Calcium, mitochondria and reperfusion injury: a pore way to die

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Abstract
When mitochondria are exposed to high Ca\(^{2+}\) concentrations, especially when accompanied by oxidative stress and adenine nucleotide depletion, they undergo massive swelling and become uncoupled. This occurs as a result of the opening of a non-specific pore in the inner mitochondrial membrane, known as the MPTP (mitochondrial permeability transition pore). If the pore remains open, cells cannot maintain their ATP levels and this will lead to cell death by necrosis. This article briefly reviews what is known of the molecular mechanism of the MPTP and its role in causing the necrotic cell death of the heart and brain that occurs during reperfusion after a long period of ischaemia. Such reperfusion injury is a major problem during cardiac surgery and in the treatment of coronary thrombosis and stroke. Prevention of MPTP opening either directly, using agents such as cyclosporin A, or indirectly by reducing oxidative stress or Ca\(^{2+}\) overload, provides a protective strategy against reperfusion injury. Furthermore, mice in which a component of the MPTP, CyP-D (cyclophilin D), has been knocked out are protected against heart and brain ischaemia/reperfusion. When cells experience a less severe insult, the MPTP may open transiently. The resulting mitochondrial swelling may be sufficient to cause release of cytochrome c and activation of the apoptotic pathway rather than necrosis. However, the CyP-D-knockout mice develop normally and show no protection against a range of apoptotic stimuli, suggesting that the MPTP does not play a role in most forms of apoptosis.

Introduction
The major role of mitochondria in most cells is the provision of ATP by oxidative phosphorylation to drive energy-dependent processes, such as ion and metabolite transport, metabolism and physical work (e.g. contraction). To fulfil this function, mitochondria must maintain a pH gradient and membrane potential. This demands that their inner membrane remains impermeable to all but a few ions and metabolites for which specific transport mechanisms are present. One ion for which specific transport mechanisms exist is the Ca\(^{2+}\) ion, which enters via an electrogenic uniporter, now known to be a channel, and is pumped out again by an Na\(^{+}/Ca^{2+}\) antiporter [1]. The activity of the Na\(^{+}/Ca^{2+}\) antiporter saturates as mitochondrial matrix Ca\(^{2+}\) increases, whereas the uniporter acts as a channel and is thus not saturated with increasing extramitochondrial Ca\(^{2+}\) concentration. Consequently, as the extramitochondrial Ca\(^{2+}\) concentration increases beyond a certain value, the mitochondria can no longer regulate their matrix Ca\(^{2+}\) concentration, and mitochondrial overload ensues [1]. In some mitochondria under ‘healthy’ conditions, this overload can occur without damage to the mitochondria. However, when the overload is also accompanied by a combination of other factors, most notably oxidative stress, high phosphate concentrations and low ad- 

Key words: apoptosis, brain, heart, ischaemia, mitochondrion, permeability transition pore
Abbreviations used: ANI, adenine nucleotide translocator; BKA, bongkrekic acid; LAT, carboxyatractylodiside; CA, cyclosporin A; CyP, cyclophilin; DOG, 2-deoxyglucose; IPC, ischaemic preconditioning; LVEDP, left ventricular developed pressure; MPTP, mitochondrial permeability transition pore; PTase, peptidyl-prolyl cis-trans isomerase; SIA, sanglifehrin A; VDAC, voltage-activated anion channel

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The molecular mechanism of the MPTP
A key observation in the elucidation of the molecular mechanism of the MPTP was made in the 1980s by Martin Crompton [4], who showed that the permeability transition...
could be inhibited by submicromolar concentrations of the immunosuppressant drug, CsA (cyclosporin A). CsA works as an immunosuppressant by binding to a small cytosolic protein, CyP (cyclophilin)-A, which exhibits PPIase (peptidyl-prolyl cis-trans isomerase) activity. Based on these observations, we proposed that there might be a similar CyP with PPIase activity within the matrix that could catalyse a conformational change in an inner membrane protein to induce pore formation. We were able to demonstrate the existence of such a PPIase activity in the matrix and showed that the ability of a range of CsA analogues to inhibit this enzyme correlated with their ability to inhibit MPTP opening [2]. We purified the PPIase protein and identified it as a unique mitochondrial isoform of CyP, CyP-D, that is nuclear-encoded and enters the mitochondria using a mitochondrial targeting sequence that is subsequently cleaved off [5,6]. The role of CyP-D in the MPTP has now been established beyond question through the use of CyP-D-knockout mice, whose mitochondria no longer show a CsA-sensitive MPTP; indeed, they behave just as control mitochondria do in the presence of CsA [7–10]. We have discovered another immunosuppressant, SfA (sanglifehrin A), to be a potent inhibitor of the PPIase activity of CyP-D that also inhibits the MPTP [11]. This drug has some advantages over CsA in that it does not exert its immunosuppressant effects through calcineurin inhibition and thus does not suffer from the same side effects as does CsA. However, SfA does show some differences from CsA in its inhibition of the MPTP. The concentration-dependence of its inhibition of the MPTP is sigmoidal compared with the linear dependence of CsA, and it does not prevent CyP-D binding to the ANT (adenine nucleotide translocase) as discussed below. Thus it would seem that SfA acts to inhibit MPTP opening not by preventing CyP-D binding, but by inhibiting the conformation change catalysed by the bound CyP-D.

