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Release of apoptogenic proteins from the mitochondrial intermembrane space during the mitochondrial permeability transition

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Abstract The Bcl-2-sensitive release of proteins such as cytochrome *c* from the mitochondrial intermembrane space into the cytosol is a critical early event in apoptosis. The mitochondrial permeability transition is also an important event in many forms of apoptotic cell death. To determine whether the permeability transition led to the release of apoptogenic proteins from mitochondria we induced the permeability transition in isolated rat liver mitochondria and characterised the proteins which were released. The permeability transition led to a generalised, non-specific release of proteins, including cytochrome *c*, from the mitochondrial intermembrane space which was prevented by an inhibitor of the permeability transition. To determine the mechanism of this protein release we measured both mitochondrial matrix swelling and protein release during the permeability transition in media of different osmolarities. Protein release correlated with mitochondrial matrix swelling, therefore the permeability transition causes release of proteins from the intermembrane space by rupturing the mitochondrial outer membrane. Supporting an apoptotic role for the proteins released by this mechanism, supernatants from mitochondria undergoing the permeability transition caused apoptotic changes in isolated nuclei. These data support the proposal that the mitochondrial permeability transition can induce apoptosis by releasing apoptogenic proteins into the cytoplasm [Skulachev, V.P., FEBS Lett. 397 (1996) 7–10].

Key words: Mitochondrion; Apoptosis; Permeability transition; Cytochrome *c*

1. Introduction

Apoptotic death occurs when a cell activates an intrinsic death programme during development or following cell injury [1,2]. Mitochondria play a critical early role in this pathway of cell death by releasing signalling proteins from their intermembrane space into the cytoplasm [3–5], where they activate cysteine proteases leading to cell death [6,7]. Apoptogenic proteins released by mitochondria include cytochrome *c* (cyt *c*) [3,5,8] and a 50 kDa protease [4,9]. Protein release from the intermembrane space is blocked by the antiapoptotic protein Bcl-2 [3,5,9,10], therefore the Bcl-2-sensitive release of proteins from the mitochondrial intermembrane space may be a critical early signalling step in apoptotic cell death [4,10,11].

The mechanism of mitochondrial intermembrane protein release during apoptosis is uncertain; one appealing possibility is that the mitochondrial permeability transition (PT) is involved [4,11]. The PT arises due to the formation of a protein pore in the mitochondrial inner membrane which depolarises mitochondria and renders them permeable to low molecular weight solutes [12]. A decrease in mitochondrial membrane potential ($\Delta\psi$) due to the PT is an early event in several types of apoptosis [12–15] and bongkrekic acid, an inhibitor of the PT, prevents both this $\Delta\psi$ decrease and the subsequent apoptosis [16]. The antiapoptotic protein Bcl-2 localises to the mitochondrial outer membrane [10] and overexpression of this protein prevents the PT [4]. Mitochondria undergoing the PT in vitro release proteins that cause apoptotic changes in isolated nuclei [17]. However, the mechanism by which the PT leads to release of apoptogenic proteins is uncertain. Furthermore, it is unclear if the PT is required for the release of cyt *c* from mitochondria as this release occurs in apoptotic cells without any apparent changes in mitochondrial $\Delta\psi$ [3,5].

To clarify the relationship between the PT and the release of apoptogenic proteins from the mitochondrial intermembrane space we induced the PT in isolated rat liver mitochondria and characterised the protein release. We then determined whether these proteins could induce apoptotic changes in isolated nuclei.

2. Materials and methods

2.1. Mitochondrial preparation and incubation

Liver mitochondria were prepared from fed, female Wistar rats (150–200 g) by homogenisation and differential centrifugation in ice-cold medium containing 250 mM sucrose, 5 mM Tris-HCl and 1 mM EGTA at pH 7.4 [18]. The final wash was in medium without EGTA and the mitochondria were resuspended at about 50 mg protein/ml as determined by the biuret method using BSA as a standard [19].

