A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection

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Abstract

In addition to their normal physiological role in ATP production and metabolism, mitochondria exhibit a dark side mediated by the opening of a non-specific pore in the inner mitochondrial membrane. This mitochondrial permeability transition pore (MPTP) causes the mitochondria to breakdown rather than synthesize ATP and, if unrestrained, leads to necrotic cell death. The MPTP is opened in response to Ca\(^{2+}\) overload, especially when accompanied by oxidative stress, elevated phosphate concentration and adenine nucleotide depletion. These conditions are experienced by the heart and brain subjected to reperfusion after a period of ischaemia as may occur during treatment of a myocardial infarction or stroke and during heart surgery. In the present article, I review the properties, regulation and molecular composition of the MPTP. The evidence for the roles of CyP-D (cyclophilin D), the adenine nucleotide translocase and the phosphate carrier are summarized and other potential interactions with outer mitochondrial membrane proteins are discussed. I then review the evidence that MPTP opening mediates cardiac reperfusion injury and that MPTP inhibition is cardioprotective. Inhibition may involve direct pharmacological targeting of the MPTP, such as with cyclosporin A that binds to CyP-D, or indirect inhibition of MPTP opening such as with preconditioning protocols. These invoke complex signalling pathways to reduce oxidative stress and Ca\(^{2+}\) load. MPTP inhibition also protects against congestive heart failure in hypertensive animal models. Thus the MPTP is a very promising pharmacological target for clinical practice, especially once more specific drugs are developed.

Introduction

David Keilin was a pioneer in the study of mitochondrial electron transfer and bioenergetics who discovered the key role of cytochromes in substrate oxidation [1]. These studies focused on the major role of mitochondria in cellular function which is the provision of ATP via oxidative phosphorylation to drive energy-dependent processes such as ion and metabolite transport, metabolism and physical work (e.g. contraction). Little could Keilin have realized that nearly 70 years later, one of the cytochromes, cytochrome c, would be recognized as a key player in cell death through apoptosis [2,3]. We now know that mitochondria also play a key role in other forms of cell death such as necrosis, involving uncontrolled cell death in response to a severe insult that leads to plasma membrane rupture and an inflammatory response [4,5], and autophagy, where a cell ‘digests’ itself in times of nutrient deprivation [6–9]. Thus the emerging picture is that mitochondria are intimately involved in both the life and death of the cell and, like Dr Jekyll, they exhibit a split personality. Upon an appropriate trigger, they can switch from their normal role of supporting the life of the cell to expose their dark side, the murdering Mr Hyde, which actually kills the cell.
The mechanisms underlying this switch in function differ in apoptosis, necrosis and autophagy. In apoptosis, a selective permeabilization of the OMM (outer mitochondrial membrane) occurs that releases cytochrome c and other apoptotic factors such as endonuclease G, Smac (second mitochondrial-derived activator of caspase)/DIABLO (direct IAP (inhibitor of apoptosis)-binding protein with low pI) and AIF (apoptosis-inducing factor). The detailed mechanism of this OMM permeabilization remains uncertain, but it is known that it usually involves the induction of pores by pro-apoptotic members of the Bcl2 oncogene family [10]. In autophagy, recruitment of proteins such as p19ARF to the mitochondria appears to target them specifically for engulfment by autophagic vacuoles and transfer to lysosomes [11]. In necrosis, and occasionally also in apoptosis, yet another mechanism operates, involving the opening of a non-specific pore in the IMM (inner mitochondrial membrane). This is known as the MPTP (mitochondrial permeability transition pore) and is the major focus of the present review.

I will first provide a brief account of the discovery of the MPTP before discussing what is known of its regulation and molecular mechanism. I will then focus on the role of the MPTP in causing the necrotic cell death of the heart that occurs during reperfusion after a long period of ischaemia. This is known as reperfusion injury and is a major problem during cardiac surgery and in the treatment of coronary thrombosis and stroke. Finally, I will indicate how the prevention of MPTP opening provides a protective strategy against acute reperfusion injury and chronic congestive heart failure.

The discovery of the mitochondrial permeability transition

The massive swelling of mitochondria that accompanies Ca\(^{2+}\) overload was first described several decades ago at which time it was sometimes referred to as ‘high-amplitude swelling’ [12–15]. The phenomenon was later given the name ‘mitochondrial permeability transition’ (MPT) and was thought by some workers to reflect non-specific damage to the IMM caused by the action of Ca\(^{2+}\)-activated PLA\(_2\) (phospholipase A\(_2\)) [16]. However, pioneering studies initiated by Hunter and Haworth in the late 1970s [17–20], and later confirmed by Crompton and colleagues in the late 1980s [21–23], showed that the MPT involved the reversible opening of a pore of defined size that allowed the passage of any molecule of <1500 Da across the IMM, and which could be rapidly closed by chelation of Ca\(^{2+}\). The extensive swelling of mitochondria that accompanies MPTP opening is the result of equilibration of all small solutes across the IMM, leaving behind high concentrations of proteins in the matrix. These exert a colloidal osmotic pressure that draws in water to cause a large increase in matrix volume. In addition, the MPTP allows rapid passage of protons across the IMM and it is this that is responsible for the observed depolarization of the mitochondria and uncoupling of oxidative phosphorylation [24,25].

It was recognized quite early on that, although Ca\(^{2+}\) alone could induce the MPTP, other factors were able to greatly sensitize pore opening to Ca\(^{2+}\) concentration. Of particular significance were three factors: oxidative stress, high phosphate concentrations and depletion of mitochondrial matrix adenine nucleotides [13,17,19,21,22,26]. As discussed further below (in the ‘Conditions occurring during reperfusion favour MPTP opening’ section), these three factors are all implicated in the damage that occurs to tissues in response to ischaemia/reperfusion. Indeed, this was recognized by Martin Crompton in the 1980s nearly 20 years before the role of the MPTP in reperfusion injury was widely accepted [21,22,27].

