Mitochondria and cell death
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Abstract
Mitochondria play a central role in both apoptosis and necrosis through the opening of the mitochondrial permeability transition pore (MPTP). This is thought to be formed through a Ca\(^{2+}\)-triggered conformational change of the adenine nucleotide translocase (ANT) bound to matrix cyclophilin-D and we have now demonstrated this directly by reconstitution of the pure components. Opening of the MPTP causes swelling and uncoupling of mitochondria which, unrestrained, leads to necrosis. In ischaemia/reperfusion injury of the heart we have shown MPTP opening directly. Recovery of hearts correlates with subsequent closure, and agents that prevent opening or enhance closure protect from injury. Transient MPTP opening may also be involved in apoptosis by initially causing swelling and rupture of the outer membrane to release cytochrome c (cyt c), which then activates the caspase cascade and sets apoptosis in motion. Subsequent MPTP closure allows ATP levels to be maintained, ensuring that cell death remains apoptotic rather than necrotic. Apoptosis in the hippocampus that occurs after a hypoglycaemic or ischaemic insult is triggered by this means. Other apoptotic stimuli such as cytokines or removal of growth factors also involve mitochondrial cyt c release, but here there is controversy over whether the MPTP is involved. In many cases cyt c release is seen without any mitochondrial depolarization, suggesting that the MPTP does not open. Recent data of our own and others have revealed a specific outer-membrane cyt c-release pathway involving porin that does not release other intermembrane proteins such as adenylate kinase. This is opened by pro-apoptotic members of the Bcl-2 family such as BAX and prevented by anti-apoptotic members such as Bcl-x\(_1\). Our own data suggest that this pathway may interact directly with the ANT in the inner membrane at contact sites.

Key words: apoptosis, cytochrome c, permeability transition, porin, reperfusion injury.
Abbreviations used: ANT, adenine nucleotide translocase; CsA, cyclosporin A; Cyp-D, cyclophilin-D; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; ΔΨ, mitochondrial membrane potential.

Introduction
The cells of most tissues have a finite lifetime with a few cells dying at any one time and being replaced by new cells. Such cell death is known as programmed cell death or apoptosis, and is strictly controlled. It is an energy-requiring process, associated with characteristic changes in cell morphology including condensation of the chromatin with nuclear fragmentation, condensation of the cytosol into apoptotic bodies and changes in the cell surface that enable recognition by macrophages. These engulf apoptotic cells enabling them to be destroyed in a non-inflammatory manner. A particularly dramatic example of apoptosis is seen in tissue remodeling during embryo development or metamorphosis such as when the tadpole loses its tail. Apoptosis also occurs following a moderate insult, insufficient to kill the cell outright but enough to cause significant cell damage. Examples include a short period of hypoxia or exposure to low doses of a chemical toxin. In contrast, if the initial damage to a cell is too severe, the precisely regulated process of apoptosis is not possible and cell death occurs by necrosis. This is characterized by changes in cell morphology quite distinct from those seen in apoptosis. The cell and its organelles swell and the plasma membrane ruptures with the loss of intracellular contents into the surrounding medium. This attracts neutrophils which cause an inflammatory response and secondary damage to the tissue. Thus cell death is a two-edged sword. In situations such as chemical poisoning of the liver or reperfusion injury that may follow clot-busting treatment of coronary thrombosis or stroke, or after cardiac surgery there is cell death that leads to irreversible damage. Conversely, in many cancers it is an impairment of apoptosis that leads to unrestrained cell proliferation and an insensitivity to chemotherapeutic agents. Clinically it would be desirable to inhibit cell death in the former situations and stimulate it in the latter. In recent years it has become apparent that mitochondria play a critical role in the mechanism of both apoptotic and necrotic cell death, in addition to their more widely recognized function in the provision of ATP to sustain cellular function. This article will briefly review how this is achieved.
For a more detailed account the reader is referred elsewhere [1-3].