Mitochondria (10 mg protein/ml) were incubated at 37°C with shaking under 95% O_2/5% CO_2 in 2 ml medium containing 110 mM KCl, 10 mM HEPES-KOH (pH 7.2), 65 nmol $CaCl_2$/mg protein, 20 mM succinate and 13 μM rotenone. For some incubations 500 nM cyclosporin A (CsA) was also present. After 1 min the PT was induced by adding 15 mM K_2HPO_4 and 2 min later the mitochondria were pelleted by centrifugation (13 000×*g* for 30 s). The pellets were retained for analysis of proteins remaining in the mitochondria and the supernatants were concentrated ~4-fold using Centricon-3 centrifugal microconcentrators (Amicon, USA), which retain molecules >3 kDa.

2.2. Characterisation of mitochondrial proteins released during the permeability transition

The release of cyt *c* into the post-mitochondrial supernatant was confirmed by scanning absorption spectroscopy, after addition of di-

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Abbreviations: CsA, cyclosporin A; cyt *c*, cytochrome *c*; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\psi$, mitochondrial membrane potential; PT, permeability transition; TPMP, methyltriphenylphosphonium cation

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thionite to reduce cyt *c*, and quantitated using ε_{550}=27.7 mM^{-1} cm^{-1}. To quantitate cyt *c* release as a proportion of the total amount present, cyt *c* was extracted from the mitochondrial pellet by addition of butanol to 20% (v/v), emulsified by shaking and incubated at 0°C for 10 min [20]. The emulsion was then mixed 4:1 with saturated ammonium sulphate, shaken and incubated at room temperature for 10 min. The two phases were separated by centrifugation (10 000×*g* for 1 min) and the lower, aqueous phase removed and its cyt *c* content determined as described above.

Adenylate kinase was assayed [21] in buffer containing 5 mM $MgSO_4$, 0.1% Triton X-100, 10 mM glucose, 1 mM ADP, 1 mM $NADP^+$, 10 U glucose 6-phosphate dehydrogenase, 10 U hexokinase and 50 mM Tris-HCl, pH 7.5, at 30°C. The change in absorbance at 340 nm ($\varepsilon = 6.22\times10^3$ M^{-1} cm^{-1}) was recorded and the background rate, measured in the presence of the specific adenylate kinase inhibitor diadenosine pentaphosphate (100 μM), was subtracted from the initial rate. Citrate synthase was assayed in buffer containing 0.1% Triton X-100, 0.4 mM oxaloacetic acid, 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid), 10 μM acetyl CoA, 100 mM Tris-HCl (pH 8), at 30°C and the increase in absorbance at 412 nm ($\varepsilon = 13.6\times10^3$ M^{-1} cm^{-1}) was recorded [22].

Proteins released by mitochondria were concentrated ~8-fold using Centricon-3 centrifugal microconcentrators, separated by discontinuous polyacrylamide gel electrophoresis (17.5% acrylamide separating gel) using a BioRad Mini Protean system and visualised by Coomassie blue staining. To measure swelling, mitochondria (2 mg protein/ml) were incubated in a 3 ml stirred cuvette and the change in absorption at 540 nm recorded continuously using an Aminco DW2000 spectrophotometer. Mitochondrial membrane potential was measured by incubating mitochondria with 1 μM (50 nCi/ml) [^{3}H]methyltriphenylphosphonium (TPMP). The uptake of TPMP was determined by pelleting mitochondria by centrifugation followed by quantitation of the [^{3}H]TPMP in the pellet and supernatant by scintillation counting. The membrane potential was calculated from the Nernst equation, assuming a mitochondrial volume of 0.5 μl/mg protein and that 60% of the intramitochondrial TPMP was membrane bound [23]. The protein released by mitochondria was quantitated using the bicinchoninic acid assay, with BSA as the standard [24].

