Cell Death and Differentiation (1999) 6, 825–832

http://www.stockton-press.co.uk/cdd

Calcium induced release of mitochondrial cytochrome *c* by different mechanisms selective for brain *versus* liver

Alexander Andreyev[1] and Gary Fiskum*[,1]

[1] Department of Anesthesiology, University of Maryland, Baltimore School of Medicine, Baltimore, Maryland, 21201, USA
* corresponding author: Gary Fiskum, Department of Anesthesiology, University of Maryland, Baltimore, MSTF 5.34, 685 W. Baltimore St., Baltimore, Maryland, 21201, USA. tel: (410) 706-4711; fax: (410) 706-2550; e-mail: gfiskum@umaryland.edu

Received 16.2.99; revised 12.4.99; accepted 15.7.99
Edited by D Green

Abstract

Increased mitochondrial Ca^{2+} accumulation is a trigger for the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol where it can activate caspases and lead to apoptosis. This study tested the hypothesis that Ca^{2+}-induced release of cytochrome *c in vitro* can occur by membrane permeability transition (MPT)-dependent and independent mechanisms, depending on the tissue from which mitochondria are isolated. Mitochondria were isolated from rat liver and brain and suspended at 37°C in a K^+-based medium containing oxidizable substrates, ATP, and Mg^{2+}. Measurements of changes in mitochondrial volume (via light scattering and electron microscopy), membrane potential and the medium free [Ca^{2+}] indicated that the addition of 0.3–3.2 μmol Ca^{2+} mg^{-1} protein induced the MPT in liver but not brain mitochondria. Under these conditions, a Ca^{2+} dose-dependent release of cytochrome *c* was observed with both types of mitochondria; however, the MPT inhibitor cyclosporin A was only capable of inhibiting this release from liver mitochondria. Therefore, the MPT is responsible for cytochrome *c* release from liver mitochondria, whereas an MPT-independent mechanism is responsible for release from brain mitochondria.

Keywords: apoptosis; membrane permeability transition; mitochondria; calcium; cytochrome *c*; immunoblot; electron microscopy

Abbreviations: BSA, bovine serum albumin; CsA, cyclosporin A; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $\Delta\Psi$, mitochondrial membrane potential; MPT, membrane permeability transition; TPP+, tetraphenylphosphonium; Tris, tris-(hydroxymethyl)-aminomethane

Introduction

Substantial evidence indicates that in many apoptotic cell death paradigms, proteins are released from mitochondria into the cytosol where, together with other factors, they form the apoptosome complex that activates the caspase cascade of cell death protease activities.[1,2] The best characterized of these mitochondrial factors is cytochrome *c*, a 12.5 kDa soluble protein normally located exclusively in the intermembrane space between the inner and outer mitochondrial membranes. Although its normal function is to transport electrons between electron transport chain complexes III and IV, when present in the cytosol, it can combine with Apaf1, dATP, and caspase 9 to form the apoptosome. Known molecular triggers for the release of cytochrome c include Ca^{2+}, BAX, and Bid.[3–10]

The mechanism(s) responsible for the release of cytochrome *c* from mitochondria in response to different inducing factors are not well characterized. Ca^{2+} can, under certain conditions, induce the release of cytochrome *c* and other proteins from isolated liver mitochondria by a mechanism that involves increased permeability of the inner membrane to small solutes, osmotic swelling of the mitochondrial matrix, and physical disruption of the outer membrane.[11–15] Under conditions where this Ca^{2+}-induced swelling phenomenon is inhibited by the immunosuppressive agent cyclosporin A (CsA), the process is normally defined as the membrane permeability transition (MPT).[16–19] We questioned the relevance of the MPT to Ca^{2+}-induced release of cytochrome *c in situ* within intact cells, since physiological levels of adenine nucleotides and Mg^{2+} are potent inhibitors of the MPT.[6,20] The MPT also results in a dramatic decrease in the high electrical potential ($\Delta\Psi$) that normally exists across the mitochondrial inner membrane, and several laboratories have reported little if any drop in $\Delta\Psi$ prior to the release of cytochrome *c* into the cytosol during apoptosis.[21–23] We reported previously that $<40\%$ of cytochrome *c* present within isolated brain mitochondria can be released in response to the energy-dependent accumulation of exogenous Ca^{2+} in the presence of physiologic concentrations of ATP and Mg^{2+} by a mechanism that is not accompanied by mitochondrial swelling and that is not inhibited by cyclosporin A.[6] However, several laboratories have reported that brain mitochondria are relatively resistant to the MPT under a variety of conditions *in vitro*.[6,24,25] The present study, directly comparing Ca^{2+}-induced cytochrome *c* release from isolated brain and liver mitochondria, was undertaken to test the hypothesis that both MPT-dependent and independent mechanisms can be responsible for cytochrome *c* release, depending on the tissue from which the mitochondria are isolated.

Results

The mitochondrial membrane permeability transition is normally accompanied by mitochondrial swelling, a precipitous drop in $\Delta\Psi$, and the spontaneous release of accumulated Ca^{2+}. Simultaneous measurements of these events after the addition of different Ca^{2+} levels to suspensions of isolated rat liver and brain mitochondria are described in Figure 1.

Mitochondria were suspended in a medium at 37°C that contained physiologically realistic concentrations of K^+, Mg^{2+}, ATP, and P_i, together with the oxidizable substrate succinate in the presence of rotenone. After 2 min, various levels of $CaCl_2$ were added to the suspension. For liver mitochondria, increased levels of added Ca^{2+} resulted in decreased light scattering, indicative of mitochondrial swelling. This decrease in light scattering was accompanied by a drop in the steady-state $\Delta\Psi$, as reflected by an increase in the medium TPP^+ concentration measured with an electrode. The addition of Ca^{2+} resulted in an immediate increase in the medium free [Ca^{2+}] that was followed by an energy-dependent decrease in the [Ca^{2+}] at all but the highest levels of added Ca^{2+}. However, at all but the lowest levels of added Ca^{2+}, the accumulation of added Ca^{2+} by mitochondria was incomplete. These results demonstrate that even in the presence of physiological levels of Mg^{2+} and ATP that are known to inhibit the MPT, increasing levels of mitochondrially accumulated Ca^{2+} can induce mitochondrial swelling, a drop in $\Delta\Psi$, and release of sequestered Ca^{2+} indicative of the MPT.