The mitochondrial permeability transition (MPT)

The mechanism of oxidative phosphorylation requires that the mitochondrial inner membrane be impermeable to all but a few selected metabolites and ions. If this permeability barrier is lost, mitochondria become uncoupled and hydrolyse ATP rather than synthesize it. Left unrestrained this would inevitably lead to cell death. It is now recognized that mitochondria contain a latent non-specific protein in their inner membrane that, when activated, causes just such an increase in membrane permeability. Known as the mitochondrial permeability transition pore (MPTP), this opens when the mitochondria are exposed to high calcium concentrations, especially when this is associated with adenine nucleotide depletion and oxidative stress [1-4]. These are exactly the conditions that accompany many cellular insults that lead to necrotic cell death [1-3,5]. The MPTP is non-specific and transports any molecule of <1500 Da. Not only does its opening prevent ATP synthesis, it also causes the loss of ions and metabolites from the mitochondrial matrix and induces extensive swelling of the mitochondria as a result of the colloidal osmotic pressure exerted by the matrix proteins. It is well established that mitochondria in necrotic cells are swollen and have greatly impaired respiration and oxidative phosphorylation [1-3,5].

The MPT was first described several decades ago, although originally it was thought to occur as the result of phospholipase A, degradation of the inner membrane. However, a major breakthrough came in 1988 when Crompton and colleagues demonstrated that the process could be inhibited specifically by sub-micromolar concentrations of the immunosuppressive drug, cyclosporin A (CsA) [6]. In this laboratory we demonstrated that this effect of CsA was exerted through inhibition of a peptidyl-prolyl cis-trans isomerase unique to the mitochondria, otherwise known as cyclophilin-D (CyP-D) because of its CsA-binding properties [7,8]. In addition we confirmed and extended data of others that the conformational state of the adenine nucleotide translocase (ANT) greatly influenced the sensitivity of the MPTP to [Ca²⁺]. This led to our proposal in 1990 that in the presence of calcium a cyclophilin-mediated conformational change of the ANT was responsible for the formation of the MPTP [7]. Subsequent data have shown that oxidative stress greatly enhances the calcium sensitivity of the MPTP by two mechanisms [9]. First, by increasing CyP-D binding to the ANT, and second, by greatly reducing the affinity of the intramitochondrial adenine nucleotide-binding site on the ANT; binding of adenine nucleotides to this site was shown to inhibit the MPTP competitively with respect to [Ca²⁺]. The evidence in support of this hypothesis has been steadily mounting [1-3] and most recently we have demonstrated directly that CyP-D binds very tightly...
and specifically to the ANT. Binding was prevented but not reversed by CsA [10]. Crompton and colleagues have reported similar data but with two important differences that may reflect the distinct detergent used [11]. First, the binding they observed was not prevented by CsA treatment and second they observed that both porin and ANT bound tightly to CyP-D, whereas in our experiments no protein other than the ANT was bound. Porin is known to be associated with the ANT at contact sites and its involvement in the MPTP is a matter of controversy [1–3].

Final proof that the MPTP is composed of just the ANT and CyP-D would require reconstitution of the pure proteins into proteoliposomes to form a CsA-sensitive calcium-activated pore. Earlier data had shown that the ANT alone could produce such a pore either when critical thiol groups were modified or when exposed to high [Ca$^{2+}$] (> 1 mM) [12]. Crompton and colleagues were able to reconstitute their ANT/porin/CyP-D complex into proteoliposomes to produce a CsA-inhibitable pore that opened at 100 μM [Ca$^{2+}$] [11] and very recently we have succeeded in doing the same with pure ANT and CyP-D (in the absence of porin). For this purpose we have developed a continuous assay for the reconstituted MPTP which should greatly assist future studies. Preliminary data are shown in Figure 1. Thus it now seems that the controversy over the identity of the MPTP may be resolved; the components are the ANT and CyP-D as originally proposed [7]. However that does not exclude a role for other proteins such as porin in the regulation of the MPTP.