2.3. Rat liver nuclei preparation and in vitro apoptosis assay

Rat liver nuclei were prepared by a modification of a published method [25]. A female Wistar rat (150–200 g) was anaesthetised using pentobarbital and its liver was perfused briefly with ice-cold PBS, removed and then homogenised in ice-cold buffer A, containing 250 mM sucrose, 1 mM dithiothreitol, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 1 μM phenylmethylsulphonyl fluoride and 15 mM PIPES (NaOH), pH 7.4. The homogenate was filtered through four layers of cheesecloth and added to 1 vol. buffer B, containing 2.3 M sucrose, 1 mM dithiothreitol, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 0.5 mM spermidine, 0.2 mM spermine, 1 μM phenylmethylsulphonyl fluoride and 15 mM PIPES (NaOH), pH 7.4. This was loaded onto a 5 ml cushion of buffer B and the nuclei were pelleted by centrifugation (22 000×*g* at 4°C for 1.5 h). The nuclear pellet was then resuspended at ~8.5×10^7 nuclei/ml in buffer A and aliquots were stored at −80°C.

For the in vitro apoptosis assays 50 μl concentrated supernatant from a mitochondrial incubation was added to 3 μl 20 mM dATP, 3 μl 20 mM $MgCl_2$ and 6 μl nuclei (~5×10^5 nuclei) and incubated for 2 h at 37°C [8]. Apoptotic changes in the nuclei were visualised by mixing 4 μl nuclear incubation with 1.5 μl Hoechst 33342 (10 μM in formalin) on a glass slide and then observed under UV illumination using a fluorescence microscope. DNA was extracted from the remaining nuclei with 500 μl buffer containing 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2 mM NaCl, 0.2% w/v SDS, and 0.2 mg/ml proteinase K and incubated at 37°C overnight. NaCl (final concentration 1.5 M) was then added and the protein was pelleted by centrifugation (16 000×*g* for 15 min). The supernatant was mixed with 1 vol. Tris-equilibrated phenol (pH 8)/chloroform (1:1), the aqueous layer removed, mixed with 1 vol. isopropanol and stored at −20°C for 20 min. The DNA was then pelleted by centrifugation (16 000×*g* for 15 min at 4°C), washed with ice-cold 70% ethanol, resuspended in 40 μl H_2O containing 200 μg/ml RNase A, and incubated for 1 h at 37°C. The DNA was separated by electrophoresis on a 2% agarose gel run at 50 V for 2 h, and the DNA was visualised by ethidium bromide fluorescence under UV illumination.

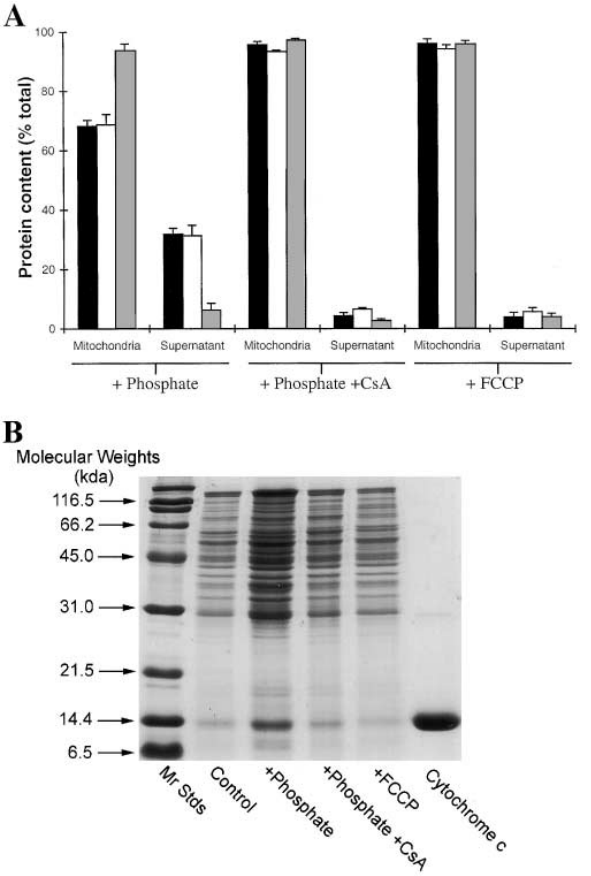

Fig. 1. Release of proteins from the mitochondrial intermembrane space during the permeability transition. Mitochondria were incubated with calcium as described in Section 2.1 and the PT was induced by addition of phosphate. These experiments were repeated in the presence of CsA, or with FCCP added instead of phosphate. A: Analysis of the release of cyt *c* (black bars), adenylate kinase (white bars), and citrate synthase (shaded bars) from mitochondria. The proportions of the proteins released and remaining in the mitochondria are given as a percentage of the total amounts in the mitochondria. Data are the mean ± S.E.M. of experiments on at least three separate mitochondrial preparations. B: Equal volumes of the concentrated supernatants from the mitochondrial incubations were separated by SDS-PAGE on a 17.5% acrylamide gel as described in Section 2.2. Purified horse heart cyt *c* (15 μg) was loaded where indicated.