In contrast to what was observed with liver mitochondria, comparable additions of $CaCl_2$ to suspensions of isolated rat brain mitochondria resulted in a net increase in light scattering (Figure 1). This phenomenon has previously been shown to be primarily the result of Ca^{2+} precipitation within the mitochondria rather than due to mitochondrial shrinkage.[6] Although increasing Ca^{2+} additions did result in a decrease in $\Delta\Psi$, the final membrane potentials were generally higher than those observed with liver mitochondria. Unlike results obtained with liver mitochondria, the decline in $\Delta\Psi$ was not associated with either swelling or a lack of Ca^{2+} retention. Although liver mitochondria could only completely accumulate <0.7 μmol added Ca^{2+} mg^{-1}, brain mitochondria completely sequestered and retained at least 3.3 μmol Ca^{2+} mg^{-1}.

Since Ca^{2+} accumulation by brain mitochondria suspended in the presence of ATP and Mg^{2+} results in an increase in light scattering due to Ca^{2+} precipitation, it is possible that this phenomenon could obscure a decrease in light scattering due to swelling. We tested this possibility by centrifuging the mitochondrial suspensions after the approximate 10 min incubation and observing the mitochondrial morphology with transmission electron microscopy. Figure 2A,D describe the normal appearance of liver and brain mitochondria, respectively, following incubation in the absence of added Ca^{2+}. Figure 2B,E provide a similar comparison for liver and brain mitochondria after exposure to 2.1 μmol Ca^{2+} mg^{-1} protein. Consistent with the light scattering results shown in Figure 1, liver mitochondria appeared grossly swollen whereas brain mitochondria appeared normal. In fact, some of the Ca^{2+}-treated brain mitochondria exhibited relatively large intermembrane

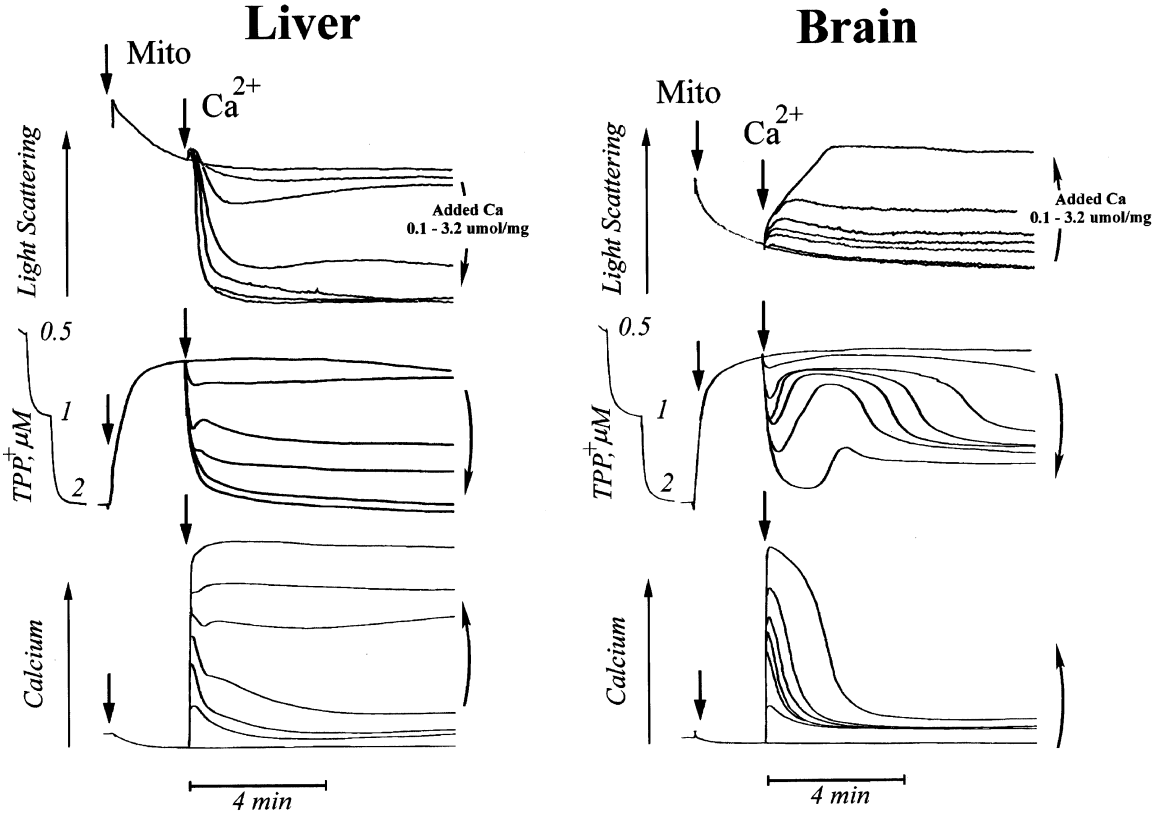

Figure 1 Effects of added Ca^{2+} on mitochondrial volume, membrane potential ($\Delta\Psi$), and Ca^{2+} transport in suspensions of rat liver *vs* brain mitochondria. Isolated mitochondria were suspended at 37°C in a K^+-based medium containing ATP, Mg^{2+}, and oxidizable substrates (see Materials and Methods). Changes in mitochondrial volume were measured by light scattering. Mitochondrial $\Delta\Psi$ was monitored with a TPP^+ electrode. Ca^{2+} transport into the mitochondria was measured by monitoring the medium [Ca^{2+}] with a Ca^{2+}-selective electrode

