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Mitochondrial membrane potential regulates matrix configuration and cytochrome *c* release during apoptosis

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Abstract

During apoptosis, the mitochondrial membrane potential (MMP) decreases, but it is not known how this relates to the apoptotic process. It was recently suggested that cytochrome *c* is compartmentalized in closed cristal regions and therefore, matrix remodeling is required to attain complete cytochrome *c* release from the mitochondria. In this work we show that, at the onset of apoptosis, changes in MMP control matrix remodeling prior to cytochrome *c* release. Early after growth factor withdrawal the MMP declines and the matrix condenses. Both phenomena are reversed by adding oxidizable substrates. In mitochondria isolated from healthy cells, matrix condensation can be induced by either denying oxidizable substrates or by protonophores that dissipate the membrane potential. Matrix remodeling to the condensed state results in cristal unfolding and exposes cytochrome *c* to the intermembrane space facilitating its release from the mitochondria during apoptosis. In contrast, when a transmembrane potential is generated due to either electron transport or a pH gradient formed by acidifying the medium, mitochondria maintain an orthodox configuration in which most cytochrome *c* is sequestered in the cristae and is resistant to release by agents that disrupt the mitochondrial outer membrane.

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Keywords: apoptosis; mitochondrial membrane potential; matrix configuration; cytochrome *c*

Abbreviations: MMP, mitochondrial membrane potential; EM, electron microscopy; TMRE, tetramethyl-rhodamine ethyl ester; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; BH3, Bcl–2 homology region-3; MIB, mitochondrial isolation buffer; MRB, mitochondrial reaction buffer.

Introduction

In addition to being the source of energy that supports life under aerobic conditions, mitochondria can also be the source of signals that initiate apoptotic cell death.[1] Mitochondria contain key regulators of caspases; a family of proteases that are major factors in many apoptotic processes.[2] The release of cytochrome *c* from the mitochondrial intermembrane space induces the assembly of the apoptosome that is required for activating downstream caspases.[3,4] Cytochrome *c* release from mitochondria is a key event in initiating apoptosis, but the actual mechanism of its release is still debatable. In particular, the relation between mitochondrial physiology and the release of cytochrome *c* and other apoptogenic factors from mitochondria is not clear.

Mitochondria utilize oxidizable substrates to produce a membrane potential in the form of a proton gradient across the mitochondrial inner membrane. It was shown recently that the supply of oxidizable substrates to mitochondria depends on the concentration of external growth factors.[5] Withdrawal of growth factors or loss of the extracellular glucose supply will lead to a decline in mitochondrial membrane potential (MMP). If growth factor or glucose deprivation persists, cells ultimately undergo apoptosis that is initiated by cytochrome *c* release from mitochondria.[5,6] Whether changes in mitochondrial physiology contribute to the initiation of cell death in response to growth factor withdrawal remains a controversial issue.[7]

It was recently suggested that structural changes in mitochondria may be required to achieve complete and rapid cytochrome *c* release.[8] In this work, we demonstrate a direct relation between mitochondrial structure and function. A decrease in MMP leads to matrix condensation and exposure of cytochrome *c* to the intermembrane space, facilitating cytochrome *c* release and cell death following an apoptotic insult.

Results

Mitochondrial matrix condenses because of substrate limitation after IL-3 deprivation

We used the IL-3-dependent FL5.12 cell line to examine mitochondrial structure following growth factor withdrawal. Electron microscopy (EM) analysis was performed at 6 and 12 h after IL-3 withdrawal and compared to electron micrographs of cells maintained with IL-3. It is important to note that the cells were still viable at both time points of analysis and more than 90% of the cells were capable of recovery, as assayed by clonogenicity in IL-3-containing medium (data not shown). Changes in mitochondrial morphology 6 and 12 h after IL-3 removal were observed by EM (Figure 1a). The mitochondria became progressively smaller than those in