More controversial than the role of CyP-D in the MPTP is the nature of the membrane component that undergoes a conformational change to produce the pore. The most widely accepted candidate for this protein, first proposed by us in 1990 [12], is the ANT. The evidence for a role for the ANT is considerable and is reviewed extensively elsewhere [13]. Early observations from several laboratories, including our own, showed that MPTP opening is enhanced by adenine nucleotide depletion and is inhibited by addition of ATP or ADP. Opening is also modulated by other specific ligands of the ANT, including CAT (carboxyatractyloside) that activates MPTP opening and overcomes the effects of adenine nucleotides, and BKA (bongkrekic acid) that inhibits pore opening [14]. CAT and BKA are known to induce two quite distinct conformation of the ANT. We were able to use immobilized glutathione S-transferase-tagged CyP-D to confirm that ANT binds to CyP-D in a CsA-sensitive manner [15] and Crompton [4] provided similar data. However, this latter study failed to detect CsA inhibition of binding and, unlike us, also found that the VDAC (voltage-dependent anion channel) bound to the CyP-D column. The difference may reflect the different detergent solubilization routine and our use of liver rather than heart mitochondria. We have recently obtained further evidence for the specific binding of CyP-D by the ANT using co-immunoprecipitation (S.J. Clarke and A.P. Halestrap, unpublished work). A polyclonal antibody raised against whole rat ANT immunoprecipitated the ANT from liver mitochondria and a monoclonal antibody against CyP-D and anionic channel) bound to the CyP-D column. The difference may reflect the different detergent solubilization routine and our use of liver rather than heart mitochondria. We have recently obtained further evidence for the specific binding of CyP-D by the ANT using co-immunoprecipitation (S.J. Clarke and A.P. Halestrap, unpublished work). A polyclonal antibody raised against whole rat ANT immunoprecipitated the ANT from liver mitochondria and a monoclonal antibody against CyP-D detected this protein in the immunoprecipitate, consistent with its inability to prevent MPTP opening and by increasing CyP-D binding [14]. Further-

Another strong line of evidence in favour of the involvement of the ANT in MPTP formation comes from the effects of oxidative stress and thiol reagents to enhance MPTP opening. We have shown that such treatments act both by decreasing adenine nucleotide binding and inhibition of MPTP opening and by increasing CyP-D binding [14]. Furthermore, we have identified Cys^160 as the critical thiol group that is responsible for overcoming the inhibitory effect of adenine nucleotides, and we have demonstrated that oxidative...
stress and the vicinal thiol reagent phenyl arsine oxide exert their effect by cross-linking Cys\textsuperscript{162} with Cys\textsuperscript{257} [16]. We have also provided evidence that the well-established inhibition of MPTP opening by high membrane potential works by increasing matrix adenine nucleotide binding to the ANT, reflecting the electrogenic nature of the ANT [14]. In contrast, the inhibitory effect of low pH on MPTP opening is probably mediated by protons competing for Ca\textsuperscript{2+} at the Ca\textsuperscript{2+} trigger site on the matrix surface of the ANT [17]. Although the identity of this site has not been established, there are several glutamate and aspartate residues on the inner surface of the ANT that are likely to be involved [13], but the recently published three-dimensional structure of the ANT in its CAT-bound form does not allow firm conclusions to be drawn on the particular residues involved [18]. However, the solved structure is for a single conformation that does not allow for the cross-linking of Cys\textsuperscript{162} with Cys\textsuperscript{257} and as such may not be helpful in establishing how the ANT forms the MPTP when in its native state. One feature that does emerge from the published structure is that it has a large channel on the cytosolic surface that penetrates deep into the membrane and is blocked by a relatively narrow gate at the bottom [18]. This would be consistent with MPTP opening being mediated by a conformation change that wedges this gate open.