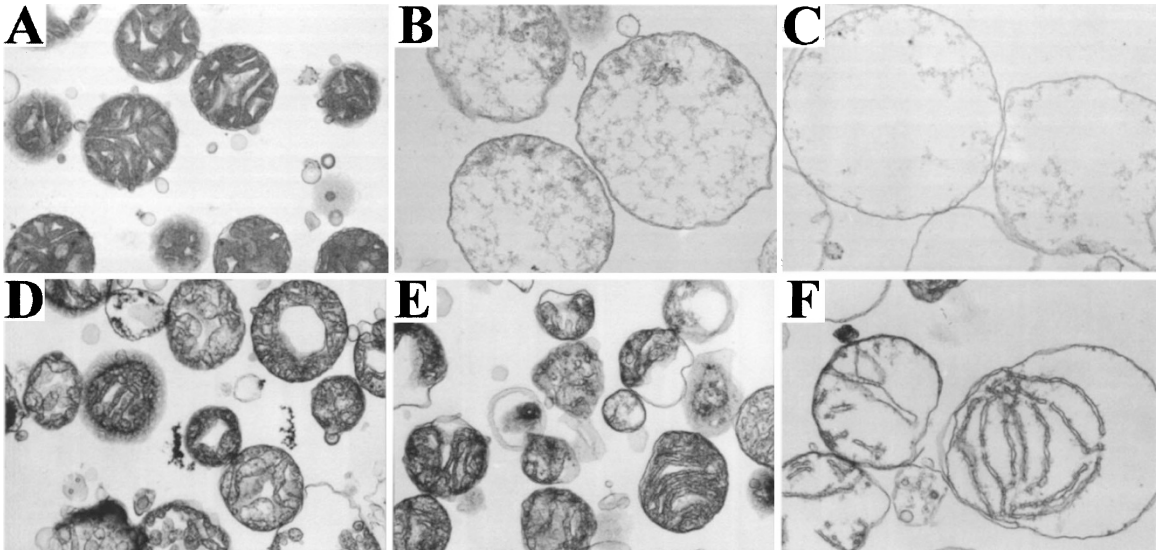

Figure 2 Electron micrographs of rat liver and brain mitochondria in the absence and presence of exposure to Ca^{2+} or to the pore-former alamethicin. At the end of incubations such as those described in Figure 1, suspensions of either rat liver mitochondria (**A–C**) or rat brain mitochondria (**D–F**) were centrifuged and processed for electron microscopy. Ca^{2+} was added at a level of 2.1 μmol mg^{-1} protein (**B**,**D**). Alamethicin was added at a concentration of 40 μg mg^{-1} protein (**C**,**E**)

spaces and highly condensed matrix space. We also tested the relative ability of liver and brain mitochondria to swell when both were exposed to alamethicin, an artificial pore former that can cause mitochondrial swelling similar to that associated with the MPT.[6] As seen in Figure 2C,F, both liver and brain mitochondria underwent large amplitude swelling approximately equivalent to that observed with liver mitochondria exposed to Ca^{2+}. These results confirm that brain mitochondria are capable of coloidosmotic swelling when there is a substantial increase in the solute permeability of their inner membrane but that no detectable swelling occurs after accumulating very large loads of Ca^{2+} in the presence of ATP and Mg^{2+}.

At the end of the experiments described in Figure 1, each mitochondrial suspension was rapidly centrifuged. The resulting supernatants and selected pellets were used for immunoblot determinations of cytochrome *c*. As shown in Figure 3, in the absence of added Ca^{2+}, there was no significant release of liver mitochondrial cytochrome *c* into the medium (i.e., supernatant). In comparison to the total cytochrome *c* present in the supernatant plus the mitochondrial pellet, the extramitochondrial cytochrome *c* represented $<1\%$ of the total. However, there was a detectable release of cytochrome *c* after the addition of 0.1 μmol Ca^{2+} mg^{-1} protein, and more extensive release at 0.3–3.2 μmol Ca^{2+} mg^{-1}. More than 80% of the total cytochrome *c* was released after the addition of 3.2 μmol mg^{-1} (mean $\pm$ S.E.=81 $\pm$ 8%, n=3). Notice that the amount of cytochrome *c* released appeared to be maximal after the addition of 0.5 μmol Ca^{2+} mg^{-1}, which is the highest amount of added Ca^{2+} that the mitochondria could completely accumulate (Figure 1).

Figure 3 also describes the immunoblot determinations of cytochrome *c* released from rat brain mitochondria at the end of the experiments shown in Figure 1. There was

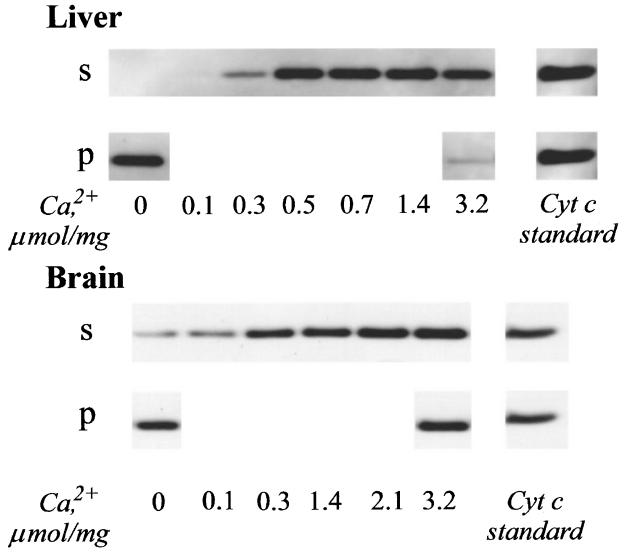

Figure 3 Western immunoblots of cytochrome *c* in suspensions of mitochondria exposed to different levels of Ca^{2+}. At the end of the experiments shown in Figure 1, mitochondrial suspensions were centrifuged and the supernatants (s) and pellets (p) used for immunoblot determinations of cytochrome *c* (see Materials and Methods). Although direct visual comparisons can be made of cytochrome *c* bands among different supernatants or among different pellets, differences in dilutions for the supernatants *vs* the pellets precludes direct comparisons between these fractions. Semi-quantitative, densitometric analysis of band intensities were made and corrected for dilution of the samples. Blots are representative of three separate experiments