**Role of the MPT in necrotic cell death**

The conditions which promote the MPT are exactly those that a cell experiences in response to a range of insults that lead to necrotic cell death. The three major consequences of its opening are an uncoupling of oxidative phosphorylation, the loss of ions and small molecules from the mitochondrial matrix and extensive swelling of the mitochondria. If the MPT remains open, ATP cannot be regenerated and damage caused by the initial insult cannot be repaired. In addition, continued perturbation of ion balances, especially calcium, will further exacerbate the damage by activating degradative enzymes such as phospholipases, proteases and nucleases. The cell is doomed to die by necrosis and the swollen, uncoupled mitochondria observed in necrotic cells is consistent with this scenario [1–3,5]. However, direct demonstration that the MPTP opens under such conditions is more difficult. In isolated cells, measurement of mitochondrial membrane potential ($\Delta\Psi$) with fluorescent probes such as tetramethylrhodamine has been used to demonstrate uncoupling of mitochondria that occurs following the MPT [2,3,5,13]. A more rigorous method uses confocal fluorescence microscopy with a fluorescent molecule such as calcein that is normally unable to permeate the inner mitochondrial membrane, but which can permeate through the MPTP. This technique can be used in conjunction with tetramethylrhodamine, enabling coincident measurement of $\Delta\Psi$. Experiments such as these have confirmed that the MPT does occur in isolated cells subjected to a variety of stresses that induce necrosis [5]. Furthermore, low pH and drugs such as CsA that inhibit the MPT protect cells from necrotic death under these conditions [1–3,5].

Fluorescence techniques are open to the criticism that photo-oxidation of the dyes occurs and causes oxidative stress to the cells which may open the MPTP in its own right [14,15]. There are also concerns over how rigorously dyes can be excluded or accumulated by mitochondria [5,15]. Furthermore, such an approach is not appropriate for use in studies of reperfusion injury of the heart, a particular interest of this laboratory. For this purpose, we have developed a procedure to measure pore opening in the perfused heart that involves loading hearts with [$^3$H]deoxyglucose and its subsequent entrapment in mitochondria when the MPTP opens [16]. This technique has provided direct evidence that the MPT occurs during reperfusion after ischaemia (but not during ischaemia itself) [16]. Such reperfusion injury is associated with necrotic damage to the heart and we have demonstrated that inhibitors of the MPT such as CsA, low pH and free-radical scavengers all offer protection [1–3,5,17,18]. Pyruvate and the widely used anaesthetic, propofol, are especially effective in this regard and the deoxyglucose technique has demonstrated directly that protection is associated with less pore opening. Even hearts that achieved 100% functional recovery from ischaemia showed some pore opening in the early stages of reperfusion, but the pores subsequently closed as reperfusion was continued [18]. Others have demonstrated a role for the MPTP in reperfusion injury and other forms of oxidative stress in the perfused liver and isolated hepatocytes and endothelial cells, again with protection offered by inhibitors of pore opening.
[1,2,5]. Thus the role of the MPT in necrosis seems well established and the ability of MPTP antagonists to protect tissues from damage caused by ischaemia/reperfusion or chemically induced oxidative stress is of considerable clinical importance.