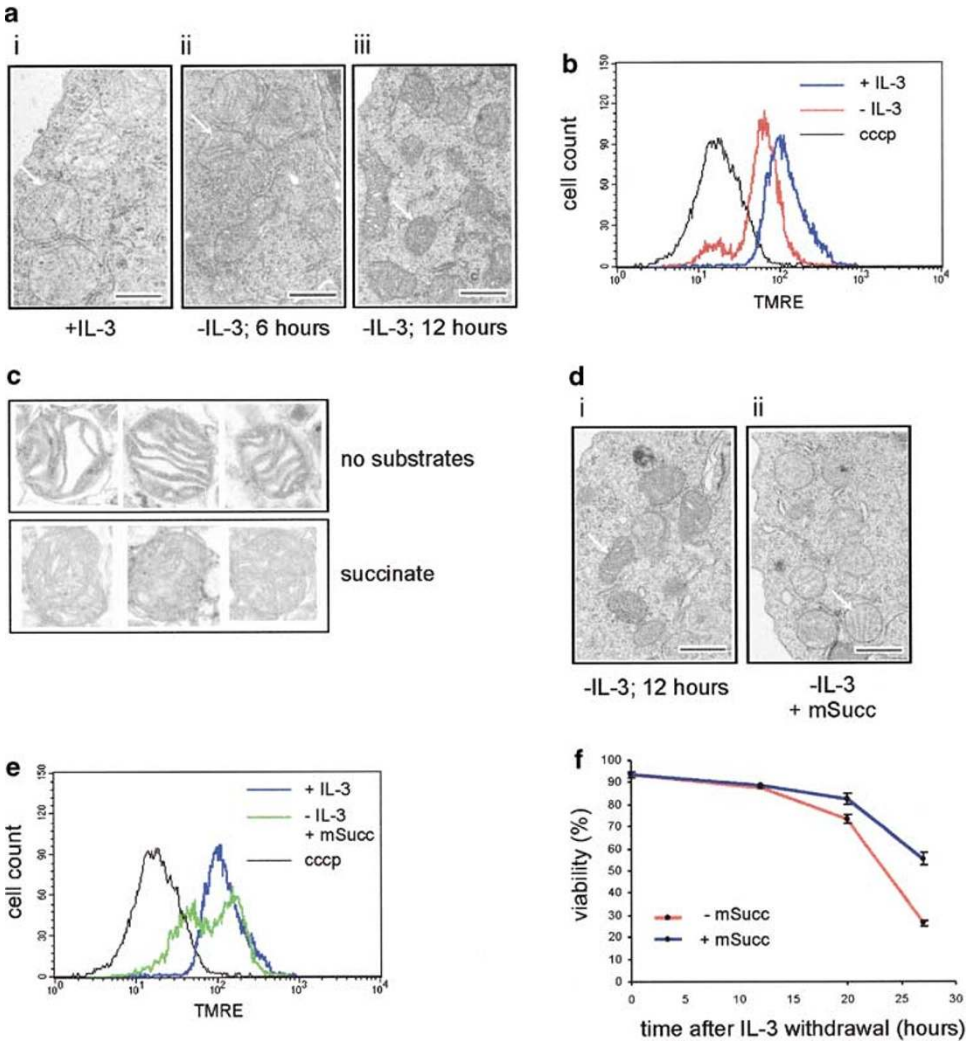


Figure 1 Mitochondrial matrix condenses because of the limitation in oxidizable substrates after IL-3 withdrawal. (**a**) FL5.12 cells were grown in the presence (i) or absence of IL-3 for 6 h(ii) or 12 h (iii) and fixed for EM analysis. The bar represents 500 nm length and the white arrows point at one representative mitochondrion in each panel. (**b**) Cells were grown in the presence or absence of IL-3 for 12 h, followed by MMP analysis with 25 nM TMRE staining followed by FACS analysis. The protonophore CCCP was used to dissipate the membrane potential and to define the baseline for the analysis of mitochondrial potential by TMRE fluorescence. 50 μM CCCP was added to cells growing in the presence of IL-3 5 min before the FACS analysis. (**c**) Mitochondria were isolated from cells growing with IL-3 and incubated in MRB with or without 7 mM succinate for 10 min before fixation and EM analysis. (**d**) Cells were incubated without IL-3 for 12 h (i, ii). Where indicated (ii), 5 mM methyl succinate (mSucc) was added to cells growing in the absence of IL-3 15 min before fixation and analysis. (**e**) A measure of 5 mM methyl succinate was added every 3 h to cells growing in the absence of IL-3. TMRE staining followed by FACS analysis was used to assess MMP. A comparative analysis of untreated (+IL-3) and treated (-IL-3 +mSucc) cells is presented. (**f**) Viability analysis of cells incubated in the absence of IL-3 either with or without methyl succinate (as in panel E). Viability was determined by propidium iodide exclusion and assessed by FACS analysis

control cells, and the matrix became increasingly more dense. This condensation correlates with a decrease in MMP, as assessed by staining with the lipophilic cationic dye tetramethyl-rhodamine ethyl ester (TMRE) (Figure 1b). In subsequent experiments, 12 h of IL-3 withdrawal was used because a reproducible decline in MMP is observed (Figure 1b). In contrast, at later time points, when cells are initiating apoptosis, paradoxical increases in MMP have been reported,[9–11] which may represent dequenching of rhodamine-based dyes or the metabolic acidosis that occurs under conditions of reduced ATP synthesis.

When mitochondria, isolated from FL5.12 cells growing in the presence of IL-3, were incubated *in vitro* in a buffer lacking oxidizable substrates, they assumed a condensed configuration (Figure 1c). Addition of succinate, an electron donor to complex II of the respiratory chain (succinate dehydrogenase), resulted in matrix expansion to the 'orthodox configuration' normally observed in healthy cells (Figure 1c), demonstrating that the availability of oxidizable substrates plays a role in regulating matrix configuration.[12] Therefore, it was plausible that in growth factor-deprived cells, substrate deficiency was the reason for matrix condensation. To address this possibility, cells were withdrawn from IL-3 for 12 h and then incubated for 15 min in the presence of methyl succinate, a membrane permeable substrate of complex II. EM analysis showed that mitochondria responded to methyl succinate by readopting an orthodox configuration (Figure 1d), indicating that matrix condensation following IL-3 with-

drawal is the result of substrate deficiency. In addition, the decrease in MMP following IL-3 withdrawal was partially prevented by methyl succinate (Figure 1e). To evaluate the importance of substrate-regulated mitochondrial configuration to apoptosis, FL5.12 cells were maintained in the absence of IL-3 with or without periodic additions of 5 mM methyl succinate every 3 h. Viability analysis demonstrated significant protection from apoptosis by methyl succinate (Figure 1f).

MMP regulates matrix configuration

The above data suggested that matrix condensation in IL-3-deprived cells is because of a decline in oxidizable substrates and a consequent drop in MMP. To test if mitochondrial structure is regulated by membrane potential, we pharmacologically manipulated the MMP of FL5.12 cells that were maintained in the presence of IL-3 and followed changes in mitochondrial structure by EM analysis. Rotenone blocks respiration at complex I of the mitochondrial respiratory chain (NADH dehydrogenase) and therefore decreases the redox-dependent proton pumping by complexes I, III and IV. Incubating cells with rotenone for 15 min resulted in a decline in MMP (Figure 2ai) that was comparable to that of IL-3-deprived cells (Figure 1b). EM analysis showed that mitochondria became condensed in response to rotenone treatment even in the presence of IL-3 (Figure 2aii and iii). Oligomycin is a blocker of ATP synthase that prevents proton passage through the enzyme, thus inducing mitochondrial membrane hyperpolarization. Treatment of cells that were maintained in the presence of IL-3 with oligomycin for 5 min resulted in mitochondrial swelling beyond the orthodox configuration (Figure 2bii). To confirm that mitochondrial swelling was because of hyperpolarization, the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was added to depolarize the mitochondria in oligomycin-treated cells (Figure 2bi). Bypassing ATP synthase with CCCP resulted in reversal of the mitochondrial swelling (Figure 2biii). However, the mitochondria of oligomycin-treated cells do not condense as completely with CCCP as the mitochondria from cells treated with rotenone. This appears to result from the associated depolarization of the plasma membrane in intact cells, because isolated mitochondria treated with CCCP in the presence of substrate undergo condensation equivalent to that achieved with rotenone treatment (Figure 3).