MPTP-dependent and -independent mechanisms of mitochondrial swelling

Research on the MPTP in this laboratory started in the late 1980s as a natural development of our investigation into the mechanisms by which hormones regulate ATP production in response to metabolic need. These studies demonstrated that an increase in mitochondrial matrix volume played a key role in the hormone-induced stimulation of respiration (see [28–30]). Our investigations into how this is achieved revealed that a hormonally induced increase in mitochondrial Ca\(^{2+}\) concentration stimulates the entry of K\(^+\) ions into the mitochondria through a K\(^+\)-channel, driven by the membrane potential. The proton-coupled co-transport of phosphate provides charge compensation and osmotically obliged water follows the uptake of K\(^+\) and Pi, causing the matrix to swell by 20–40%. This was found to be much less than the massive swelling that accompanies the permeability transition [15,31]. We showed that the Ca\(^{2+}\)-mediated increase in K\(^+\) entry into the mitochondria and resulting volume increase correlated with the matrix content of PP, [32,33], whose breakdown by pyrophosphatase is strongly inhibited by micromolar Ca\(^{2+}\) concentration [34]. Furthermore, a modest increase in matrix volume also occurred in a Ca\(^{2+}\)-independent manner when matrix PP, concentration was increased by the provision of butyrate, whose activation to butyryl-CoA releases PP, [33].

Other laboratories had provided evidence that the binding of ligands such as ADP, BKA (bongkrekic acid) and atractylloside to the ANT (adenine nucleotide translocase) might somehow influence the matrix volume [35–37], perhaps enabling the ANT itself to act as a pathway for K\(^+\) entry [38]. This led us to propose that the Ca\(^{2+}\)-induced rise in matrix PP, concentration might cause a displacement of adenine nucleotides from the ANT causing it to become leaky to K\(^+\) ions. The large (negative) membrane potential would then drive K\(^+\) entry into the matrix along with phosphate as a compensatory anion and osmotically obliged water to cause swelling. A new steady state would be reached when the increased matrix volume stimulated the K\(^+\)/H\(^+\) antiport to balance K\(^+\) efflux with K\(^+\) influx [15,28,31]. In addition to this mechanism of Ca\(^{2+}\)-induced K\(^+\) entry, results have suggested the presence of a Ca\(^{2+}\)-activated K\(^+\) channel (mitochondrial K\(_{Ca}\) channel) in the IMM that is similar to the BK-type K\(_{Ca}\) channel of the plasma membrane [39,40]. This might provide
Figure 1 | The original model proposed for the mechanism of the MPTP in 1990

The model is redrawn from [15] and shows how the ANT was proposed to become either a K⁺ channel when a modest increase in matrix Ca²⁺ concentration (e.g. upon hormone stimulation) or the non-specific MPTP at higher (pathological) matrix Ca²⁺. The former involved Ca²⁺ inhibiting matrix pyrophosphatase to increase matrix PP_i concentration, which, together with Pi, displaced adenine nucleotides from the ANT, inducing a leaky conformation that allowed K⁺ entry. The latter involved a Ca²⁺-triggered conformational change that is facilitated by the PPiase activity of a mitochondrial cyclophilin now identified as CyP-D.

Properties of the MPTP

The open pore is a non-selective channel

Haworth and Hunter, and later Crompton, measured the permeation of a range of solutes of increasing size into mitochondria that had undergone the MPT and demonstrated that the MPTP allowed entry of any molecule of <1.5 kDa. This implied that the MPTP is a non-specific pore with a diameter of approx. 2.3 nm [17,27]. Furthermore, patch-clamp studies identified the presence of a megachannel within the IMM under conditions that induced the MPT. The electrophysiological properties of this channel matched those predicted for the MPTP [45,46], although such megachannel behaviour may also reflect other molecular entities [47]. Electrophysiological studies also suggested that the MPTP may rapidly switch between fully open, full closed and intermediate sub-states [45,46]. If such partially open states do occur, they could allow passage of H⁺ (to produce uncoupling) or Ca²⁺ (to promote rapid Ca²⁺ release) without equilibration of low-molecular-mass metabolites and thus uncertainties and areas of ignorance, considerable progress has been made, as discussed below.
without swelling [48,49]. However, whether these intermediate conductance states exist under physiological conditions remains controversial. Kinetic measurements of sucrose permeation into mitochondria have shown that the MPTP can rapidly oscillate between fully open and closed states without evidence for intermediate states [27]. Furthermore, in isolated mitochondria, induction of MPTP opening normally produces a population of mitochondria that are either 'normal' or massively swollen, without any intermediate states [see 50]. This 'all or none' swelling can be explained as follows. Once a single pore has opened in a mitochondrion, it will become depolarized and such depolarization is known to sensitize the MPTP to Ca^{2+} concentration [51]. This will induce additional pore opening in the same mitochondrion to produce swelling and release its accumulated Ca^{2+} which will be taken up by other mitochondria to induce their MPT and swelling [50]. Within a population of isolated mitochondria, individual mitochondria appear to possess different sensitivities to MPTP opening, perhaps reflecting their age and consequent exposure to oxidative stress, a potent activator of MPTP opening as described below (in the 'Ca^{2+} triggers MPTP opening, but this is regulated by many additional factors' section).

The situation for mitochondria in vivo may be more complex since a modest trigger for MPTP opening can induce a slow CsA-sensitive permeation of large molecules such as calcine without an observable fall in membrane potential as determined with fluorescent dyes such as TMRE/ TMRM (tetramethylrhodamine ethyl ester/tetramethylrhodamine methyl ester) [52,53]. This can best be explained by transient opening of the MPTP in a few mitochondria at any one time. When open, each individual mitochondrion takes up calcine and is depolarized. However, on resealing, the calcine remains trapped, but the membrane potential is re-established. If the open time is very short and the percentage of mitochondria that are 'open' and uncoupled at any one time is small, a significant fall in membrane potential (mitochondrial) will not be observed despite calcine progressively entering the mitochondria.

\textbf{Ca}^{2+} \textbf{triggers MPTP opening but this is regulated by many additional factors}

The primary trigger for opening of the MPTP is elevated intramitochondrial Ca^{2+} concentration, whereas rapid closure can be induced by chelation of matrix Ca^{2+} [17,21,22,27,54]. Other bivalent cations such as Sr^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+} do not induce MPTP opening and can act as inhibitors of the Ca^{2+} trigger site [17,55,56], as can H^{+} (protons) [17]. The latter may account for the potenti inhibition of MPTP opening by low pH [57,58]. It has also been reported that bivalent cations (including Ca^{2+}) can act as inhibitors of the MPTP by binding to an additional site outside the matrix [59]. Many other factors that regulate MPTP opening do so by changing the Ca^{2+}-sensitivity of the MPTP. Thus oxidative stress and phosphate enhance the Ca^{2+} sensitivity of the MPTP, whereas adenine nucleotides (ATP or ADP), CsA and SfA (sanglifehrin A) decrease the sensitivity [22,60–63]. This is shown diagrammatically in Figure 2, which highlights how it is possible to cause MPTP opening without an increase in matrix Ca^{2+} concentration if one of the other regulatory parameters changes appropriately. Oxidative stress may be especially important in this regard, as has been shown for ischaemia/reperfusion [64,65], which is discussed further in the 'Conditions occurring during reperfusion favour MPTP opening' section. MPTP opening can also be modulated by changes in the rate of mitochondrial Ca^{2+} influx (e.g. by membrane potential or Mg^{2+}) or efflux (e.g. by Na^{+}) [66]. There appears to be an additional regulatory effect of membrane potential on MPTP opening that occurs independently of Ca^{2+} uptake. Thus mitochondria that are loaded with insufficient Ca^{2+} to induce MPTP opening on its own can be triggered to undergo the permeability transition by addition of an uncoupler [51,54]. This had led some to consider the MPTP to be a voltage-regulated pore [67], although our own data suggest that the effect of membrane potential is mediated through potential-dependent binding of adenine nucleotides to the ANT [60] as discussed further in the 'The adenine nucleotide translocase' section.