detectable cytochrome *c* in the supernatant after incubation in the absence of added Ca^{2+}; however, when compared to the content of the mitochondrial pellet, it was less than 5% of the total. Further release of cytochrome *c* occurred after

addition of 0.1 μmol Ca^{2+} mg^{-1} protein and considerably more was released after the addition of 0.3 μmol mg^{-1}. Notice that 0.3 μmol Ca^{2+} mg^{-1} protein is less than 10% of the maximum Ca^{2+} that can be accumulated by brain mitochondria under these conditions, as indicated by the observations provided in Figure 1. In contrast to liver mitochondria, the maximum level of cytochrome *c* that could be released from brain mitochondria by Ca^{2+} under these conditions was less than 30% of the total, even at 3.2 μmol Ca^{2+} mg^{-1} protein (mean $\pm$ S.E.=25 $\pm$ 4%, n=3).

We previously reported that the release of cytochrome *c* from brain mitochondria in response to the addition of high levels of Ca^{2+} was insensitive to the MPT inhibitor cyclosporin A.[6] As a further test of the mechanisms involved in Ca^{2+}-induced cytochrome *c* release from both brain and liver mitochondria, we measured the release of cytochrome *c* in suspensions of both types of mitochondria in the absence and presence of MPT inhibitors after the addition of a relatively low and a relatively high level of Ca^{2+} (Figure 4). At 0.3 μmol Ca^{2+} mg^{-1} protein, 1 μM cyclosporin A completely inhibited the cytochrome *c* released from liver mitochondria. At 2.1 μmol Ca^{2+} mg^{-1}, cyclosporin A inhibited release by approximately 70%. The additional inclusion of 3 mM ADP elevated the inhibition of cytochrome *c* release to greater than 90%. In contrast to liver mitochondria, there was no significant inhibition of cytochrome *c* release from brain mitochondria at either level of added Ca^{2+} by cyclosporin A in the absence or presence of added ADP.

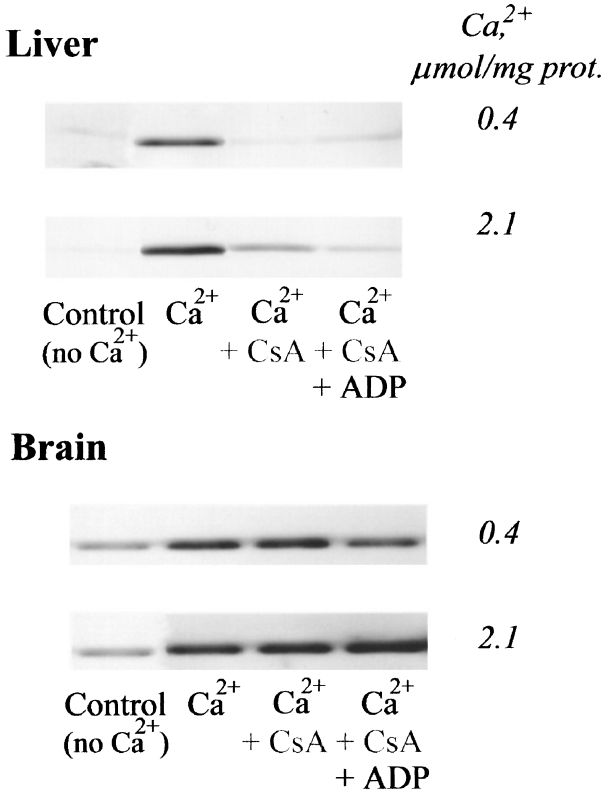

Figure 4 Effects of inhibitors of the membrane permeability transition (MPT) on the release of cytochrome *c* evoked by Ca^{2+} in suspensions of mitochondria. Immunoblot determinations of cytochrome *c* were performed on supernatants obtained following centrifugation of suspensions of rat liver and brain mitochondria after incubations similar to those described in Figure 1. Control samples contained no added Ca^{2+}. The other incubations contained either 0.4 or 2.1 μmol Ca^{2+} mg^{-1} protein in the absence of either 1 μM cyclosporin A alone or 1 μM cyclosporin A plus 3 mM ADP

Discussion

The release of cytochrome *c* from mitochondria into the cytosol is thought to be a key event in the formation of the apoptosome and the initiation of the caspase cascade in many, but not all, apoptotic cell death paradigms.[1] As apoptosis and mitochondrial dysfunction are known to contribute significantly to neuronal death in both acute neurodegenerative disorders, e.g., global and focal ischemia and traumatic brain injury, and in chronic neurodegenerative diseases, e.g., Alzheimer's and Parkinson's diseases,[26] the involvement of mitochondrial dysfunction in neural cell apoptosis is being actively investigated.[27] Particular attention has focused on the effects of Ca^{2+} on brain and neural cell mitochondria, as substantial evidence indicates that mitochondrially accumulated Ca^{2+} rather than cytosolic Ca^{2+} is the most important determinant of excitotoxicity.[28–31] Evidence also suggests that mitochondrial Ca^{2+} accumulation at least contributes to the early induction of molecular cell death cascades in several different paradigms.[32,33]