To avoid cytosolic effects on mitochondria during treatment, and to explore the direct relations between MMP and mitochondrial configuration, we turned to an *in vitro* analysis of isolated mitochondria, in which membrane potential could be regulated by manipulating the availability of oxidizable substrates to the respiratory chain and by protonophores. Mitochondria were isolated from FL5.12 cells growing in the presence of IL-3 and MMP was analyzed *in vitro* using the rhodamine 123 quenching technique.[13] Matrix configuration was determined either by EM analysis, or by light scattering – the more condensed the matrix, the more light the mitochondria diffract.[13,14] When mitochondria were incubated in the absence of oxidizable substrates and in the presence of rotenone, light scattering and rhodamine 123 fluorescence were high, indicating matrix condensation and low MMP, respectively (Figure 3ai). Upon addition of succinate, an

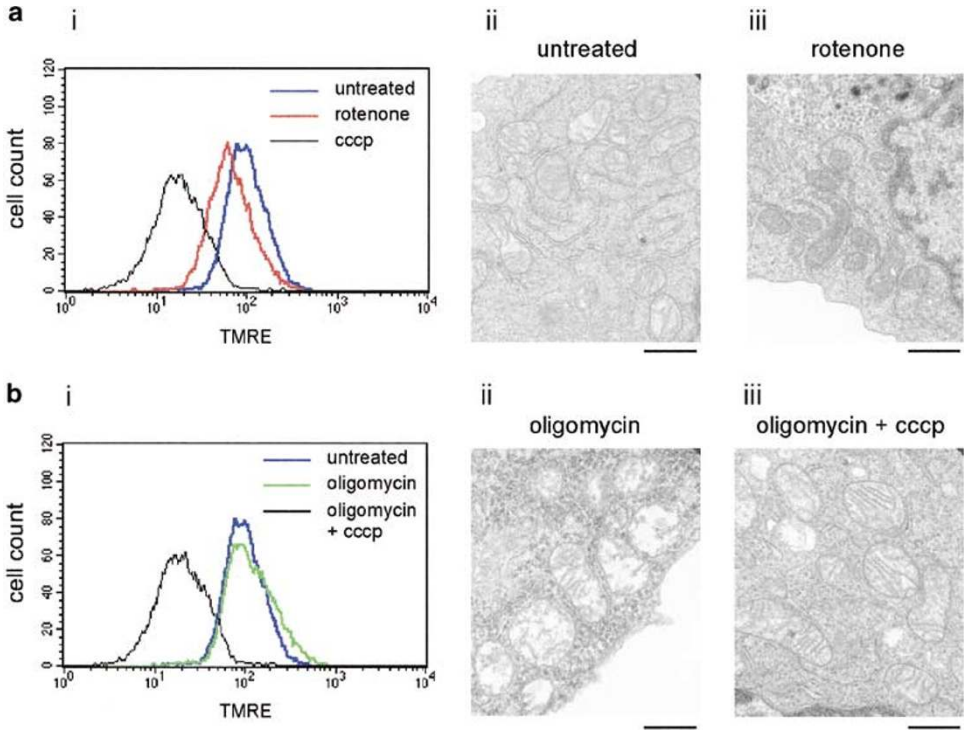


Figure 2 MMP regulates matrix configuration in cells. (**a**) Cells growing in the presence of IL-3 were either left untreated or treated with 5 μM rotenone for 15 min prior to TMRE staining and FACS analysis (i) or EM (ii, iii) analysis as in Figure 1. (**b**) Cells growing in the presence of IL-3 were treated with 10 μg/ml oligomycin for 5 min, with or without 50 μM CCCP and analyzed by FACS (i) or EM (ii, iii). The bar represents 500 nm length