\textbf{The molecular mechanism of the MPTP}

\textbf{The role of CyP-D}

The demonstration by Martin Crompton in 1988 that the MPT could be inhibited by submicromolar concentrations of the immunosuppressant drug CsA [41] was critical for the elucidation of the molecular mechanism of the MPTP. CsA was already known to act as an immunosuppressant by binding to a small cytosolic protein, CyP-A (cyclophilin A),
whose complex with CsA mediates immunosuppression by inhibiting the Ca\(^{2+}\)-activated protein phosphatase calcineurin [68]. CyP-A was known to be a PPIase, which led us to investigate whether a similar PPIase might be present in the mitochondrial matrix and be responsible for facilitating a CsA-sensitive conformational change of an IMM protein to form the MPTP. We confirmed the existence of such a PPIase activity and showed that the ability of a range of CsA analogues to inhibit this enzyme correlated with their ability to inhibit MPTP opening [15,69]. Subsequently, we purified the protein responsible and identified it as a distinct 18 kDa isoform of cyclophilin, now known as CyP-D [70]. CyP-D is encoded by a nuclear gene (PPIF) and has an N-terminal mitochondrial targeting sequence that is cleaved following translocation of the protein into the matrix [70–72]. CsA analogues such as 6-methyl-Ala-CsA, 4-methyl-Val-CsA, \(N\)-methyl-4-isoleucine-CsA (NIM811) and \(N\)-3-methyl-Ala-4-ethyl-Val-CsA (Debio-025) also bind to CyP-D and inhibit MPTP opening [69,73–75], as does the unrelated MPTP inhibitor Sfa [61]. Importantly, unlike CsA, binding of these drugs to CyP-A does not cause inhibition of calcineurin which makes them preferable for use as MPTP inhibitors in vivo. Recently, a CsA analogue conjugated to the hydrophobic cation triphenylphosphonium has been synthesized that allows membrane potential-driven targeting of CsA to the mitochondria [76].

The generation of CyP-D-knockout mice has now established a role for CyP-D in facilitating MPTP opening beyond doubt. Mitochondria from these animals fail to exhibit a CsA-sensitive MPTP and require much higher Ca\(^{2+}\) loading to elicit the permeability transition, behaving identically with control mitochondria in the presence of CsA [77–80]. Overall, the data on the role of CyP-D in MPTP opening are consistent with it inducing pore opening by facilitating a Ca\(^{2+}\)-triggered conformational change in a membrane protein that can occur independently of CyP-D if the stimulus is large enough.

The membrane component(s) of the MPTP

The identity of the IMM component of the MPTP remains controversial, although two candidate proteins have been proposed: ANT and the P/EC (mitochondrial phosphate carrier). However, He and Lemasters [81] have suggested that the search for a unique membrane protein responsible for MPTP formation may be futile. Rather they suggest that the pore is formed from aggregated and misfolded integral membrane proteins that have been damaged by oxidant and other stresses. In this model, it is proposed that CyP-D binds to these protein aggregates to block pore formation until the protein clusters exceed the CyP-D available. The apparent involvement of the ANT and P/EC merely reflects the abundance of these proteins in the IMM and their susceptibility to oxidative damage. However, it is not entirely clear how Ca\(^{2+}\) and CsA would act to activate and inhibit pore formation respectively, nor how such pores could exhibit a defined diameter.

The adenine nucleotide translocase

As noted above (‘MPTP-dependent and -independent mechanisms of mitochondrial swelling’ section), we first proposed that the ANT might be the membrane component of the MPTP in 1990 [15], and we have reviewed the evidence for this extensively elsewhere [44,82]. In brief, MPTP opening is enhanced by adenine nucleotide depletion and CAT (carboxyatractyloside), a specific ligand of the ANT, and inhibited by ATP, ADP and BKA, another ligand of the ANT that induces a conformation of the protein distinct from that induced by CAT. The sensitivity of MPTP opening to inhibition by a range of purine nucleotides matched their ability to act as substrates of the ANT [60]. These regulatory effects all act by modulating the Ca\(^{2+}\) sensitivity of the MPTP as discussed above (see also Figure 2). Subsequently, we and Crompton’s laboratory demonstrated binding of the ANT to immobilized GST (glutathione transferase)–CyP-D which in our experiments (but not Crompton’s) was blocked by CsA [83,84].

The involvement of the ANT in MPTP formation was supported by our studies on the mechanism by which oxidative stress and thiol reagents stimulate MPTP opening. We demonstrated that a major effect of these activating treatments was to impair the ability of adenine nucleotides to inhibit MPTP opening [62]. We were able to demonstrate that oxidative stress and the vicinal thiol reagent phenylarsine oxide achieved this by cross-linking Cys\(^{160}\) with Cys\(^{257}\) on the ANT and that modification of Cys\(^{160}\) alone with eosin 5-maleimide could induce the same effect [85]. Furthermore, our data provided an explanation for the established inhibition of MPTP opening by the membrane potential [51,67], since the ANT mediates electrogenic exchange of adenine nucleotides implying a potential-dependent change in protein conformation [60]. We have also provided strong evidence that increased matrix Ca\(^{2+}\) concentration can induce the ‘c’ conformation of the ANT [15,86] which would be consistent with the Ca\(^{2+}\) trigger site being associated with the ANT. Although there are no established Ca\(^{2+}\)-binding motifs on the ANT, there are several glutamate and aspartate residues in the matrix-facing loops of the ANT whose carboxy groups could play such a role [87].