Our previous observations using isolated rat forebrain and canine frontal cortex mitochondria indicated that Ca^{2+} elicits the release of cytochrome *c* *in vitro* by a mechanism that is not associated with mitochondrial swelling and that is not inhibited by the MPT inhibitor cyclosporin A.[6] Most of these experiments were conducted in the presence of physiologically realistic concentrations of ATP and Mg^{2+} that are known to inhibit the MPT in various types of mitochondria. However, we also observed a lack of inhibition of cytochrome *c* release from brain mitochondria by cyclosporin A in the absence of these inhibitors where the MPT was demonstrated to be active. These results suggested that an MPT-independent mechanism of Ca^{2+}-induced cytochrome *c* release was operative with isolated brain mitochondria. Although a number of studies have documented the role of either large-amplitude swelling or, more recently, the cyclosporin A-sensitive MPT in mediating Ca^{2+}-induced cytochrome *c* release from isolated liver mitochondria, virtually all of these experiments have been performed under *in vitro* conditions that favor the induction of the MPT.[3–5,7] We therefore conducted the present study to provide a direct comparison of the release of cytochrome *c* from liver *vs* brain mitochondria under conditions that are at least somewhat inhibitory toward the MPT but are more physiologically realistic than those commonly employed with isolated mitochondria.

The measurements of light scattering, $\Delta\Psi$, and Ca^{2+} movements described in Figure 1 indicate that even in the presence of mM concentrations of ATP and Mg^{2+}, liver mitochondria can undergo a typical MPT which is dependent on the amount of mitochondrially accumulated Ca^{2+}. In contrast, under identical conditions, brain mitochondria accumulate and retain much more added

Ca^{2+} in the absence of the decrease in light scattering indicative of swelling. The accumulation of large loads of Ca^{2+} by brain mitochondria does result in a decrease in $\Delta\Psi$; however, this change is not associated with the release of accumulated Ca^{2+} and is due to factors other than the induction of the classical MPT. Although the comparisons made in the present study were conducted in the presence of the oxidizable substrate succinate, we have reached similar conclusions for brain mitochondria respiring on the NADH-linked substrates malate plus glutamate.[6] The classical MPT exhibited by liver mitochondria can occur in the presence of either FADH2- or NADH-linked substrates. However, evidence that there is a direct involvement of the mitochondrial NADH-CoQ oxidoreductase (Complex I) in regulating MPT warrants future studies comparing cytochrome *c* release from liver and other types of mitochondria utilizing different respiratory substrates.[34]

Further evidence that the MPT was evoked by Ca^{2+} in liver but not brain mitochondria comes from the electron micrographs shown in Figure 2. Although both Ca^{2+} and the artificial pore former alamethicin both caused an approximate 400% increase in the two dimensional area occupied by individual rat liver mitochondria, there was no increase in the size of brain mitochondria exposed to Ca^{2+}, but a 400% increase in mitochondrial area after exposure to alamethicin.

Under the conditions used in our study, approximately 80% of the total cytochrome *c* could be released from liver mitochondria after the addition of Ca^{2+}. This efflux of cytochrome *c* was Ca^{2+} dose-dependent with substantial extramitochondrial cytochrome *c* evident at a level of added Ca^{2+} equivalent to 0.3 μmol mg^{-1} protein. Maximal release occurred at approximately 0.5 μmol Ca^{2+} mg^{-1}, which is approximately the maximum Ca^{2+} uptake capacity for liver mitochondria under these conditions. Substantial cytochrome *c* was also released from brain mitochondria at 0.3 μmol Ca^{2+} mg^{-1} protein, which is less than 10% of the maximum amount of Ca^{2+} they can accumulate under these conditions. Although it is difficult to determine if cytochrome *c* release was saturated at 0.3 μmol Ca^{2+} mg^{-1}, it is clear that there was not extensive additional release after the accumulation of much more Ca^{2+}. The observation that the maximal amount of cytochrome *c* that could be liberated from brain *vs* liver mitochondria was 25 *vs* 80% of the total present indicates that the Ca^{2+} releaseable pool and the mechanisms of release are different for the two types of mitochondria.

Additional evidence that the cytochrome *c* release mechanisms are different for liver *vs* brain mitochondria comes from the immunoblots described in Figure 4 indicating that the MPT inhibitor cyclosporin A can largely or completely inhibit release from liver mitochondria but has no effect on release by brain mitochondria. In addition to these results arguing against the role of the MPT in mediating Ca^{2+}-induced cytochrome *c* release from brain mitochondria, they also indicate that the MPT is at least the primary mechanism responsible for Ca^{2+}-mediated release from liver mitochondria. Although the residual cytochrome *c* release apparent with liver mitochondria in the presence of cyclosporin A at 2.1 μmol Ca^{2+} mg^{-1} protein could be interpreted to be due to an MPT-independent mechanism, most of this extramitochondrial cytochrome *c* was eliminated by the further addition of 3 mM ADP, which is also known to inhibit MPT. Therefore, the mechanism responsible for Ca^{2+}-induced cytochrome *c* release from liver mitochondria is the MPT even when release is measured in the presence of physiological concentrations of ATP and Mg^{2+} that suppress MPT activity.

The striking difference between the characteristics of Ca^{2+}-evoked cytochrome *c* release from liver and brain mitochondria may help explain some of the discrepancies that have been observed in apoptotic cell paradigms. Although several studies have demonstrated a substantial drop in $\Delta\Psi$ concurrent with intracellular cytochrome *c* redistribution and (or) inhibition by cyclosporin A of cytochrome *c* release *in situ* within cells in the early stages of apoptosis,[15,35] a number of other studies have found no evidence for mitochondrial swelling or other measures of the MPT until after cytochrome *c* is released and caspases are activated.[8,21–23,36–39] Although Lemasters and colleagues have obtained perhaps the best evidence for the role of the MPT in apoptosis, their work has focused on hepatocytes whose mitochondria are the primary model for the MPT.[33,40–42] The findings that brain mitochondria are relatively resistant to the MPT together with our observations that cytochrome *c* is released from brain mitochondria by an MPT-independent mechanism suggests that mitochondria from other tissues and cells may also share similar characteristics.