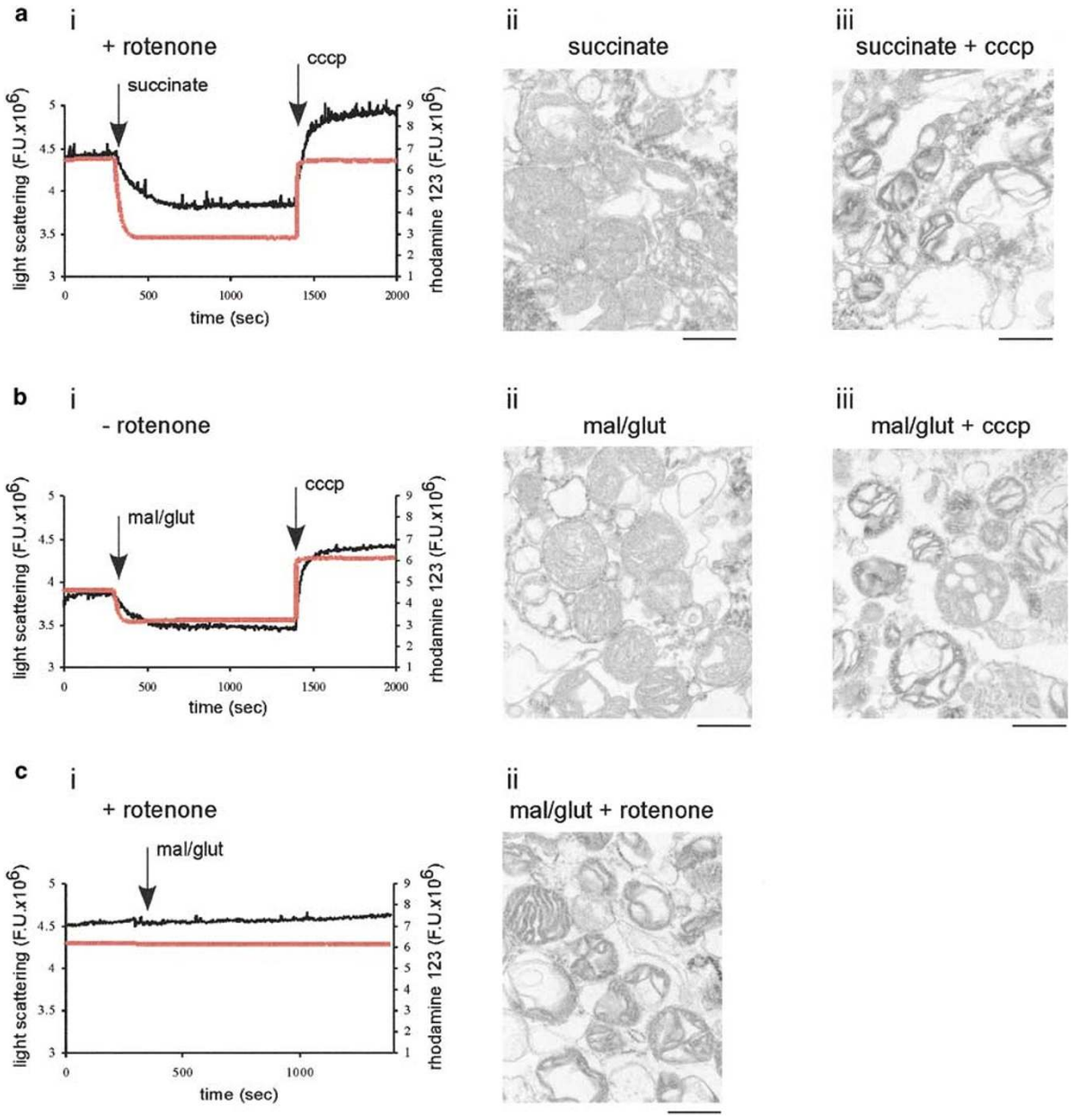


Figure 3 MMP regulates matrix configuration *in vitro*. (**a–c**) Mitochondria were isolated from FL5.12 cells growing in the presence of IL-3 and analyzed for membrane potential using rhodamine 123 quenching (red lines) and for matrix configuration using light scattering (black lines) in a spectrofluorometer (i) or by EM (ii and iii) under the indicated conditions. The bar represents 500 nm length; FU=fluorescent units; mal/glut=malate and glutamate

immediate increase in MMP was detected followed by a delayed change in light diffraction (Figure 3ai). This change in diffraction resulted from conversion of the matrix to the orthodox configuration as was confirmed by EM (Figure 3aii). Addition of CCCP to the assay resulted both in a dissipation of the membrane potential and in an increase in mitochondrial light diffraction (Figure 3ai). EM analysis confirmed that the matrix recondensed (Figure 3aiii). The same results were observed when mitochondria were treated with malate and glutamate that supply electrons to complex I of the respiratory chain by increasing mitochondrial NADH (Figure 3b). In this case, rotenone was omitted from the assay to enable complex I activity, therefore mitochondria maintained some of their membrane potential even without exogenous substrates, because of endogenous NADH (Figure 3bi). This partial potential could be dissipated with rotenone (compare the initial level of fluorescence in Figure 3bi to 3ai). When malate and glutamate were added to mitochondria, a rapid increase in MMP was detected and followed by a configuration change (Figure 3bi and ii). As was the case when succinate was used as an electron donor, adding CCCP resulted in a decline of the membrane potential and in matrix condensation (Figure 3bi and iii). In contrast, when malate and glutamate were added to mitochondria in the presence of rotenone, no change in membrane potential or matrix configuration was detected (Figure 3c). This indicates both that these mitochondrial

modifications were not because of changes in ionic strength caused by the addition of substrates, and that matrix condensation can occur independently of the respiratory rate; since malate- and glutamate-energized mitochondria respire at their maximal rate with CCCP, and do not respire in the presence of rotenone (data not shown).

To verify that following mitochondrial depolarization the matrix becomes more condensed, a morphometric analysis was performed. In the EM analysis, increased opacity of the matrix resulted from increased diffraction of electrons, making the matrix appear darker in the image. Therefore, the more condensed the matrix, the more electrons it diffracts and the darker it appears on the micrograph. Using Scion Image for Windows (Scion Corporation), electron micrographs were converted to a density plot, in which the *Z*-axis is representative of the density of each pixel. Digital pixel density is numerically expressed from 0 to 255, and a color gradient from white to red was then applied to better distinguish between areas of different densities. In a typical mitochondrion that was incubated in the presence of succinate (orthodox configuration), the matrix is less dense and occupies more area (in a 3D image – this will represent volume) than matrix from a mitochondrion incubated with succinate and CCCP (Figure 4ai and ii). A density threshold above 50 was used to define the matrix area and 25 mitochondria from each treatment were analyzed for matrix density and the percent of image area occupied by matrix. These results showed a significant increase in matrix density ($P<0.0001$; Student's *t*-test) and a decrease in matrix area ($P<0.0001$) in depolarized mitochondria as compared to energized mitochondria, providing objective evidence that matrix volume decreases and density increases when mitochondria are depolarized (Figure 4b and c).