Although there are reports that the MPTP can be reconstituted from partially purified solubilized IMM proteins or purified ANT and CyP-D, none is very convincing (see [82]). Probably the best data come from Brustovetsky and Klingenberg [88] who reconstituted bovine ANT and demonstrated the formation of a non-specific channel at high (mM) Ca\(^{2+}\) concentration. Reconstituted ANT from Neurospora crassa exhibited a similar Ca\(^{2+}\)-induced channel, whose open probability was increased at high membrane potential by oxidative stress in the presence of \(N\). crassa cyclophilin [89]. However, there is no evidence that \(N\). crassa mitochondria demonstrate a conventional MPTP, and work with Saccharomyces cerevisiae mitochondria suggest that, although there may be a Ca\(^{2+}\)-activated permeability transition under appropriate conditions, it is not CsA-sensitive [83,90–93].
Genetic ablation has provided the most convincing evidence that the ANT plays an important role in MPTP opening, but this role is probably regulatory, rather than as an essential structural component. Thus mouse liver mitochondria lacking ANT1 and ANT2 were found to exhibit a CsA-sensitive permeability transition which was less sensitive to Ca\(^{2+}\) concentration than wild-type mitochondria and was insensitive to ligands of the ANT such as ADP, CAT and BKA [94]. It is probable that the mitochondria employed in these studies did contain a small amount of ANT, since proteomic studies have demonstrated that there is some ANT4 expressed in mouse liver mitochondria [95] and this was not knocked out. Indeed, this may explain why liver function of these mice was normal, which would not be expected if ANT was totally absent [96]. Nevertheless, ANT4 is unlikely to account for the adenine nucleotide-insensitive MPTP opening observed in these mitochondria, implying the presence another pore-forming protein. This could either take the place of the ANT when it is absent or be the major pore-forming protein, relegating the ANT to a purely regulatory role. Recent studies from this laboratory support the latter possibility and have provided strong evidence that it is PiC that forms the MPTP [62,97].

**The mitochondrial phosphate carrier**

The ability of phosphate to activate the MPT has been known for more than 50 years [13,22,26] and, until recently, was assumed to reflect the ability of phosphate to enhance Ca\(^{2+}\) uptake while maintaining matrix pH [54,98,99]. However, our recent studies have suggested that phosphate acts by binding to the PiC [62]. These studies were initiated in response to our observation that CAT did not prevent the activation of the MPTP by PAO (phenylarsine oxide), but it did prevent binding of ANT in solubilized IMM to an immobilized PAO column. Thus PAO must have an activating site other than that which cross-links Cys\(^{165}\) to Cys\(^{297}\) of the ANT. In order to establish the identity of the protein involved, we passed detergent-solubilized IMMs of CAT-treated beef heart mitochondria through a PAO column and found that only four proteins bound. These were identified by MS, and one was identified as the PiC [62]. Importantly, binding of the PiC was prevented by pre-treatment of the mitochondria with UQo (oxidized ubiquinone) and Ro 68-3400, which Bernardi’s laboratory had shown previously to be potent inhibitors of the MPTP [100,101]. Ro 68-3400 had been shown by these authors to bind to a protein of approx. 32 kDa, a similar size to the PiC, which they originally proposed to be VDAC (voltage-dependent anion channel) 1 [102]. However, they later disproved this by demonstrating the same protein to be labelled in mitochondria from VDAC1-knockout mice [103]. We were able to show that UQo and Ro 68-3400 inhibited mitochondrial phosphate transport with the same potency as they inhibited MPTP opening, a property shared by the established inhibitor of the PiC NEM (N-ethylmaleimide) [62]. Furthermore, pre-treatment of mitochondria with UQo and Ro 68-3400 prevented activation of MPTP opening by PAO in isolated mitochondria, consistent with their effect to prevent binding of the PiC to the PAO column. In addition, we were able to demonstrate binding of CyP-D to the PiC directly using co-immunoprecipitation and GST–CyP-D pull-down experiments. As expected, binding was prevented by CsA but increased by oxidative stress and to a lesser extent by CAT, both of which sensitize MPTP opening to Ca\(^{2+}\) concentration [62]. More recently, Bernardi’s laboratory has shown that phosphate is required for inhibition of MPTP opening by CsA or CyP-D knockdown [99], which is consistent with a key role for the PiC [63]. Furthermore, knockdown of the PiC in HeLa cells reduces their sensitivity to apoptosis induced by staurosporine [104], which is thought to be mediated by MPTP opening [105], whereas apoptosis was induced by overexpression of the PiC [104].

**Other components of the MPTP**

In 1992, it was reported that VDAC (also known as porin) and PDBR (peripheral benzodiazepine receptor) co-purified in a complex with the ANT under some conditions [106]. This led Zoratti and Szabo [46] to suggest that these proteins might form part of the MPTP complex, which is not inconceivable because the same proteins are thought to interact at contact sites, points of intimate contact between the IMM and OMM (see [107]). Indeed, Crompton et al. [84] demonstrated that GST–CyP-D could pull down both ANT and VDAC from detergent-solubilized heart mitochondria, although our own data showed no binding of VDAC under similar conditions [83]. A role for VDAC1 in MPTP formation and regulation has now been ruled out since Bernardi and colleagues showed normal MPTP behaviour in mitochondria from VDAC1-knockout mice [103]. This was confirmed by Baines et al. [108] who demonstrated that MPTP opening could also occur in mitochondria lacking all three isoforms of VDAC. Thus it would seem that VDAC is not an essential component of the MPTP, although this does not rule out a role in its regulation under some conditions. The role of the PDBR, now more often known as the 18 kDa mitochondrial transporter such as the PiC is unlikely to be possible and, secondly, purification and reconstitution of active mitochondrial carriers is extremely difficult owing to their instability once solubilized [82].
Bcl-2 and Bax. However, the role, if any, of these components in MPTP formation remains unclear [97,117–120]. Evidence is quite strong for the presence of Bcl-2 family members at contact sites, perhaps in association with VDAC, where they are thought to play a role in the OMM permeabilization that leads to release of pro-apoptotic proteins such as cytochrome c [4,121,122]. Subsequent MPTP opening could then occur following the loss of cytochrome c [78]. The evidence for HK playing some role in the regulation of the MPTP is quite strong. Thus GSK3β (glycogen synthase kinase 3β) has been reported to phosphorylate VDAC1 in cancer cells and this was associated with a reduction in HK-II binding to mitochondria and inhibition of MPTP opening [123]. Apoptosis in these cells was enhanced by dissociation of HK from VDAC through the use of competing peptides [124].