Role of the MPT in apoptotic cell death

In recent years there has been a flood of data implicating the mitochondria in apoptotic cell death [2,3,5,13,19,20]. It is now clearly established that in many if not all apoptotic cells an early event is the release of proteins from the intermembrane space of mitochondria. The protein whose release appears most critical is cytochrome c which, in the presence of ATP and dATP, forms a complex with apoptosis activating factor 1 (APAF-1) and procaspase 9. This induces cleavage of procaspase 9 with release of active caspase 9 that cleaves and activates procaspase 3. The active caspase 3 then induces proteolytic cleavage of a range of target proteins responsible for the rearrangements of the cytosol, nucleus and plasma membrane that are characteristic of apoptosis [20–23]. However, the mechanism of cytochrome c release remains controversial. One proposed method is through induction of the MPT, leading to mitochondrial swelling and outer-membrane rupture. Data in support of this has relied on measurements of a drop in $\Delta \Psi$ during apoptosis and the protective effects of inhibitors of the MPTP [13,19]. However, neither measurement is without criticism. The determination of $\Delta \Psi$ with fluorescent dyes is a controversial area fraught with problems [24,25] and the time course of the drop in $\Delta \Psi$ is also critical, since if it occurs after caspase activation and changes in plasma-membrane morphology it may reflect secondary necrosis rather than apoptosis. This would not occur in vivo where apoptotic cells are rapidly removed by phagocytes. However, in culture phagocytes are not present and the continued progression of apoptosis with an ever-increasing loss of cytochrome c is likely to increase reactive oxygen species by mitochondria leading to the permeability transition and thus necrosis [26]. In Figure 2 we present some of our own data demonstrating that caspase 3 activation in the murine fibrosarcoma cell line WEHI-164 is initiated 6–8 h after tumour necrosis factor $\alpha$ addition, at which time we could detect no decrease in $\Delta \Psi$. Indeed a decrease in $\Delta \Psi$ was not detected even

![Figure 2](image_url)
after 12 h, at which time other measures of apoptosis, such as DNA fragmentation detected by the Tunel assay, show a large number of cells to be apoptotic. After 24 h changes in ΔΨ did become apparent, although at this stage the morphology of the cells suggested that they were undergoing secondary necrosis.

The inhibitory action of antagonists of the MPT (e.g. CsA and bongkrekic acid) on apoptosis under some conditions has been taken as supporting a role for the MPTP, as has the ability of agents that stimulate opening of the MPTP to induce apoptosis (e.g. oxidative stress and high concentrations of atractyloside). However, none of these reagents is specific [2]. For example, CsA is a potent inhibitor of the calcium-sensitive protein phosphatase calcineurin and there is now strong evidence that calcineurin is involved in the dephosphorylation of BAD, a pro-apoptotic member of the Bcl-2 family [19,20,27] that binds to the mitochondria and induces cytochrome c release only when dephosphorylated [28]. There is also the possibility that CsA exerts calcineurin-mediated effects on the cell-survival pathways that work in opposition to apoptosis. In the experiments shown in Figure 2, CsA was without effect on the activation of caspase 3, although trifluoperazine, another inhibitor of the MPT, did prevent caspase activation. Trifluoperazine is also a calmodulin antagonist and thus its inhibitory effects on caspase activation may involve inhibition of an additional calcium/calmodulin-mediated event in the complex signalling pathways of apoptosis and cell survival.

Despite the reservations noted above, there is good evidence to suggest that apoptosis caused by mild oxidative stress may be mediated by transient opening of the MPTP. This would cause a limited swelling of the mitochondria, sufficient to disrupt the outer membrane and release cytochrome c, while enabling the maintenance of the mitochondrial membrane potential and oxidative phosphorylation [1,2]. We have demonstrated directly that such transient opening of the MPTP can occur in the perfused heart subjected to reperfusion injury, and this may explain the presence of a ring of apoptotic cells that is observed around the necrotic area of a myocardial infarct [18]. These apoptotic cells are likely to have experienced less severe ischaemia and hence reperfusion injury than the cells at the centre of the infarct. A similar situation occurs in the brain following a period of transient ischaemia or hypoglycaemia. Under these conditions specific neurons in the hippocampus die by apoptosis several days after the initial insult, and appear to have swollen mitochondria characteristic of those that have undergone the permeability transition [29–31]. CsA and its non-immunosuppressive analogues can prevent both the cell death and mitochondrial swelling, an effect not seen with FK506, another immunosuppressive drug that acts via calcineurin inhibition while having no effect on the MPTP [29,31]. Interestingly, mitochondria isolated from hippocampal neurons, which are most susceptible to apoptosis, are more sensitive to calcium-induced MPTP opening than mitochondria from the cortex or cerebellum which do not show apoptosis under these conditions [30].