3. Results

3.1. Induction of the permeability transition releases proteins from the mitochondrial intermembrane space

To determine whether the PT released proteins from the mitochondrial intermembrane space, mitochondria were incubated with calcium as described in Section 2.1 and the PT was induced by addition of phosphate. These conditions are effective inducers of the PT [12] and this was confirmed under our conditions by parallel measurement of the mitochondrial $\Delta\psi$ (data not shown). These $\Delta\psi$ measurements also confirmed that CsA prevented the PT following phosphate addition. The supernatants from these mitochondrial incubations were concentrated ~4-fold and proteins released from the mitochondria during the PT were characterised and quantitated (Fig. 1). To determine whether cyt *c* was released from mitochondria during the PT a scanning absorption spectrum of the concentrated supernatant was obtained (data not shown).

This showed the characteristic absorption spectrum of cyt *c* and this cyt *c* release was blocked by CsA, which prevents the PT. The amount of cyt *c* lost from the mitochondria into the cytoplasm was about 30% of the total present (Fig. 1A). To determine whether this protein release during the PT was selective for cyt *c*, or was general for all intermembrane space proteins, the concentrated supernatant was assayed for adenylate kinase, a mitochondrial intermembrane space enzyme (Fig. 1A). The PT led to the loss of about 30% of adenylate kinase from the intermembrane space to the supernatant, about the same proportion lost as for cyt *c* (Fig. 1A). This loss of adenylate kinase was prevented by CsA, as was the case for cyt *c* (Fig. 1A). The mitochondrial matrix enzyme citrate synthase was not released from the mitochondria during the PT (Fig. 1A), showing that the protein release was confined to intermembrane space proteins and was not due to a generalised breakdown of mitochondrial membranes. Addition of the mitochondrial uncoupler FCCP did not result in the release of intermembrane space proteins (Fig. 1A). To characterise further the proteins released from the mitochondria during the PT, equal volumes of the concentrated supernatants from the mitochondrial incubations were analysed by SDS-PAGE (Fig. 1B). In control incubations, where mitochondria were incubated with calcium, the proteins in the supernatant were due to contamination of the mitochondrial preparation (Fig. 1B). When the PT was induced by addition of phosphate there was a substantial increase in the amount of protein released from the mitochondria and all the proteins visible in the control sample were present in increased amounts (Fig. 1B). By comparison with purified cyt *c* it is clear from the protein gel that cyt *c* is one of the proteins released from mitochondria during the PT (Fig. 1B), corroborating the evidence for cyt *c* release shown in Fig. 1A. This protein release was blocked by CsA (Fig. 1B). Protein assays of the supernatant from mitochondria undergoing the PT contained about 60 μg more protein per mg mitochondrial protein added than was found in control incubations or when the PT was blocked by CsA. Addition of the uncoupler FCCP to the mitochondrial suspension did not release mitochondrial proteins into the supernatant (Fig. 1B).

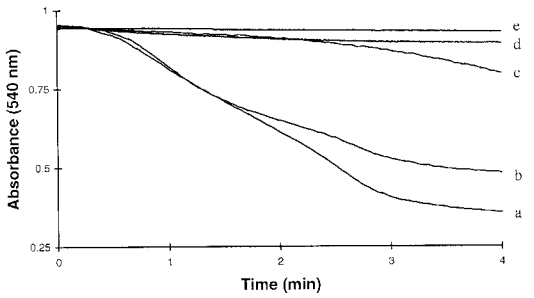

Fig. 2. Mitochondrial swelling during the permeability transition. Mitochondria were incubated in the presence of calcium as described in Section 2.2 and the absorbance at 540 nm was measured continuously. The PT was induced at time zero by addition of phosphate. In trace a the medium was 195 mM mannitol, 25 mM sucrose and 10 mM HEPES-KOH (pH 7.2). In traces b, d and e the medium was 110 mM KCl and 10 mM HEPES-KOH (pH 7.2), as for Fig. 1. In trace c the KCl concentration was increased to 400 mM. For trace d, FCCP was added at time zero in place of phosphate, and for trace e, CsA was present. All traces are typical experiments repeated on at least three separate mitochondrial preparations.