A working model for the MPTP

Figure 3 shows a working scheme for the molecular mechanism of the MPTP that is consistent with the data described above. In this model, it is proposed that dimers can form between the ANT and PiC as described by Pedersen and co-workers within the ‘ATP synthasome’ [125,126]. It is suggested that CyP-D binds to the PiC and, using its PPIase activity, this facilitates a conformational change in one or both of these membrane transporters that is triggered by an increase in matrix Ca²⁺ concentration. Oxidative stress enhances CyP-D binding as does the ANT when in the ‘c’ conformation, which could account for the decreased Ca²⁺-sensitivity of the MPTP in mitochondria from ANT-knockout mice [94]. The conformational change is enhanced by P₁P binding to the PiC, but inhibited by the binding of adenine nucleotides to the ANT, effectively overcoming the stimulatory interaction between the ANT and the PiC. Oxidative stress also activates MPTP opening by preventing adenine nucleotides binding to the ANT, allowing the ANT to exert its fullest stimulatory interaction with an enhancement of CyP-D binding. CsA inhibits the MPTP by preventing CyP-D binding to the PiC complex, whereas SFA inhibits the PPIase activity of CyP-D without causing its dissociation [61]. It is proposed that ubiquinone analogues and NEM inhibit MPTP opening both by enhancing the ‘m’ conformation of the ANT and by binding to the PiC to inhibit the conformational change responsible for pore formation. An important feature of the model is that it provides an explanation of why most regulators of the MPTP act via shifting the Ca²⁺ sensitivity of the pore (see Figure 2). It also explains why CsA does not prevent pore opening if the stimulus is too great [60,77,127]. However, until appropriate PiC-knockdown and reconstitution experiments provide more definitive proof of the model, it remains entirely possible that another key component of the MPTP awaits identification. It is also possible that there is no single member of the mitochondrial transporter family that forms the MPTP, but that any member sharing the common family structure [128,129] might be capable of undergoing an appropriate conformational change to produce a pore. Since the ANT and PiC are the most abundant members of the family, they would still be revealed as playing the dominant role.
The role of the MPTP in reperfusion injury

Conditions occurring during reperfusion favour MPTP opening

When a tissue is subject to ischaemia (loss of blood flow), the lack of oxygen prevents mitochondrial respiration and oxidative phosphorylation, leading to a rapid decline in tissue ATP concentration and an increase in ADP, AMP and P, concentrations. Although glycolysis is stimulated, this is unable to provide sufficient ATP to support metabolically active tissues such as the heart and brain. Furthermore, the loss of blood flow leads to the build up of lactic acid produced by glycolysis. This causes the intracellular pH to fall leading to activation of NHE1 (Na+/H+ exchanger 1), which loads the cell with Na+. Once the ATP levels become too low, this cannot be pumped out via the Na+/K+-ATPase, leading to a reversal of the Na+/Ca2+ antiporter and an accumulation of intracellular Ca2+ concentration (Ca2+ overload). A prolonged period of ischaemia will lead to significant activation of Ca2+-sensitive enzymes, including nucleases, phospholipases such as PLA2 and proteases such as calpains that degrade essential intracellular components. Any possibility of damage repair during the ischaemic period is prevented by the lack of ATP. In addition, the hydrolysis of ATP leads to the formation of AMP that is broken down further via the purine degradation pathway, leading to depletion of total adenine nucleotides. Ultimately, the energy compromise and Ca2+-mediated breakdown of cellular components leads to necrotic cell death with rupture of the plasma membrane and subsequent inflammatory responses as the tissue is invaded by neutrophils [119,130,131].

Clearly, to salvage an ischaemic tissue, it is necessary to restore the blood flow (reperfusion) as soon as possible. Clinically, this is important for the brain in the treatment of stroke and for the heart during surgical procedures that require its beating to be blocked or in the treatment of a coronary thrombosis (a clot in one of the coronary arteries that is the major cause of heart attack) by clot-busting enzymes or angioplasty. Short periods of ischaemia are tolerated by most tissues which can eventually recover fully on reperfusion. In the case of the heart, some functional impairment is observed initially (known as stunning), but, given sufficient time, full haemodynamic recovery is observed [132–136]. However, if the period of ischaemia is longer, restoration of the blood flow causes additional damage to the tissue, known as reperfusion injury, and the extent of this can have a significant effect on the clinical outcome. The major cause of this reperfusion injury is now known to be opening of the MPTP, and its inhibition can provide significant protection of tissues from damage [119,131,137]. Upon reperfusion, oxygen and substrate supplies are restored to the tissue, and the respiratory chain can restart, leading to mitochondrial re-energization. This enables the mitochondria to take up the Ca2+ that has accumulated during ischaemia, but, at the same time, the restoration of oxygen causes a surge in oxygen free radical production by the mitochondria (oxidative stress). The combination of high matrix Ca2+ concentration and oxidative stress, together with the elevated phosphate and depletion of adenine nucleotides, provide ideal conditions for MPTP opening, except for one restraining feature. That is the low pH generated during ischaemia which can inhibit MPTP opening [17,57]. Thus it is only as the restored blood flow allows the intracellular pH to return to normal through the loss of lactic acid and the operation of pH regulatory pumps that opening of the MPTP would be predicted to occur, and, as outlined below, this is what is observed. These events are summarized schematically in Figure 4.