Different mechanisms of cytochrome *c* release may explain variations in the response of mitochondria in different cell systems to apoptotic stimuli and may also explain differences in the degree to which MPT inhibitors like cyclosporin A are capable of interfering with apoptosis. The lack of sensitivity of isolated brain mitochondria to cyclosporin A is, however, apparently inconsistent with the reported neuroprotective effects of this drug in animal models of cerebral ischemia and hypoglycemia.[43,44] It is therefore possible that the permeability transition is more active in brain mitochondria as they exist *in situ* within neural cells and tissues. It is also likely that the potential for involvement of the MPT varies between neuronal and non-neuronal brain cells and between cellular subtypes. For example, we observe a robust MPT in digitonin-permeabilized cortical astrocytes in the presence of ATP and Mg^{2+} that is not apparent in permeabilized cerebellar granule neurons.[45] Friberg *et al*.[46] recently reported differences in the sensitivity to MPT by non-synaptosomal mitochondria isolated from different regions of the rat brain. These differences were observed in the absence of exogenous Mg^{2+} and adenine nucleotides and attributed to variations in endogenous adenine nucleotides present in isolated mitochondria. As mitochondrial proteins, e.g., Bcl-2 and cyclophilins, can additionally affect the sensitivity of mitochondria to the MPT and to other Ca^{2+}-evoked responses,[47–50] relationships between the levels of such proteins and Ca^{2+}-

induced cytochrome *c* release among different types of mitochondria should also be pursued.

The mechanism by which the MPT evokes cytochrome *c* release from either isolated mitochondria or mitochondria present within cells is most likely due to the physical rupture of the mitochondrial outer membrane that occurs in response to the expansion of the normally highly invaginated inner membrane.[5,13–15] The MPT-independent mode of cytochrome *c* release exhibited by isolated brain mitochondria could be mediated by a limited, albeit swelling-independent disruption in the continuity of the outer membrane. This disruption could potentially occur as a consequence of Ca^{2+}-stimulated phospholipase activity.[51] Although phospholipase inhibitors were not included in our experiments, the fact that brain mitochondrial cytochrome *c* is not released by exogenous Ca^{2+} in the absence of transport into the matrix argues against the direct involvement of phospholipase activity in outer membrane permeability changes.[6] Alternatively, release of cytochrome *c* might be mediated by activation of a pore in the outer membrane that is large enough for the passage of cytochrome *c*. The most likely known candidate for such a pore is mitochondrial porin, also known as the voltage dependent anion channel (VDAC). Porin has an internal pore diameter of approximately 2.8–3.0 nm whereas the dimensions of native cytochrome *c* are approximately 3.0×3.4 nm.[52] Cytochrome *c* has been demonstrated to bind to porin and, in light of porin's β-barrel structure, such binding could possibly induce a conformational change sufficient to allow cytochrome *c* through the pore.[52] The findings that the anti-apoptotic protein Bcl-2 inhibits Ca^{2+}-induced cytochrome *c* release[36,37] and that Bcl-2 is co-localized with porin at mitochondrial inner-outer membrane contact sites[54] are further indications that porin may be involved in the apoptotic cytochrome *c* release phenomenon. The recent study by Shimizu *et al*.[53] supporting the role of VDAC in cytochrome *c* release elicited by the binding of BAX to mitochondria provides additional support for assessing its role in Ca^{2+}-induced cytochrome *c* release.

Materials and Methods

Isolation of mitochondria

Liver mitochondria were prepared from male Sprague Dawley rats that had been fasted overnight. The procedure was a slight modification of the method of Schnaitman and Greenwalt[55] where the pH of the isolation media was maintained at 7.2. Rat brain mitochondria were isolated according to the procedure of Rosenthal *et al*.[56] yielding a mixture of non-synaptosomal plus synaptosomal mitochondria. In brief, the pellet from the first $12\,000 \times g$ centrifugation that contains non-synaptosomal mitochondria plus synaptosomes is treated with a low concentration of digitonin (0.01%) to disrupt the cholesterol-rich synaptosomal membranes, releasing the mitochondria trapped within. Additional differential centrifugation is employed to further purify the mixture of mitochondria. Mitochondrial protein was determined by a modified biuret reaction.[57] Mitochondria so isolated exhibit excellent respiratory rates and respiratory control ratios compared to brain mitochondria isolated by other procedures.[56]

Measurements of mitochondrial volume changes, membrane potential ($\Delta\Psi$) and Ca^{2+} transport

Mitochondria were incubated at 37°C at 0.5 mg protein ml^{-1} in medium containing 125 mM KCl, 2 mM K_2HPO_4, 3 mM ATP, 4 mM $MgCl_2$, 5 mM HEPES-KOH (pH 7.0) and 5 mM succinate plus 2 μM rotenone. Mitochondrial volume changes were followed by measuring the light scattering of suspensions via the absorbance of light at 660 nm. In some experiments, relative volumes were compared using transmission electron microscopy. Centrifuged samples of the mitochondrial suspensions were fixed overnight in a solution containing 4% formaldehyde plus 1% glutaraldehyde, and postfixed in 1% osmium tetroxide. Dehydration was performed in a series of ethanol and propylene oxide extractions, prior to sample embedding in Polibed® 812. Sections were cut at 0.1 μm and stained with uranyl acetate plus lead citrate. Electron micrographs were obtained using a JOEL 1200 EX electron microscope. Mitochondrial transmembrane potential was monitored simultaneously with light scattering using a TPP^+-selective electrode and 2 μM TPP-Cl. Ca^{2+} fluxes were also monitored simultaneously using a Ca^{2+}-selective electrode to measure changes in the medium [Ca^{2+}].