The above results suggest a connection between MMP and mitochondrial matrix configuration. To confirm that the rhodamine 123 quenching assay measures changes in MMP and not mitochondrial volume, we examined the relation of mitochondrial volume to MMP in purified mitochondria in which the volume is manipulated by stepwise addition of sucrose to the medium. Mitochondria were initially incubated in 100 mM sucrose buffer. Light scattering and rhodamine 123 fluorescence were analyzed during

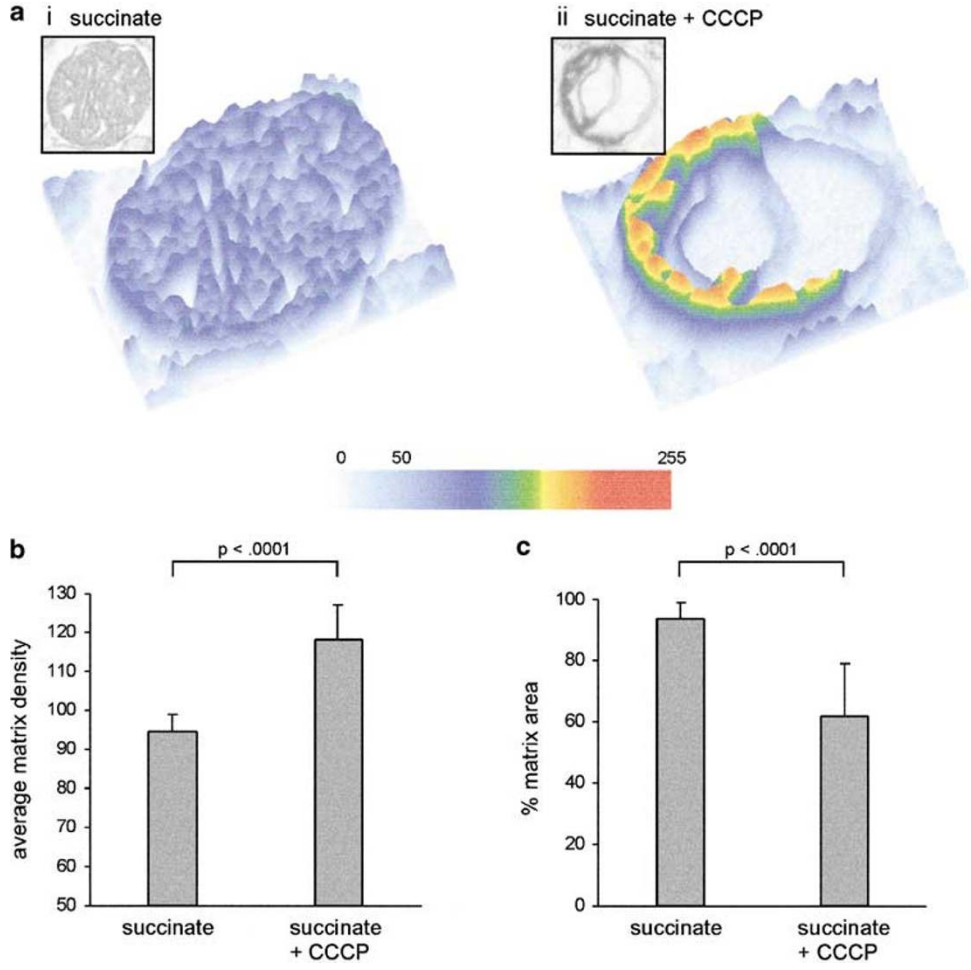


Figure 4 Depolarized mitochondria adopt a condensed configuration. (**a**) Mitochondria were incubated in MRB with 5 μM rotenone and 7 mM succinate with (ii) or without (i) 50 μM CCCP for 10 min. Following fixation and EM analysis, the digital images of individual mitochondria were analyzed by Scion Image for Windows to display color-mapped density on the *Z*-axis. Representative mitochondria of each treatment are presented. The matrix area was defined as a density unit above 50. (**b** and **c**) Based on the analysis described in panel A, 25 mitochondria from each treatment were analyzed for the average density of the matrix (**b**) or the percentage of the area that the matrix occupied in each image (**c**)

consecutive increases in the concentration of sucrose (50 mM increments). While light scattering was significantly affected by the addition of sucrose (indicating a more condensed matrix), there was no apparent change in rhodamine 123 fluorescence (Figure 5a). This indicates that changes in matrix volume alone were not responsible for the changes in rhodamine 123 fluorescence we observed through manipulation of electron transport substrates and uncoupling agents. To confirm a direct relation between MMP and matrix configuration, mitochondria were incubated without oxidizable substrates in a slightly acidic environment (pH 6.4). By increasing the proton concentration outside the mitochondria, an artificial potential is created even when, because of lack of substrates, mitochondria are incapable of electron transport. EM analysis showed that substrate-deprived, condensed mitochondria underwent decondensation of their matrix when incubated at pH 6.4 for 10 min, as compared to mitochondria at physiological pH (7.4) (Figure 5b), demonstrating that the membrane potential can control mitochondrial configuration directly.