**An MPT-independent role for mitochondria in apoptosis**

As outlined above, cytochrome c release from mitochondria plays a central role in apoptosis under many conditions that are not associated with the permeability transition. The mechanism of this release pathway remains uncertain although there is an emerging picture. Bcl-2 is a 26-kDa protein whose over-expression in some tumours blocks apoptosis induced by a wide range of stimuli [27]. Cytochrome c release is inhibited by Bcl-2 and related family members such as Bcl-xL, whereas other Bcl-2 family members such as BAD, BAX and most recently BID enhance apoptosis and can stimulate cytochrome c release [22,27,32–36]. These proteins interact with each other in the outer mitochondrial membrane to maintain the required balance between cell survival and proliferation on the one hand and apoptosis on the other; but exactly how this is achieved is unclear. Proteolytic modifications and phosphorylations of the different family members have been suggested to provide the link between some cell-surface receptors and stimulation of cytochrome c release [20,28]. In particular, there is strong evidence for cytokine-receptor-mediated death-domain-dependent activation of caspase 8 followed by cleavage of a BID precursor to form active BID which migrates to the mitochondria and through interaction with BAX causes cytochrome c release [32–36].

What is still missing from the picture is how BID, BAX and BAD cause the release of cytochrome c from the intermembrane space. Two possibilities have been proposed [2,19]. The first mechanism involves outer-membrane rupture, either as a result of the MPT (see above) or by MPT-independent mechanisms [37], while the
second mechanism proposes that BAX, alone or in combination with other proteins such as BAD or BID, may form a specific pathway (channel) for cytochrome c transport \[34,36\]. The former mechanism would be associated with release of other intermembrane proteins such as adenylate kinase, and under some conditions this may be observed, probably reflecting an opening of the MPTP \[38\]. However, outer-membrane rupture would seem to be an imprecise mechanism for the release of a specific apoptotic signal and data from our own laboratory suggests that another mechanism is involved. We have demonstrated that isolated rat liver mitochondria contain some BAX dimer. When these mitochondria were incubated in KCl medium under energized conditions a loss of cytochrome c to the medium was observed over several minutes. This release was not associated with mitochondrial swelling or with release of adenylate kinase (measured enzymically). Neither was it prevented by CsA, confirming that the MPT was not involved. However release was greatly reduced by increasing the colloidal osmotic pressure of the incubation medium with 5% dextran. This contrasts with the effects of increasing the matrix volume using hypo-osmotic media, where a 50% increase in matrix volume caused substantial cytochrome c release that was also associated with extensive adenylate kinase release whether or not dextran was present. Data are illustrated in Figure 3.

The effect of increasing the colloidal osmotic pressure with dextran is to bring the inner and outer mitochondrial membranes into closer proximity, and to increase the number of contact sites \[39\]. This suggests that disruption of an interaction between the inner and outer membrane, perhaps at the contact sites, may open up a permeability pathway for cytochrome c. In this context it is of interest that Bcl-2 is preferentially

![Figure 3](image-url)

**Figure 3**

The effect of osmolarity and dextran on cytochrome c and adenylate kinase release from isolated rat liver mitochondria