Measurements of cytochrome *c* release

For measurements of cytochrome *c*, an aliquot of the experimental suspension was centrifuged at $13\,000 \times g$ for 2 min. An 0.75 ml aliquot of supernatant was carefully removed and supplemented with 15 μl of Protease Inhibitor Cocktail (Sigma), frozen on dry ice and stored at −70°C. The mitochondrial pellet was resuspended in 1 ml of the medium and stored likewise. Comparison of results obtained following centrifugation of suspensions at 2, 5, and 10 min verified that complete mitochondrial sedimentation occurred after 2 min. For immunoblots, 16 μl aliquots of the samples (diluted to 0.12 mg mitochondrial protein ml^{-1} for the pellets) were run on to 4–20% Tris-Glycine gradient gels. Proteins were electrotransferred to PVDF membranes, that were then rinsed with imidazole-buffered saline with 0.02% Tween 20 (Kirkegaard & Perry Labs, MD, USA) buffer and blocked overnight in buffer supplemented with 1% BSA and 1% dry milk. Cytochrome *c* was immunostained with primary 7H8 mouse anti-cytochrome *c* antibody (PharMingen) plus secondary anti-mouse Ig bound to horseradish peroxidase (Amersham) (1 : 2000 dilution each). Peroxidase activity was detected using Enhanced ChemiLuminescence detection kit (Amersham) and X-ray film. Band intensities were analyzed densitometrically using GelExpert system (NucleoTech). Semi-quantitative comparisons were made between the band intensities measured for samples obtained under different experimental conditions.

Acknowledgements

This work was supported by NIH grant NS34152 and a grant from the Bayer corporation. We wish to thank Mr. S Russell for expert technical assistance and Dr. A Kowaltowski for helpful discussions.

References

1. Green DR and Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309–1312
2. Susin SA, Zamzami N and Kroemer G (1998) Mitochondria as regulators of apoptosis: doubt no more. Biochim. Biophys. Acta. 1366: 151–165
3. Kantrow SP and Piantadosi CA (1997) Release of cytochrome c from liver mitochondria during permeability transition. Biochem. Biophys. Res. Commun. 232: 669–671