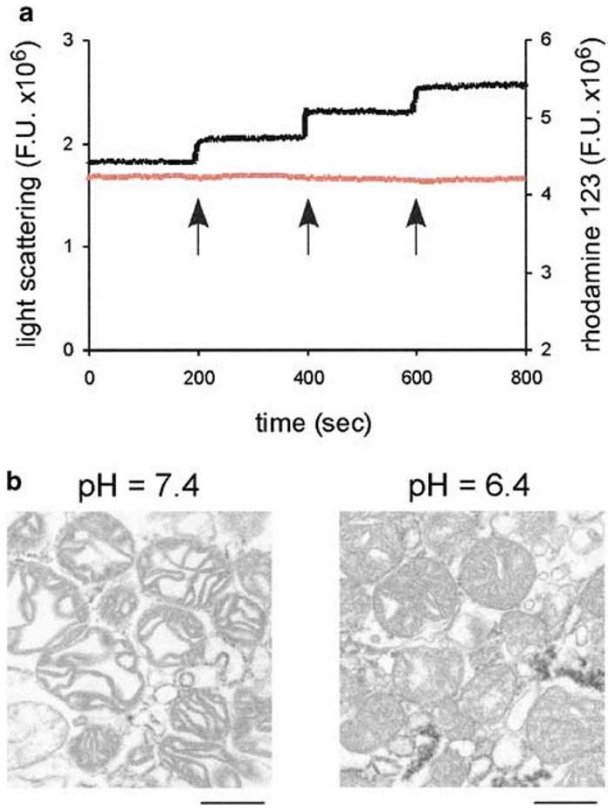
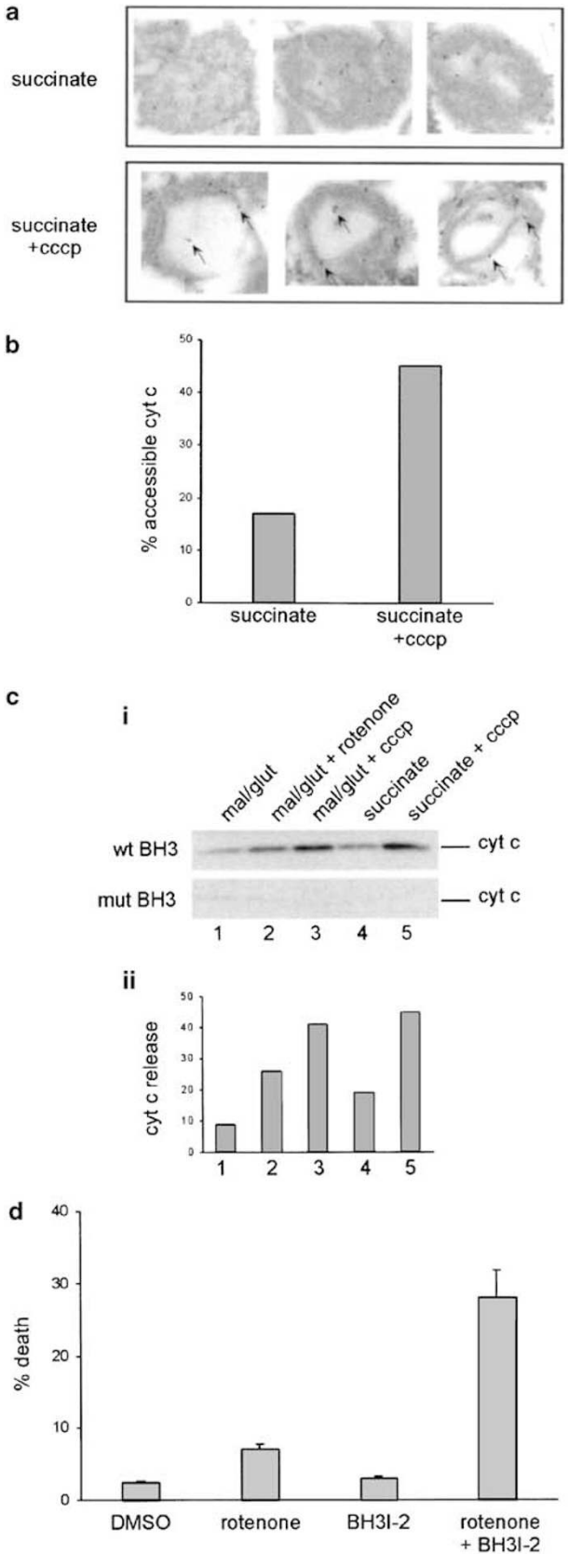


Figure 5 (**a**) Change in matrix volume but not in MMP does not affect rhodamine 123 quenching. Mitochondria were depolarized with 50 μM CCCP and 50 nM valinomycin and incubated in 100 mM sucrose buffer and light scattering (black line) or rhodamine 123 quenching analysis (red line) was performed as in Figure 3. Sucrose levels were increased in 50 mM increments every 200 s (arrows) to follow changes in matrix volume and rhodamine 123 fluorescence. (**b**) An artificial membrane potential in the absence of oxidizable substrates induces orthodox configuration. Isolated mitochondria were incubated without oxidizable substrates in MRB at normal pH (7.4) or at acidic pH (6.4) creating a respiration-independent proton gradient across the inner membrane. Following 10 min incubation, mitochondria were fixed and analyzed by EM. The bar represents 500 nm length

Matrix condensation facilitates cytochrome *c* release from mitochondria

The fact that adding methyl succinate to IL-3-deprived cells resulted in mitochondrial matrix expansion and protection from apoptosis (Figure 1), suggested that mitochondrial structural changes may contribute to the subsequent step of initiating apoptosis through cytochrome *c* release. It was suggested recently that most of the cytochrome *c* in mitochondria is enclaved in the cristae.[15] To test whether matrix condensation leads to unraveling of the cristae and contributes to exposure of cytochrome *c* to the intermembrane space, we performed immuno-EM analysis using anticytochrome *c* antibodies. When mitochondria were maintained in the orthodox configuration in the presence of succinate, cytochrome *c* was compartmentalized in cristal regions (Figure 6a top panel). In contrast, when CCCP was added to mitochondria energized with succinate, to induce a condensed configuration, a higher percentage of cytochrome *c* detected by immuno-gold staining was facing the expanded intermembrane space regions (Figure 6a bottom panel). Since the matrix undergoes condensation, communication between intermembrane space and the intracristal space becomes more extensive. Owing to limitations in the fixation process of the samples for immuno-EM, these samples are not stained with osmium tetroxide to efficiently visualize matrix condensation. Therefore, in order to confirm that mitochondria adopted the orthodox or condensed configuration for each treatment, a parallel sample from each mitochondria prep was fixed and analyzed by standard EM analysis (data not shown). Moreover, it is still apparent from the immuno-EM analysis that when compared to succinate-energized mitochondria, depolarized mitochondria displayed larger nonmatrix areas (intermembrane space) as a result of matrix condensation (Figure 6a). To analyze the change of cytochrome *c* distribution in mitochondria more than 50 mitochondria of each treatment were analyzed, and the percentage of detectable cytochrome *c* in the immuno-EM that are oriented towards the intermembrane space was calculated. A three-fold increase in cytochrome *c* that is accessible to the intermembrane space was detected in mitochondria depolarized (condensed) with CCCP (Figure 6b).