Direct demonstration of MPTP opening during reperfusion, but not ischaemia

In order to confirm these predictions directly, it is necessary to devise a method to determine the extent of MPTP opening in situ. In isolated cells, it is possible to use confocal microscopy to determine the mitochondrial content of fluorescent dyes that detect the membrane potential (red-fluorescing TMRE or TMRM) or the IMM permeability (green-fluorescing calcine). Different protocols have been used, but, essentially, opening of the MPTP causes a loss of TMRM from the mitochondria coincident with the entry of calcine into the matrix [53,64,138–141]. Using these dyes, it has been confirmed that simulated ischaemia and reperfusion does cause MPTP opening in heart and liver cells [64,138,141]. Although it is now possible to apply this technique for the measurement of MPTP opening in whole tissues by the use of two-photon confocal microscopy [143–146], this is not applicable to a beating heart. In order to measure MPTP opening in the perfused heart, we developed a radiotracer technique sometimes known as the ‘Hot-DOG’ technique [127,147]. Here the heart is loaded with DOG (2-deoxy[1H]glucose) whose rapid metabolism through HK produces DOG-6P (DOG 6-phosphate) that is not metabolized further. DOG-6P cannot enter the mitochondria unless the MPTP opens, and the magnitude of [1H]DOG-6P uptake into the mitochondria reflects the extent of MPTP opening. The measurement of the [1H]DOG-6P within the mitochondria requires their rapid isolation in the presence of EGTA to chelate Ca2+ and so close the MPTP to prevent the loss of accumulated [1H]DOG-6P. Using this method, we demonstrated that MPTP opening occurs after approx. 2 min of reperfusion when the pH has returned to normal, just as predicted [127,147]. However, a drawback of the technique is that it cannot discriminate between mitochondria in which the MPTP has opened and then closed again and those in which the MPTP stays open. To solve this problem, we developed the ‘post-loading’ technique in which hearts are perfused with [1H]DOG after a period of reperfusion to allow some recovery of function. By comparison of the pre-loading and post-loading data, we were able to demonstrate that a significant proportion of the mitochondria that experienced MPTP opening during the initial phase of reperfusion, subsequently recovered their IMM permeability, and that this closure correlates well with haemodynamic recovery of the heart [148]. As an alternative to the Hot-DOG...
DiLisa et al. [149] have utilized the loss of mitochondrial NAD\(^+\) that accompanies opening of the MPTP during reperfusion as a surrogate indicator of pore opening. Further confirmation that MPTP opening plays a critical role in reperfusion injury has been provided by the cardioprotection offered by drugs that target CyP-D such as CsA and SfA or in CyP-D-knockout mice as described in the next section.

**Inhibition of the MPTP protects against ischaemia/reperfusion injury**

**Targeting CyP-D**

Crompton and colleagues were the first to show that CsA could protect isolated cardiac myocytes from reoxygenation injury [150] in 1991, whereas work in my laboratory in 1993 demonstrated such protection in the Langendorff perfused heart [151]. Subsequently, protection by CsA has been confirmed in many laboratories using both global and regional ischaemia models of reperfusion injury and a range of indicators of injury, including haemodynamic function, enzyme release (lactate dehydrogenase and creatine kinase) and infarct size (the area of necrosis) [61,152–154]. CsA has also been demonstrated to protect the brain from damage following ischaemia/reperfusion [155–158] and hypoglycaemia [159]. The hippocampus is the most vulnerable region of the brain to ischaemic and hypoglycaemic damage, and we demonstrated that isolated hippocampal mitochondria are more susceptible to MPTP opening than cortex mitochondria [160]. In the rat heart, we and others have demonstrated that SfA also offers protection from reperfusion injury [61,153,161,162] and similar data have been provided for human heart cells undergoing simulated ischaemia/reperfusion [163]. Furthermore, the heart and brain of CyP-D-knockout mice show a substantial decrease in infarct size following ischaemia/reperfusion [79,80,164].

Although most published work has shown cardioprotection by SfA and CsA (and its non-immunosuppressant analogues such as NIM-811) when they are added before the ischaemic episode, a significant decrease in infarct size has also been demonstrated in the isolated perfused rat heart when the drugs are only added at the start of reperfusion [152,153]. Similar data were obtained in an *in vivo* mouse in which hearts were subject to 25 min regional ischaemia before reperfusion in the presence or absence of Debio-025, a non-immunosuppressant CsA analogue, for 24 h or 30 days [75]. Under these conditions, it is likely that adding the CsA early in reperfusion does not stop the initial MPTP opening, but rather prevents a progressive pore opening that causes
the infarct to increase in size over several hours [165]. This progressive MPTP opening may be maintained by ‘ROS-induced ROS release’ whereby opening of the MPTP induces ROS (reactive oxygen species) production that then sensitizes other mitochondria to MPTP opening [166–168]. Whatever the cause, these data are clearly an encouragement towards developing drugs that target the MPTP for the treatment of MI (myocardial infarction) with thrombolysis or PCI (percutaneous coronary intervention). Indeed, the first small clinical trials do confirm improved recovery following CsA treatment of patients undergoing PCI treatment following a coronary thrombosis [169]. However, pharmacological targeting of CyP-D is not ideal for two reasons: first because MPTP opening can occur in the absence of CyP-D when the stimulus is sufficiently large (see the ‘Ca²⁺ triggers MPTP opening, but this is regulated by many additional factors’ section) and, secondly, because drugs that target CyP-D are likely to bind to the other cyclophilins such as CyP-A. When CsA binds to CyP-A, it leads to inhibition of the Ca²⁺-sensitive protein phosphatase calcineurin, which has direct effects on heart function [170] and also undesirable immunosuppressive activity [68]. Although non-immunosuppressant CsA analogues such as NIM811 and Debio-025 or the unrelated SFA can be employed to inhibit MPTP opening and induce cardioprotection [61,127,152–154], there is still the concern as to what other effects the inhibition of the PPIase activity of cyclophilins may have. In an attempt to circumvent this potential problem, Crompton and co-workers have recently described a CsA derivative conjugated to the lipophilic triphenylphosphonium cation that does specifically target the mitochondrial CyP-D and can protect against necrotic cell death in a neuroblastoma cell line [76]. However, as yet, there are no published data on the effectiveness of this drug on protecting against reperfusion injury of the heart.

Targeting other components of the MPTP

At present, CyP-D is the only unequivocal component of the MPTP for which appropriate specific inhibitors exist. It is possible to inhibit the MPTP by targeting the ANT with BKA, and this can protect cells from cell death under some conditions [82,119]. However, this is not an appropriate strategy for protection of metabolically active tissues such as the heart from reperfusion injury, since they require rapid export of mitochondrially synthesized ATP to the cytosol using the ANT [82]. Targeting the phosphate carrier is also inappropriate for similar reasons; indeed, we have found ubiquinone analogues to be damaging to the heart rather than cardioprotective [119]. It remains possible that other components of the MPTP may be identified that are suitable targets, or that drugs targeting the proposed regulatory interface between the ANT and PiC may be found that have no effect on the transporter activity of either carrier itself. Until this is achieved, our data suggest that the most effective way of targeting the MPTP is indirectly by reducing levels of those factors responsible for MPTP opening such as matrix Ca²⁺ concentration and oxidative stress or increasing natural inhibitors such as H⁺ (i.e. maintaining a low pH). A variety of effective cardioprotective protocols are available that act in this way as described below and summarized in Figure 4.