Despite the strong circumstantial evidence for the involvement of the ANT in MPTP formation, definitive proof is lacking. There have been several reports of reconstitution of the MPTP from partially purified solubilized inner membrane proteins that include the ANT and CyP-D, and other reports of reconstitution using purified ANT and CyP-D. However, none of these reports are that persuasive, as reviewed elsewhere [18]. Perhaps the most convincing data come from Brustovetsky and Klingenberg [19], who have shown that reconstituted bovine ANT can produce a non-specific channel at very high Ca\textsuperscript{2+} concentrations. This would be consistent with the observation from many laboratory groups that the MPTP can open at very high Ca\textsuperscript{2+} concentrations in both normal mitochondria in the presence of CsA [14] and in CyP-D-deficient mitochondria [7–10]. Using reconstituted ANT from Neurospora mitochondria, an effect of a Neurospora CyP to change the voltage gating of a Ca\textsuperscript{2+}-activated pore formed by purified and reconstituted Neurospora ANT was demonstrated [20]. Whether this represents the MPTP remains uncertain, since Neurospora mitochondria do not demonstrate a conventional MPTP.

It remains possible that the MPTP does not involve the ANT as an essential component, and some evidence for this comes from Wallace’s group [21]. It was reported that mitochondria from the livers of mice in which both major ANT isoforms had been knocked out still showed a CsA-sensitive MPTP, although pore opening required a substantially higher Ca\textsuperscript{2+} concentration and was not sensitive to ligands of the ANT [21]. Although at first sight this might seem to provide conclusive proof against the ANT playing a crucial role in MPTP opening, careful consideration of the data presented shows this not to be the case [22]. Thus it would be anticipated that the metabolism of the liver of animals without any ANT would be totally compromised, especially with regard to urea synthesis and gluconeogenesis. Yet these mice have no obvious disturbances of their liver metabolism. It seems likely that there remains a small amount of ANT activity, quite possibly an additional isoform that has recently been identified [23]. Secondly, even if the ANT is not essential for MPTP formation, this does not mean that it is not the normal inner membrane component. The ANT is a member of a large family of mitochondrial carriers with common structural motifs [24], and thus it is possible that all members are capable of forming the MPTP. However, because the ANT is present in very much larger quantities than the other members, it probably provides the normal CyP-D-binding partner.

The role of the MPTP in reperfusion injury

The conditions that occur during reperfusion following a period of ischaemia are exactly those that favour MPTP opening [3]. Thus the ischaemic period has led to a profound drop in ATP/ADP, adenine nucleotide depletion and the build-up of lactic acid, with a consequent drop in intracellular pH. In an attempt to rectify this, the cell utilizes the Na\textsuperscript{+}/H\textsuperscript{+} antiporter, but this loads the cell with Na\textsuperscript{+} and, because the ATP levels are severely compromised, this cannot be pumped out. As a result, the cell loads with Ca\textsuperscript{2+} through the reversal of the Na\textsuperscript{+}/Ca\textsuperscript{2+} antiporter. Upon reperfusion, this Ca\textsuperscript{2+} enters the re-energized mitochondria, and, at the same time, the replenished oxygen supply leads to the formation of oxygen free radicals. Conditions are now set for pore opening, but for one inhibitory factor: the low pH. The MPTP is potently inhibited by low pH, and thus only as the pH returns to normal through the loss of lactic acid and the operation of pH regulatory pumps would the MPTP be expected to open. So much for theory, but how can this be demonstrated in practice? For this purpose, we introduced the ‘Hot-DOG’ (DOG is 2-deoxylglucose) technique to measure MPTP opening in the perfused heart [25]. The heart is loaded with \[^{3}\text{H}\]DOG tracer that is metabolized to DOG-6P (2-deoxylglucose 6-phosphate). This remains in the cytosol, unless the MPTP opens, at which point the DOG-6P enters the mitochondria. By rapidly isolating the mitochondria and measuring their \[^{3}\text{H}\] content relative to their citrate synthase activity, it is possible to determine the extent of pore opening. Using this method, we have confirmed that MPTP opening does not occur during ischaemia, but does occur after approx. 2 min of reperfusion when the pH has returned to normal, just as predicted [3]. By a slight variation in this technique, it is possible to determine how many mitochondria undergo MPTP opening followed by subsequent closure. In this case, the \[^{3}\text{H}\]DOG loading is also performed after the heart has been reperfused (‘post-loading’) in order to determine how many mitochondria remain open and so load with DOG-6P after reperfusion. By comparison of this value with that obtained with DOG loading before ischaemia, it is possible to assess the extent to which mitochondria that were open at reperfusion were subsequently closed. Using this technique, we were able to demonstrate that a significant
Inhibition of MPTP opening protects against reperfusion injury