To investigate the relation between matrix configuration and cytochrome *c* release, we analyzed whether the condensed state of mitochondria, because of depolarization, could contribute to cytochrome *c* release following treatment with a peptide from the Bcl–2 homology region-3 (BH3) of Bid, a proapoptotic Bcl-2 family member. Indeed, manipulations that induced matrix condensation significantly enhanced cytochrome *c* release from mitochondria following the addition of BH3 peptides (Figure 6c). This release was BH3-dependent since a mutant BH3 peptide with two amino-acid substitutions did not cause release of cytochrome *c* from mitochondria under any condition (Figure 6c), indicating that matrix condensation alone does not induce cytochrome *c* release. Accelerated BH3-induced cytochrome *c* release was observed in mitochondria depolarized due to either a respiration block by rotenone in the presence of malate and glutamate, or to uncoupling of electron transport from the generation of a membrane potential by treatment with CCCP. These results



emphasize that cytochrome *c* release is not affected by the rate of respiration, but rather by the potential.

Combined with the observation that in IL-3-deprived cells methyl succinate restored an orthodox configuration and delayed apoptosis, the *in vitro* results indicate that mitochondrial configuration, determined by changes in membrane potential, may play an important role in regulating cytochrome *c* release and the susceptibility of cells to apoptosis. To test this possibility, cells were treated with rotenone to induce mitochondrial matrix condensation (Figure 2aiii) and viability was analyzed in the presence or absence of the cell-permeant BH3-mimetic compound BH3I-2.[16] Cells that were incubated with 10 μM BH3I-2 or 5 μM rotenone alone for 12 h showed very little signs of death (Figure 6d). On the other hand, a combined treatment with rotenone and BH3I-2 had a profound synergistic effect, and a significant acceleration of cell death was observed 12 h after treatment (Figure 6d).

Discussion

A decrease in MMP has been observed in response to many apoptotic stimuli,[10,17,18] but the relation between an MMP decrease and the initiation of apoptosis remained unclear. It was recently shown that disruption of the mitochondrial outer membrane is not sufficient to achieve complete cytochrome *c* release.[8,19] Studies of mitochondrial structure have suggested that most of the cytochrome *c* protein is sequestered in the cristae[15] and in order to achieve complete release of cytochrome *c*, as is generally observed during apoptosis,[20] structural remodeling of the inner membrane is required.[8] We have shown in this work that structural changes of mitochondria, namely, matrix condensation and cristal unraveling, are a consequence of the decline in MMP. These structural changes result in the redistribution of cytochrome *c* from the cristae to the intermembrane space, making it more susceptible to release.

The MMP can be decreased by several mechanisms including deficiency of oxidizable substrates for the mitochondria (Figure 1), blockage of respiration (Figure 2a and 3c), or

Figure 6 Matrix configuration regulates the efficiency of cytochrome *c* release and the susceptibility to apoptosis. (**a**) Mitochondria isolated from FL5.12 cells growing in the presence of IL-3 were incubated in MRB with 5 μM rotenone and 7 mM succinate with or without 50 μM CCCP for 10 min. Immuno-EM analysis using anticytochrome *c* antibodies was performed. Three representative images of each treatment are shown. Arrows indicate cytochrome *c* facing the intermembrane space in condensed mitochondria. (**b**) The number of immunodetected cytochrome *c* particles was tallied from more than 50 mitochondria of each treatment. The percentage of cytochrome *c* available to the intermembrane space is presented. (**c**) Isolated mitochondria were incubated with the indicated substrates for 10 min at room temperature followed by treatment with 5 μM wild type or mutant BH3 peptide for 30 min at 37°C. (i) Cytochrome *c* release from these mitochondria was assessed using Western blot analysis. (ii) Quantification of cytochrome *c* release of each wild-type BH3-treated sample by analyzing the different cytochrome *c* proteins bands with Scion Image for Windows. The densities of equivalent areas from the mutant BH3-treated samples were deducted from the corresponding wild-type BH3-treated bands. (**d**) FL5.12 cells were incubated with or without 5 μM rotenone or 10 μM BH3I-2 as indicated. Cell death was analyzed 12 h later by propidium iodide exclusion and assessed by FACS analysis. The results are the average and standard deviation of triplicate samples. cyt c=cytochrome *c*; wt BH3 and mut BH3=wild type and mutant BH3 peptides respectively; mal/glut=malate and glutamate

uncoupling of the inner membrane (Figure 3a and b). In each case, the consequence of the decline in MMP is condensation of the matrix. Still, the most compelling evidence for the direct relation between MMP and matrix configuration comes from the ability of an artificially created ΔpH to convert mitochondria from a condensed to an orthodox configuration (Figure 5b). These results could also suggest that mitochondrial matrix swelling, observed in many pathological conditions,[7,14,21,22] may be a consequence of cytosol acidification, a phenomenon frequently observed during necrotic cell death.[23–27]

The question remains as to what role does matrix configuration play in cells in which depolarization of the inner membrane is not observed prior to cytochrome *c* release.[28] It will be interesting to see whether mitochondrial structural changes, brought about by other mechanisms such as mitochondrial fission,[29] or tBid-mediated matrix remodeling,[8] will have a role in potential-independent mitochondria reorganization during apoptosis.