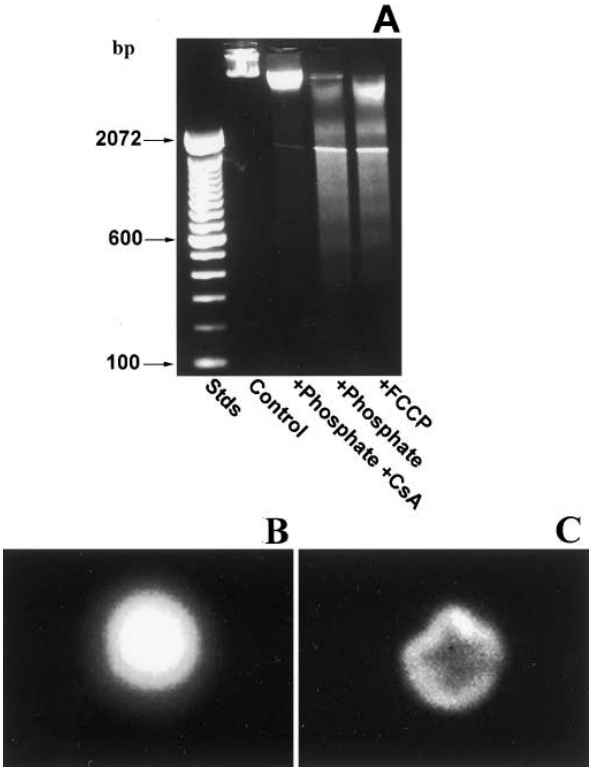

Fig. 3. Apoptogenic effects on isolated nuclei of proteins released from mitochondria. Concentrated supernatants were prepared as described in Section 2.1 from mitochondrial incubations in the presence of calcium alone (control), or in which the PT was induced by phosphate, blocked by CsA or in which FCCP was added instead of phosphate. These supernatants were incubated with isolated nuclei which were then assayed for apoptotic changes as described in Section 2.3. A: DNA was extracted from nuclei incubated with the mitochondrial supernatants and separated on a 2% agarose gel. B and C: Nuclei were stained with Hoechst 33342 and viewed by fluorescence microscopy. B: A typical non-apoptotic nucleus after incubation with the supernatant from mitochondria incubated with CsA. C: A typical apoptotic nucleus after incubation with supernatant from mitochondria undergoing the PT. Nuclei incubated with the supernatant from control mitochondrial incubations were similar to the one shown in B (data not shown) while nuclei incubated with the supernatant from mitochondrial incubations treated with FCCP were similar to the one shown in C (data not shown). All panels show typical data repeated on at least three separate mitochondrial preparations.

3.2. Protein release from the mitochondrial intermembrane space correlates with mitochondrial swelling

The data described above show a generalised release of proteins from the mitochondrial intermembrane space during the PT. As the PT is associated with swelling of the mitochondrial matrix the most likely cause of this protein release is rupture of the outer membrane following swelling of the mitochondrial matrix [11]. To determine whether this caused the protein release, we measured changes in the mitochondrial volume during the PT. This was done by measuring light scattering by mitochondria spectrophotometrically where a decrease in optical density at 540 nm indicates mitochondrial swelling [26]. Mitochondria suspended in a mannitol-sucrose buffer underwent large-amplitude swelling during the PT (Fig. 2, trace a). Similar mitochondrial swelling occurred when the PT was induced in 110 mM KCl buffer (Fig. 2, trace b) which was prevented by CsA (Fig. 2, trace e). When the KCl concentration was increased to 400 mM, swelling during the PT

was greatly reduced (Fig. 2, trace c). However, the CsA-sensitive PT still occurred under these conditions as was shown by parallel measurements of $\Delta\psi$ (data not shown), therefore the decreased swelling is not due to inhibition of the PT. Addition of the mitochondrial uncoupler FCCP did not lead to mitochondrial swelling (Fig. 2, trace d). The amount of adenylate kinase released during the PT was measured to determine how the decreased swelling at higher KCl concentrations affected protein release from the intermembrane space (data not shown). The higher KCl concentration decreased the CsA-sensitive release of adenylate kinase by about 67%, therefore the protein release from the mitochondrial intermembrane space during the PT correlated with swelling.