In addition to the protective effects of CsA and SfA against reperfusion injury, other protocols that reduce MPTP opening are also protective. These include reducing oxidative stress with free radical scavengers, maintaining a lower pH during reperfusion and decreasing mitochondrial overload during ischaemia/reperfusion [3]. An example of an agent that acts by decreasing oxidative stress is the anaesthetic propofol that has shown to improve haemodynamic function following ischaemia/reperfusion of the Langendorff and working perfused rat hearts. Protection was associated with decreased MPTP opening in vitro as determined using mitochondrial DOG-entrapment, and also inhibition of MPTP opening in mitochondria isolated from the treated hearts [34]. One of the most potent protective agents of a range of ischaemic/reperfused tissues is pyruvate. In the perfused heart, we showed that, after 30 min of ischaemia followed by reperfusion, untreated hearts only recovered approx. 30–40% of their LVDP (left ventricular developed pressure), while the LVDP of those treated with 10 mM pyruvate recovered to 100% of their pre-ischaemic values. Using the mitochondrial DOG-entrapment technique, it was possible to show that this increased recovery is accompanied by less MPTP opening at reperfusion and complete subsequent MPTP closure as reperfusion progresses [26]. It was proposed that pyruvate works by acting as a free radical scavenger and by maintaining a lower pH during reperfusion, although another factor in its favour is that it is an excellent respiratory substrate that spares fatty acid oxidation. Fatty acid oxidation is known to be detrimental to heart function during reperfusion [35].

Two other protective regimes for which inhibition of MPTP opening has been demonstrated are the use of Na+/H+ antiporter inhibitors, such as cariporide [36], and IPC (ischaemic pre-conditioning) [37–40]. The former decreases Na+ and therefore Ca2+ loading during ischaemia and also slow the Restoration of the intracellular pH during reperfusion [41]. IPC is one of the most effective protective regimes and involves exposing the heart to several short ischaemic episodes, followed by recovery, before the prolonged ischaemia. We have used the mitochondrial DOG-entrapment method to confirm that IPC is associated with less MPTP opening [37] and others have used measurement of mitochondrial membrane potential in an isolated cardiac myocyte model of ischaemia/reperfusion [40]. A variety of pharmacological agents mimic IPC, such as those causing receptor-mediated protein kinase C activation, and KATP channel openers, such as diazoxide. In all cases, it seems likely that MPTP inhibition is the end effector for protection [3,42]. More recently, ‘post-conditioning’ has been recognized, whereby brief ischaemic episodes are introduced during the reperfusion phase following prolonged ischaemia and here too, significant protection against necrotic damage is associated with inhibition of MPTP opening [44]. Although there is general consensus that the protection provided by all these ‘conditioning’ protocols involves inhibition of MPTP opening as the end effector, the mechanisms that are responsible for this inhibition remain unclear. Our own data suggest that inhibition is secondary to reduced Ca2+ overload and production of reactive oxygen species, but how this is achieved is unknown [3].

Overall, it seems well established that if the MPTP can be inhibited during reperfusion, the heart (or other tissues) will be protected from damage. We have recently extended this into the clinical setting and have shown that in a pig model of open heart surgery that requires cardioplegic arrest and ischaemia, propofol can provide significant protection with improved recovery on completion of the surgical procedure [45]. Thus the development of effective regimes to inhibit MPTP opening during reperfusion after ischaemia is likely to provide considerable benefit in cardiac surgery and in the treatment of coronary thrombosis and stroke using angioplasty and clot-busting drugs.

MPTP opening and apoptosis

In addition to the uncoupling of mitochondria, the permeability transition also leads to massive swelling of the matrix. This occurs as all small-molecular-mass solutes equilibrate across the inner membrane, while proteins remain within the matrix at high concentration and exert a colloidal osmotic pressure. The resulting movement of water causes swelling of the matrix that can accommodate the increase in volume by unfolding of the cristae. However, this is not possible for the outer membrane, and, as swelling proceeds, the outer membrane will rupture, leading to loss of intermembrane components to the cytosol [3]. These include...
The extent and reversibility of MPTP opening may determine whether a cell dies by apoptosis or necrosis.

![Scheme 1](Image)

As Bax and Bid induce changes in the permeability of the outer mitochondrial membrane that lead to cytochrome c release. Exactly how this is achieved remains unclear. Some groups believe that Bax itself forms channels in the outer membrane, while other groups propose an interaction with VDAC at points where the inner and outer membranes come into contact [4,46]. Whatever the exact mechanism, it preserves the integrity of the inner membrane and allows the ATP production required for apoptosis to be maintained.

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References


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