Isolated mitochondria (1 mg of protein) were incubated for 1 min at 25 °C in medium (pH 7.2) containing 20 mM Mops, 10 mM Tris, 2 mM EGTA, 0.2 μM rotenone, 1 mM succinate and 0–150 mM KCl in the presence or absence of 5% (w/v) dextran (40 kDa) as indicated. Following sedimentation of mitochondria by centrifugation, cytochrome c content and adenylate kinase activity of supernatants were assayed by Western blotting and enzymic assay respectively. Data for the adenylate kinase activity are expressed as a percentage of the adenylate kinase activity in 50 mosmol medium without added dextran and are given as means of two independent experiments. These showed an identical pattern of adenylate kinase and cytochrome c release with changing osmolarity. The inset is a Western blot with anti-BAX antibodies of rat liver mitochondrial proteins separated by SDS/PAGE, and demonstrates the presence of BAX dimers.
located at contact sites [13] where porin and the outer-membrane benzodiazepine receptor (BenzdR) are also concentrated [13,40]. Contact sites are thought to involve an interaction between the ANT and porin that allows the communication between the inner and outer membranes [4,39]. We have demonstrated (E. Doran and A. P. Halestrap, unpublished work) that polyaspartate, which is known to bind to porin and induce the low anion-conductance state [41], increases the release of cytochrome c from isolated liver mitochondria. This suggests that the low anion-conductance state of porin may activate a cytochrome c release pathway by some protein–protein interaction within the contact sites. This conclusion is supported by the observation that apoptosis induced by growth-factor withdrawal is associated with impaired ATP/ADP exchange into mitochondria [42], which closure of porin is known to induce [43]. In contrast, Bcl-xL over-expression facilitates mitochondrial ATP/ADP exchange and inhibits cytochrome c release [42], suggesting that it stabilizes the high conductance state of porin associated with low cytochrome c permeability. In contrast, it would be envisaged that BAX might interact with porin to favour the low-conductance conformation and so enhance cytochrome c release. In this context it may be significant that both BAX and Bcl-2 have been shown to be concentrated at the contact sites [13,40,44].

In contradiction to these data, Shimizu et al. [45] have reported that when porin is reconstituted into proteoliposomes, the addition of either BAX or BAK enables cytochrome c translocation across the membrane, which is blocked by Bcl-xL. No cytochrome c permeation occurred in the presence of porin alone. It is hard to reconcile these data with those described above and with data of Priault et al. [46], who have demonstrated that BAX can induce cytochrome c release in porin-deficient yeast.

Whatever the exact mechanism, our data imply that there is a specific cytochrome c release mechanism whose activity is modulated by the interaction of inner and outer membranes at the contact sites. This process may involve porin either directly as the cytochrome c transporter, or indirectly. In the latter case the association of porin with ANT at the contact sites may enable their respective conformations to regulate cytochrome c release without themselves being directly involved. Since the MPTP may also be associated with contact sites and be formed by a conformational change in the ANT, possibly in association with porin [1–4,10,11], this hypothesis provides an explanation for how Bcl-2 may inhibit both the specific (MPTP-independent) cytochrome c release mechanism and the MPTP-dependent mechanism.

Conclusions

Whatever the final picture to emerge, one thing is now clear. Mitochondria play a central role in both the life and death of a cell. The molecular mechanism of the MPTP is becoming clear and this may aid in the design of drugs to protect tissues from cell death caused by a range of insults such as ischaemia/reperfusion and some chemical toxins. Conversely, triggering the opening of the MPTP might provide another target for chemotherapy. Once the molecular mechanism of cytochrome c release is also elucidated yet another pharmacological target will become available. The role of mitochondria in cell death looks likely to remain a hot area of research for a few years to come!

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References


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The mitochondrial trifunctional protein: centre of a \( \beta \)-oxidation metabolon?

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Abstract

The trifunctional enzyme comprises three consecutive steps in the mitochondrial \( \beta \)-oxidation of long-chain acyl-CoA esters: 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase. Deficiencies in either 3-hydroxyacyl-CoA dehydrogenase activity, or all three activities, are important causes of human disease. The dehydrogenase and thiolase have a requirement for NAD\(^+\) and CoA respectively, whose levels are conserved within the mitochondrial and thus provide possible means for control and regulation of \( \beta \)-oxidation. Using analysis of the intact CoA ester intermediates produced by the complex, we have examined the sensitivity of the complex to NAD\(^+\)/NADH and acetyl-CoA. We consider the evidence for channelling within the trifunctional protein and propose a model for a \( \beta \)-oxidation 'metabolon'.

Introduction

The mitochondrial trifunctional protein of \( \beta \)-oxidation was purified to homogeneity after the