3.3. *Proteins released from mitochondria during the permeability transition induce apoptotic changes in isolated nuclei*

To determine whether the proteins released from mitochondria during the PT were apoptogenic, supernatants from mitochondria undergoing the PT were concentrated and incubated with isolated rat liver nuclei. Addition of proteins released from mitochondria during the PT led to chromatin condensation (Fig. 3C) and DNA fragmentation (Fig. 3A) in the isolated nuclei, suggesting that these proteins were apoptogenic. In contrast, supernatants from mitochondrial incubations in which the PT was prevented by CsA did not cause apoptotic changes in the nuclei (Fig. 3A,B). Interestingly, supernatants from mitochondria incubated with the uncoupler FCCP also induced apoptotic changes in nuclei, even though FCCP did not cause mitochondrial swelling (Fig. 2B) or release of proteins from the mitochondrial intermembrane space (Fig. 1A,B).

4. Discussion

The aim of this study was to characterise the release of apoptogenic proteins from mitochondria during the PT. The data shown in Fig. 1 demonstrate that the PT leads to the generalised release of proteins from the mitochondrial intermembrane space, and not to the selective release of particular proteins. This is supported by our finding that the same proportion ($\sim$30%) of two intermembrane proteins, adenylate kinase and cyt *c*, was released during the PT. Further quantitative and qualitative measurements of the released proteins also showed that the PT in vitro led to generalised protein release. During this work another report was published showing cyt *c* release from mitochondria in vitro during the PT [27] and our study confirms and extends this observation.

This generalised release of intermembrane proteins probably occurs by rupture of the mitochondrial outer membrane, due to osmotic swelling of the mitochondrial matrix during the PT [11]. This was supported by our finding that the protein release correlated with mitochondrial swelling, with both protein release and mitochondrial swelling decreasing in medium of high osmotic strength (Fig. 2). Together these findings demonstrate that the PT in vitro releases proteins from the mitochondrial intermembrane space. However, it remains to be seen whether the PT in vivo also ruptures the mitochondrial outer membrane, causing the generalised release of intermembrane space proteins. In particular, it is now important to determine whether the release of cyt *c* from the mitochondria within intact cells during apoptosis occurs as a consequence of the PT, or by some other mechanism [3,5,6]. In some studies cyt *c* release occurred without a decrease in $\Delta\psi$ [3,5,6], suggesting that the PT was not involved. One possibility is that a specific release pathway exists for some mitochondrial proteins which operates independently of the PT. However, it should be remembered that the PT is reversible [12]. Therefore the PT may cause an individual mitochondrion to swell, releasing apoptogenic proteins and then reverse the PT and regenerate its $\Delta\psi$. Such a process would enable the PT to release cyt *c* in vivo without any measurable change in mitochondrial $\Delta\psi$ and may explain the apparent discrepancy in the literature.

The proteins released from the mitochondrial intermembrane space by the PT induced apoptotic changes in isolated nuclei. This is consistent with the large amount of data showing that the release of intermembrane proteins leads to apoptogenic changes in isolated nuclei. Therefore, the Bcl-2-sensitive induction of the PT may be an important event in apoptotic signalling. Surprisingly, in our experiments the uncoupler FCCP also released factors from mitochondria which led to apoptotic changes in isolated nuclei, even though FCCP did not cause significant swelling or protein release: we are investigating this finding further. In summary, we have shown that induction of the PT leads to the generalised release of mitochondrial intermembrane space proteins by rupturing the mitochondrial outer membrane. These proteins can induce apoptotic changes in isolated nuclei.

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