In addition to providing a link between the structure and function of mitochondria and apoptosis, our results may have implications for future administrations of cytochrome *c* releasing drugs to induce apoptosis *in vivo*. BH3 peptides are potent cytochrome *c* releasing agents, and molecules that can mimic BH3 peptides' structure and function are candidate drugs for treating tumors that overexpress Bcl-2. In this setting, mitochondrial depolarizing drugs or other drugs that lead to matrix condensation may augment cytochrome *c* release and facilitate apoptosis.

Materials and Methods

Cell culture and analysis

The murine IL-3-dependent cell lines FL5.12 were cultured at 37°C and 5% CO_2 in RPMI medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol and 300 pg/ml IL-3. For apoptosis induction by IL-3 withdrawal, cells were washed 3 times in serum free RPMI medium and resuspended in complete medium with or without IL-3. For apoptosis induction by BH3I-2, cells were incubated in complete medium with IL-3 and treated for 12 h with either DMSO (control), 5 μM rotenone, 10 μM BH3I-2, or rotenone and BH3I-2 together. Viability was determined by propidium iodide (1 μg/ml) exclusion and analyzed by FACS. To analyze MMP in cells, cells were treated as described in the text and incubated with 25 nM TMRE for 30 min followed by FACS analysis.

Mitochondrial isolation

Mitochondria were isolated using the differential centrifugation method. Cells (2.5×10^8–5×10^8) were harvested and placed on ice for 15 min, centrifuged at $500 \times g$ for 5 min at 4°C, washed with ice-cold PBS and subsequently washed with ice-cold mitochondrial isolation buffer (MIB) (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, 0.5 mg/ml BSA; pH=7.4). Cells were resuspended in ice-cold MIB and then homogenized in a syringe-driven cell disruptor. The lysate was spun at $800 \times g$ for 10 min at 4°C. Supernatants were removed and spun at $10\,000 \times g$ for 10 min at 4°C. Pellets were resuspended in MIB and protein concentration was defined using the Bradford Reagent (Bio-Rad Laboratories).

EM preparation

Cells grown in the presence or absence of IL-3 were washed once with a fixative solution (2% glutaraldehyde in 0.1 M sodium cacodilate; pH=7.4) and spun down immediately. Fixative was then added to the pellets and samples were kept at 4°C overnight then embedded and sectioned (70 nm) in Epon following analysis by standard EM procedures. For mitochondria analysis, 200 μg of mitochondria were added to 1 ml of mitochondrial reaction buffer (MRB) (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 3 mM KH_2PO_4, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.5 mg/ml BSA; pH=7.4) with or without 7 mM succinate or 7 mM (each) malate and glutamate. Samples were incubated for 10 min at 30°C. Mitochondria were spun at $10\,000 \times g$ for 10 min at 4°C. Buffer was replaced with 1 ml of fixative and samples were fixed overnight at 4°C and analyzed.

For immuno-EM analysis, following isolation and treatments, mitochondria were fixed in 2% paraformaldehyde, 0.1% gutaraldehde in 0.1 M sodium cacodilate overnight, then dehydrated, embedded and sectioned (90 nm) in LR white. Sections were washed in Tris-buffered saline (TBS) and incubated in blocking buffer (1% ovalbumin, 0.2% cold water fish skin gelatin (Sigma, St. Louis, MO, USA) in PBS) for 1 h at 4°C, washed again and incubated with rabbit polyclonal anticytochrome *c* antibodies (10 μg/ml) (Santa Cruz, CA, USA) or control serum, overnight at 4°C. After washing with TBS, sections were incubated with 1 : 10 dilution of secondary antibodies (gold (5 nm)-conjugated goat anti-rabbit) (Sigma, St. Louis, MO, USA) for 1 h at room temperature, washed in PBS and analyzed by EM.

MMP and matrix configuration analysis *in vitro*

Isolated mitochondria (500 μg) were diluted in 3 ml of MRB at room temperature, in the presence or absence of 5 μM rotenone. For membrane potential analysis, the rhodamine 123 quenching technique was used.[13] Mitochondria were incubated with 0.3 μM rhodamine 123 and analyzed in a spectrofluorometer using an excitation and emission wavelength of 500 ± 2.5 and 525 ± 2.5 nm, respectively. Baseline fluorescence was recorded for 300 s followed by the sequential addition of oxidizable substrates (7 mM succinate or 7 mM (each) malate and glutamate) and 50 μM CCCP as indicated in the text. For matrix configuration analysis 90° light scattering technique was used.[13] Mitochondria were placed in the spectrofluorometer and light diffraction was detected by setting both the excitation and the emission wavelength to 520 ± 2 nm. To study the effects of volume changes on rhodamine 123 fluorescence in the absence of changes in MMP, mitochondria were depolarized with 50 μM CCCP and 50 nM valinomycin, incubated in 100 mM sucrose buffer (100 mM sucrose, 20 mM HEPES, 1 mM EGTA, 0.5 mg/ml BSA; pH=7.4) and 90° light scattering or rhodamine 123 quenching technique were used. Sucrose concentration was increased every 200 s by 50 mM to follow changes in matrix volume and rhodamine 123 fluorescence.

Cytochrome *c* release analysis

Isolated mitochondria (100 μg) were placed in 100 μl of MRB for 8 min at room temperature with 7 mM succinate or 7 mM (each) malate and glutamate. Where indicated (in Figure 6c) 5 μM rotenone or 50 μM CCCP were added. After 2 more minutes incubation at room temperature, 5 μM wild-type (wt) BH3 peptide derived from human Bid (amino acids 80–99) or 5 μM mutant (mut) BH3 peptide with two point mutations (L90A and D95A) were added and mitochondria were incubated for 30 min at 37°C. Then, mitochondria were spun down ($10\,000 \times g$, 4°C, 10 min) and the supernatant was collected and used for Western blot analysis using anticytochrome *c* antibody clone 7H8.2C12 (BD Pharmingen).

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