4. Scarlett JL and Murphy MP (1997) Release of apoptogenic proteins from the mitochondrial intermembrane space during the mitochondrial permeability transition. FEBS Lett 418: 282 – 286
5. Petit PX, Goubern M, Diolez P, Susin SA, Zamzami N and Kroemer G (1998) Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. FEBS Lett 426: 111 – 116
6. Andreyev AY, Fahy B and Fiskum G (1998) Cytochrome c release from brain mitochondria is independent of the mitochondrial permeability transition. FEBS Lett. 439: 373 – 376
7. Yang JC and Cortopassi GA (1998) Induction of the mitochondrial permeability transition causes release of the apoptogenic factor cytochrome c. Free Radic. Biol. Med. 24: 624 – 631
8. Eskes R, Antonsson B, Osen-Sand A, Montessuit S, Richter C, Sadoul R, Mazzei G, Nichols A and Martinou JC (1998) Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg^{2+} ions. J. Cell. Biol. 143: 217 – 224
9. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P and Korsmeyer SJ (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J. Biol. Chem. 274: 1156 – 1163
10. Han Z, Bhalla K, Pantazis P, Hendrickson EA and Wyche JH (1999) Cif (Cytochrome c efflux-inducing factor) activity is regulated by bcl-2 and caspases and correlates with the activation of Bid. Mol. Cell. Biol. 19: 1381 – 1389
11. Jacobs EE and Sanadi DR (1960) Phosphorylation coupled to electron transport mediated by high potential electron carriers. Biochim. Biophys. Acta. 38: 12 – 33
12. Chappell JB and Crofts AR (1965). Calcium ion accumulation and volume changes of isolated liver mitochondria. Biochem. J. 95: 387 – 392
13. Pfeiffer DR, Gudz TI, Novgorodov SA and Erdahl WL (1995) The peptide mastoparan is a potent facilitator of the mitochondrial permeability transition. J. Biol. Chem. 270: 4923 – 4932
14. Skulachev VP (1997) Membrane-linked systems preventing superoxide formation. Bioscience Reports 17: 347 – 257
15. Kroemer G, Zamzami N and Susin SA (1997) Mitochondrial control of apoptosis. Immun. Today 18: 44 – 51
16. Broekemeier KM, Dempsey ME and Pfeiffer DR (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J. Biol. Chem. 264: 7826 – 7830
17. Zoratti M and Szabò I (1995) The mitochondrial permeability transition. Biochim. Biophys. Acta. 1241: 139 – 176
18. Bernardi P (1996) The permeability transition pore. Control points of a cyclosporin A-sensitive mitochondrial channel involved in cell death. Biochim. Biophys. Acta. 1275: 5 – 9
19. Vercesi AE, Kowaltowski AJ, Grijalba MT, Meinicke AR and Castilho RF (1997) The role of reactive oxygen species in mitochondrial permeability transition. Biosci. Rep. 17: 43 – 52
20. Hunter DR, Haworth RA and Southard JH (1976) Relationship between configuration, function, and permeability in calcium-treated mitochondria. J. Biol. Chem. 251: 5069 – 5077
21. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT and Thompson CR (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 91: 627 – 637
22. Bossy-Wetzel E, Newmeyer DD and Green DR (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J. 17: 37 – 49
23. Zhuang J, Dinsdale D and Cohen GM (1998) Apoptosis, in human monocytic THP.1 cells, results in the release of cytochrome c from mitochondria prior to their ultracondensation, formation of outer membrane discontinuities and reduction in inner membrane potential. Cell Death Differ. 5: 953 – 962
24. Kristal BS and Dubinsky JM (1997) Mitochondrial permeability transition in central nervous system: induction by calcium cyclin-dependent and -independent pathways. J. Neurochem. 69: 524 – 538
25. Berman SB, Watkins SW and Hastings TG (1998) Evidence that brain mitochondria do not readily undergo permeability transition. Soc. Neurosci. Abstr. 24: 553
26. Fiskum G, Murphy AN and Beal MF (1999) Mitochondria in neurodegeneration: Acute ischemia and chronic neurodegenerative diseases. J. Cereb. Blood Flow Metab. 19: 351 – 369
27. Murphy AN, Fiskum G and Beal MF (1999) Mitochondria in neurodegeneration: Bioenergetic function in cell life and death. J. Cereb. Blood Flow Metab. 19: 231 – 245
28. Dugan LL, Sensi SL, Canzoniero LMT, Handran SD, Rothman SM, Lin T-S, Goldberg MP and Choi DW (1995) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. J. Neurosci. 15: 6377 – 6388
29. Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA and Nicotera P (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron. 15: 961 – 973
30. Budd SL and Nicholls DG (1996) Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells. J. Neurochem. 67: 2282 – 2291
31. Stout AK, Raphael HM, Kanterewica BI, Klann E and Reynolds IJ (1998) Glutamate-induced neuronal cell death is blocked by inhibition of mitochondrial calcium uptake with FCCP. Nature Neurosci. (in press)
32. Kruman II and Mattson MP (1999) Pivotal role of mitochondrial calcium uptake in neural cell apoptosis and necrosis. J. Neurochem. 72: 529 – 540
33. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA and Herman B (1998b) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta. 1366: 177 – 196
34. Fontaine E, Eriksson O, Ichas F and Bernardi P (1998) Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation by electron flow through the respiratory chain complex I. J. Biol. Chem. 273: 12662 – 12668
35. Walter DH, Haendeler J, Galle J, Zeiher AM and Dimmeler S (1998) Cyclosporin A inhibits apoptosis of human endothelial cells by preventing release of cytochrome C from mitochondria. Circulation 98: 1153 – 1157
36. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP and Wang X (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275: 1129 – 1132
37. Kluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275: 1132 – 1136
38. Cai J and Jones DP (1998) Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. J. Biol. Chem. 237: 11401 – 11404
39. Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG and Green DR (1999) Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. J. Biol. Chem. 274: 2225 – 2233
40. Bradham CA, Qian T, Streetz K, Trautwein C, Brenner DA and Lemasters JJ (1998) The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. Mol. Cell. Biol. 18: 6353 – 6364
41. Lemasters JJ, Qian T, Elmore SP, Trost LC, Nishimura Y, Herman B, Bradham CA, Brenner DA and Nieminen AL (1998a) Confocal microscopy of the mitochondrial permeability transition in necrotic cell killing, apoptosis and autophagy. Biofactors 8: 283 – 285
42. Qian T, Herman B and Lemasters JJ (1999) The mitochondrial permeability transition mediates both necrotic and apoptotic death of hepatocytes exposed to Br-A23187. Toxicol. Appl. Pharmacol. 154: 117 – 125
43. Uchino H, Elmer E, Uchino K, Li PA, He QP, Smith ML and Siesjö BK (1998) Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat. Brain Res 812: 216 – 226
44. Friberg H, Ferrand-Drake M, Bengtsson F, Halestrap AP and Wieloch T (1998) Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. J. Neurosci. 18: 5151 – 5159
45. Fiskum G and Andreyev A (1999) Brain mitochondrial heterogeneity in Ca^{2+}-induced release of apoptogenic cytochrome c. J. Neurochem. 72: S52C
46. Friberg H, Connern C, Halestrap AP and Wieloch T (1999) Differences in the activation of the mitochondrial permeability transition among brain regions in the rat correlate with selective vulnerability. J. Neurochem. 72: 2488 – 2497
47. Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M and Kroemer G (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med. 184: 1331 – 1341

48. Murphy AN, Bredesen DE, Cortopassi G, Wang E and Fiskum G (1996) Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. Proc. Natl. Acad. Sci. USA 93: 9893 – 9898
49. Shimizu S, Eguchi Y, Kamiike W, Funahashi Y, Mignon A, Lacronique V, Matsuda H and Tsujimoto Y (1998) Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. Proc. Natl. Acad. Sci. USA 95: 1455 – 1459
50. Halestrap AP, Kerr PM, Javadov S and Woodfield KY (1998) Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. Biochim. Biophys. Acta. 1366: 79 – 94
51. Pastorino JG, Simbula G, Yamamoto K, Glascott Jr PA, Rothman RJ and Farber JL (1996) The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition. J. Biol. Chem. 271: 29792 – 29798
52. Mannella CA (1998) Conformational changes in the mitochondrial channel protein, VDAC, and their functional implications. J. Struct. Biol. 121: 207 – 218
53. Shimizu S, Narita M and Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature 399: 483 – 487
54. Reed JC, Jurgensmeier JM and Matsuyama S (1998) Bcl-2 family proteins and mitochondria. Biochim. Biophys. Acta. 1366: 127 – 137
55. Schnaitman C and Greenwalt JW (1968) Enzymatic properties of the inner and outer membranes of rat liver mitochondria. J. Cell. Biol. 38: 158 – 175
56. Rosenthal RE, Hamud F, Fiskum G, Vardhese PJ and Sharpe S (1987) Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. J. Cereb. Blood. Flow Metab. 7: 752 – 758
57. Skarkowska L and Klingenberg M (1963) On the role of ubiquinone in mitochondria. Biochem. Z. 338: 674 – 697