Targeting regulators of the MPTP

Reducing oxidative stress

Since oxidative stress is one of the most potent activators of MPTP opening, reducing oxidative stress during reperfusion represents a potential target for cardioprotection. It has been known for many years that agents which reduce oxidative stress can reduce reperfusion injury in some experimental models, but the effects have not been very impressive and have not translated into clinical practice [171]. However, some ROS scavengers have shown greater promise. The widely used general anaesthetic propofol is known to be a free radical scavenger and was shown to inhibit MPTP opening in isolated mitochondria [172,173] and mitochondria from propofol-treated hearts [174]. Furthermore, we demonstrated that propofol treatment is cardioprotective in both the Langendorff and working rat heart models and used the HotDOG technique to confirm that this was associated with reduced MPTP opening [174]. We went on to show that propofol is also protective in an in vivo pig model of cardiopulmonary bypass with warm blood cardioplegia that closely matches current clinical practice [175]. Other anaesthetics such as isoflurane and desflurane may also offer protection that is associated with less ROS formation and Ca²⁺ overload [176–180].

More recently, ROS scavengers such as MitoQ that are specifically targeted to mitochondria through a positively charged hydrophobic moiety have been developed [181]. These are likely to be especially effective because our data suggest that it is matrix ROS that activates MPTP opening through modification of thiol groups on the ANT [85], whereas increases in cytosolic ROS can play a cardioprotective role through activation of protective signalling pathways [168,182]. Thus, by restricting the antioxidant function to the matrix, the protective signalling by ROS in the cytosol is maintained while the damaging ROS in the mitochondrial matrix are removed. Indeed, MitoQ has been shown to protect against reperfusion injury in the Langendorff perfused rat heart [183] and to improve endothelial function and attenuate cardiac hypertrophy in hypertension [184]. It has also been shown to protect against organ damage in a lipopolysaccharide–peptidoglycan model of sepsis [185] and to reduce endotoxin-induced cardiac dysfunction [186] that are both likely to involve ROS-induced MPTP opening [187]. Protection from reperfusion injury that is associated with reduced MPTP opening has also been demonstrated for trimetazidine, an ‘anti-ischaemic’ drug used for the treatment of heart failure [188]. This protection may also involve less oxidative stress because the drug acts to switch the fuel metabolism of the heart from fatty acid oxidation (which produce more ROS) to carbohydrate oxidation [189].
Reduced Ca\(^{2+}\) loading and pH

Since MPTP opening is stimulated by increased matrix Ca\(^{2+}\) concentration and inhibited by low pH, targeting either of these should be cardioprotective. Inhibition of NHE1 has the potential to have both effects because the intracellular pH of the heart decreases during ischaemia as a result of lactic acid accumulation. The myocytes endeavour to restore intracellular pH by activating NHE1 and this increases intracellular Na\(^+\) concentration allowing Ca\(^{2+}\) to enter by reversal of NHE1 [190,191]. Indeed, amiloride derivatives such as cariporide that inhibit NHE1 have been shown to protect the heart from reperfusion injury [192] and, using the Hot-DOG technique, to reduce MPTP opening in situ [193–195]. Another protective mechanism that may involve the maintenance of a more acid pH during reperfusion is ‘post-conditioning’ in which very brief (10 s) intermittent ischaemic periods are included within the first few minutes of reperfusion [196–198]. Post-conditioning has been shown to reduce MPTP opening and oxidative stress [199–201], and has obvious clinical potential in the treatment of coronary thrombosis by angioplasty [202].

The extremely potent cardioprotection offered by pre-treatment of hearts with pyruvate before ischaemia may also involve the maintenance of a lower pH during reperfusion [148,203]. However pyruvate also acts as a free radical scavenger and is an excellent respiratory substrate to replenish ATP during reperfusion [203]. Whatever the mechanism, we found that pre-treatment with 10 mM pyruvate led to 100% recovery of cardiac function that, using the Hot-DOG technique, we showed to be associated with inhibition of MPTP opening at the start of reperfusion and total closure of those pores that open initially as reperfusion continues [148].

Reducing cytosolic and mitochondrial Ca\(^{2+}\) overload with antagonists of plasma membrane or mitochondrial Ca\(^{2+}\) channels such as verapamil and Ruthenium Red has also been shown to protect hearts from reperfusion injury [204–211], but a direct demonstration that this involves inhibition of the MPTP has not been reported. A similar mechanism probably applies to the protection seen in hearts reperfused with elevated extracellular Mg\(^{2+}\) concentration (>8 mM) [212–214].

Preconditioning

If hearts are exposed to brief periods of ischaemia (2–5 min) interspersed with periods of normal perfusion before the prolonged ischaemia, they exhibit considerable protection from reperfusion injury. This protocol is known as IP (ischaemic preconditioning) and was first described in the dog heart by Murry et al. in 1986 [215] but has since been confirmed in all species including humans [216–218]. Preconditioned hearts exhibit a smaller infarct size, less release of intracellular enzyme (indicators of necrotic cell death) and fewer arrhythmias [215,219], while contractile function is preserved [220]. If ischaemia is initiated more than 1–2 h after the preconditioning protocol, protection is lost, but re-emerges again after approx. 24 h and lasts for up to 3 days. This is termed the second window of preconditioning and probably involves up-regulation of a range of protective proteins, including heat-shock proteins, cell-survival proteins and enzymes involved in protection against oxidative stress [217,221,222]. The exact mechanisms involved in the first window of preconditioning are still uncertain, but there is general agreement that the end effect is inhibition of MPTP opening [168,223]. We have used the Hot-DOG technique to demonstrate directly that IP reduces MPTP opening during the early phase of reperfusion and also increases subsequent pore closure as reperfusion continues. Indeed, there is a good correlation between reduced pore opening and cardioprotection [224]. We and others have also demonstrated that the increased sensitivity to Ca\(^{2+}\) concentration of the MPTP in mitochondria isolated following ischaemia or reperfusion is reduced by IP [224–228]. However, this protective effect is not seen in mitochondria isolated immediately after the IP protocol, suggesting that protection develops during ischaemia and reflects an effect of IP to reduce the sensitization to Ca\(^{2+}\) that occurs in control mitochondria during the ischaemic period [224,226–228].

