The ETC regulates the MPT and mitochondrial release of OPA1 and cytochrome c for apoptosis. A, CEM parental ρ^+ cells were treated for 4–6 weeks with 50 ng/ml ethidium bromide, after which time they were designated CEM ρ^0 cells. Cell lysates from ρ^+ cells and ρ^0 cells were separated by SDS-PAGE and Western blotted for cytochrome oxidase II protein subunit expression (top left). ρ^+ and ρ^0 cells were treated with THG or STS for the times indicated, and OPA1 and cytochrome c release was determined as described in the legend to Fig. 1 (top right and bottom). B, ρ^+ and ρ^0 cells were treated with THG for 36 h or STS for 9 h, and ΔΨm, together with mitochondrial ultrastructure were determined as described in the legend to Fig. 1. C, caspase-3 activity was determined in ρ^+ and ρ^0 cells after treatment with THG for 36 h or STS for 9 h as described in the legend to Fig. 2. D, cell cycle analysis of ρ^+ and ρ^0 cells was performed, and the percentage of cells in the sub-G1 fraction was determined before and after treatment with either THG for 36 h or STS for 9 h as described in Fig. 2. Insets, representative ρ^+ and ρ^0 cells treated with THG or STS and stained with DAPI as described under “Experimental Procedures.”
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![Figure 6](https://via.placeholder.com/150)

**A** and cytochrome c from isolated mitochondria and from mitochondria of CEM cells. Similarly, the role of the VDAC as a structural component of the MPT has been under scrutiny, since it was shown that Cyp-D bound to complexes of the ANT and VDAC (25). However, our results indicate, at least in isolated mitochondria, that the MPT is exclusively an inner membrane phenomenon, and remodeling of the CJ for cytochrome c release occurs independently of the VDAC channel as well as the outer membrane, in agreement with recent genetic evidence showing that VDACs are not structural components of the MPT (38).

Previous reports have clearly shown that the mitochondrial ETC is dispensable for apoptosis (39, 40); however, these studies used STS as the apoptotic stimulus, and it is known that STS-induced apoptosis is Cyp-D-independent and therefore not regulated by the MPT (9, 10). Also, previous reports have found that the MPT is regulated by complexes I and III of the ETC (41, 42). Thus, we investigated a possible role for the ETC in regulation of the MPT during ER stress. CEM ρ0 cells, lacking a functional ETC (21), were resistant to the release of OPA1 and cytochrome c and did not undergo apoptosis, indicating that electron transport was necessary for MPT activation. However, ρ0 cells are known to possess a significantly lower ΔΨm compared with parental cells (27), which could desensitize the MPT by decreasing electrophoretic uptake of Ca2+ by the uniporter. Thus, we compared THG-induced mitochondrial Ca2+ levels in both ρ+ and ρ0 cells. We also considered that differences in Bax activation and the UPR could account for differences in sensitivity of the ρ0 cells to THG-induced apoptosis. Therefore, we compared Bax activation and translocation to mitochondria and the induction of Bip/GRP78 (1–3) and the transcription factor CHOP (5) in ρ+ and ρ0 cells (Figs. 6, 1, D, E, and G). We found that there was no significant difference in mitochondrial Ca2+ levels after THG treatment using three independent measures for monitoring mitochondrial Ca2+ levels, including LSCM, fluorescence spectrofluorimetry, and FACS analysis (Fig. 6, A and B) (FACS analysis data not shown). Furthermore, semiquantitative estima-

The structure of the MPT has been the subject of intense investigation for a number of years (7, 8). Recent genetic evidence has shown that mitochondria lacking all ANT isoforms were still able to undergo the MPT, suggesting that the ANT, although it was still considered an MPT regulator, was not an essential structural component of the MPT pore (37). Our results also indicate that the ANT is an important regulatory component of the MPT, since its inhibition with the matrix surface inhibitor BGK effectively blocked the release of OPA1 and cytochrome c from isolated mitochondria and from mitochondria of CEM cells. Similarly, the role of the VDAC as a structural component of the MPT has been under scrutiny, since it was shown that Cyp-D bound to complexes of the ANT and VDAC (25). However, our results indicate, at least in isolated mitochondria, that the MPT is exclusively an inner membrane phenomenon, and remodeling of the CJ for cytochrome c release occurs independently of the VDAC channel as well as the outer membrane, in agreement with recent genetic evidence showing that VDACs are not structural components of the MPT (38).

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tion of the $\Delta \psi_m$ of $p^0$ cells, which is required to drive the $Ca^{2+}$ uniporter, indicated a $\Delta \psi_m$ of $\sim 110$ mV compared with an idealized estimate of $130$ mV for parental CEM cells (22) (Fig. 6C). The fact that $Ca^{2+}$ uptake by the uniporter reaches a plateau at $\sim 110$ mV (28) suggests that $Ca^{2+}$ uptake in these cells was not limiting and, thus, could not explain the failure to undergo MPT in response to THG treatment. Although, $p^0$ cells were slightly resistant to STS-induced release of cytochrome $c$ compared with $p^+$ cells (Fig. 5A, bottom), we considered that this could be the result of increased antioxidant defenses that $p^0$ cells possess (43, 44). This property, however, was not considered responsible for the lack of apoptosis in $p^0$ cells treated with THG, since both $p^+$ and $p^0$ cells were equally sensitive to STS-induced apoptosis (Fig. 5D).

Since the MPT is known to be regulated by ROS (29, 30), we determined whether THG treatment increased ROS production in CEM cells. Using two different ROS-sensitive probes, including dihydroethidine for superoxide anion and dichlorofluorescein diacetate for GSH oxidation and hydrogen peroxide ($H_2O_2$) (31), we observed rapid ROS production over 4 h in response to THG treatment. The ROS source was considered to

![FIGURE 7. The MPT induced by ER stress is not redox-regulated.](image)

![FIGURE 8. Role of the mitochondrion in ER stress-induced apoptosis.](image)
Role of MPT in Cytochrome c Release

be mitochondrial, because ROS production was absent in ρ₀ cells (31). Although antioxidant treatment abolished ROS production, it did not prevent sensitivity to MPT activation and induction of apoptosis by THG. Since ROS, especially H₂O₂ (10, 45), and Ca²⁺ (6–8, 10) are both known to be activators of the MPT, our results suggest possible differences in the MPT trigger mechanism in different settings. For example, in ischemia and reperfusion injury, ROS are crucial activators of the MPT, whereas Ca²⁺ is not considered to be involved (45). In summary, we show that the MPT regulates the structural remodeling of the CJ, allowing cytochrome c release for apoptosis during ER stress. This process is dependent on a functional ETC but does not depend on mitochondrial ROS production and therefore is not redox-regulated (Fig. 8).

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