There is a huge literature, but no consensus on the signalling pathways and mechanisms involved in the inhibition of the MPTP by IP and we have reviewed this elsewhere [168]. Several studies have investigated the possibility that the MPTP is inhibited by phosphorylation of one of its component proteins [168,228], and most recently it has been suggested that CyP-D phosphorylation via the Akt–ERK (extracellular-signal-regulated kinase)–GSK3 axis might modulate MPTP activity [229,230]. However, our own extensive phosphoproteomic analysis of mitochondria from control and IP hearts revealed no change in phosphorylation of any protein that correlated with inhibition of MPTP opening. The only modification of mitochondrial proteins that we did find to correlate with the inhibition of MPTP opening and cardioprotection was their carbonylation, a surrogate marker for oxidative stress [227,228]. These data revealed that the mitochondria from IP hearts experienced less oxidative stress during ischaemia/reperfusion than those from control hearts. This could readily account for their reduced sensitivity to Ca\(^{2+}\)-induced MPTP opening. Furthermore, we and others have shown that three other preconditioning protocols, treatment with urocortin [231], apomorphine [232] or exposure to several intermittent brief hypothermic episodes before index ischaemia (temperature preconditioning) [227], are also associated with mitochondria that develop less oxidative stress during ischaemia/reperfusion and are less sensitive to Ca\(^{2+}\)-induced MPTP opening. Our discovery that temperature preconditioning is even more potent than IP in reducing oxidative stress, MPTP opening, necrotic damage and arrhythmias suggests this protocol may have considerable clinical implications if it can be mimicked pharmacologically [227].

Studies in isolated cardiac myocytes have shown that IP desensitizes the mitochondria to pore opening induced by...
oxidative stress [233,234] and experiments performed with two-photon microscopy in the perfused heart have yielded similar data [235]. Thus one mechanism by which IP reduces the oxidative stress experienced by mitochondria may be via an increased ability of mitochondria to scavenge ROS, although the mechanisms involved remain unclear [168].

Since MPTP opening itself produces ROS and leads to ROS-induced ROS release [167], it is possible that IP initially increases ROS scavenging, which subsequently leads to less ROS production and thus less progressive MPTP opening. This would provide an explanation as to how IP has such a profound effect on the infarct size that develops over several hours. This explanation would also fit well with the proposal that the second window of IP may involve up-regulation of enzymes involved in protection against oxidative stress [222].

In addition to its effects on oxidative stress, IP may also reduce the detrimental effects of ischaemia on sarcoplasmic Ca2+ release and uptake, thus leading to decreased Ca2+ overload during ischaemia/reperfusion [236–239].

There is no agreed consensus on which signalling pathways are involved in mediating the inhibition of MPTP and how they interact. Activation of PKCε (protein kinase Ceε) by ROS and/or other factors (e.g. adenosine, bradykinin or noradrenaline) released during the preconditioning protocol seems probable and preconditioning is abolished in PKCe ε−/− knockout mice [240–242]. There are also many studies that have implicated NO and PKG (protein kinase G), mitoKATP (mitochondrial KATP) channels and the Akt/GSK3β pathway. Readers are referred elsewhere to review the evidence on which these claims are made [168,243–245]. However, a summary of the conclusions drawn from experiments performed in my laboratory is presented below. Further details can be found in [168,227,228,231].

Our studies have shown no translocation of protein kinases such as PKCe and GSK3β to mitochondria following preconditioning, despite several reports to the contrary. Nor were we able to demonstrate IP-mediated phosphorylation of any mitochondrial proteins [228] or connexin43 translocation to mitochondria [168], which has been described by others [246,247]. However, we have been able to reproduce the data of Zurbier et al. [248] that show IP to prevent the ischaemia-induced loss of HK from its binding site on mitochondria (P. Pasdois and A.P. Halestrap, unpublished work). As noted above in the ‘Other components of the MPTP’ section), HK binding has been associated with inhibition of MPTP opening, although the mechanism by which this is achieved remains unclear. VDAC had been implicated in this binding [249], and it was proposed that VDAC1 might be phosphorylated by PKCe to increase HK-II binding to mitochondria [250]. However, more recent data make this unlikely, since HK binding is still observed in VDAC-knockout mice [251]. Nevertheless, it has been reported that GSK3β disrupts HK-II binding to mitochondria [123], and, since IP has been reported to cause phosphorylation and inhibition of GSK3β with concomitant inhibition of the MPTP [252], this would still be consistent with a role for HK-II in IP. Furthermore, pharmacological inhibition of GSK3β during reperfusion has been shown to be cardioprotective [253].

In conclusion, although we cannot totally dismiss phosphorylation of components of the MPTP, our data suggest that the decrease in oxidative stress mediated by IP is more important. How the many signalling pathways implicated in IP converge to decrease in ROS levels during ischaemia/reperfusion remains to be elucidated. However, activation of PKCe ε, either via receptor-mediated events or through transient increases in ROS during the IP protocol, and inhibition of GSK3β induced by activation of Akt and other survival kinases are likely to be involved [252,254]. Additional signalling pathways involving kinases such as PKG and PKA (protein kinase A) may show cross-talk with this primary mechanism [168]. We would propose that none of these kinases is required to enter the mitochondria, but rather they probably phosphorylate cytosolic or OMM proteins to reduce HK-II binding. This, by some currently ill-defined mechanism, modulates oxidative stress and MPTP opening. As we have discussed fully elsewhere, we can find no evidence for a role for mitoKATP channels in IP that has been proposed by others and have even questioned the existence of these channels [168,255–257].

If IP reduces MPTP opening by preventing the loss of HK-II binding to mitochondria during ischaemia, then an important question is how HK-II binding modulates the MPTP. We are currently exploring the possibility that this may involve an effect of HK-II on the permeability of the OMM to cytochrome c. Cytochrome c release during prolonged ischaemia has been reported by others [258] and we have confirmed this, but unlike that study, we have shown this occurs without MPTP opening (P. Pasdois and A.P. Halestrap, unpublished work). This is in agreement with published data of our own and others [127,235,259]. Any loss of cytochrome c will slow electron transfer from complex III to complex IV of the respiratory chain which has the potential to increase ROS production from both complex I and complex IV as has been shown to occur follow cytochrome c loss in apoptosis [260]. It has been reported that the pro-apoptotic factor Bax translocates to mitochondria during ischaemia [261] and this, in conjunction with tBid [truncated (cleaved) Bid], could be responsible for the cytochrome c release. It is possible that the major signal responsible for this Bax translocation to the mitochondria during ischaemia and hence loss of cytochrome c is the dissociation of HK and that preconditioning, by preventing this dissociation, reduces cytochrome c loss [262–264], leading to less ROS production and less MPTP opening.

Heart failure in cardiac hypertrophy may involve MPTP opening

Following MI or in chronic hypertension (high blood pressure) the heart undergoes compensatory hypertrophy to restore some measure of cardiac output. However, over time, this progresses into a decompensated state that is
The perturbation of normal Ca\(^{2+}\) handling results in reduced systolic contraction, a delay in diastolic elevation and prolongation of diastolic Ca\(^{2+}\) concentration. This leads to reduced systolic contraction, a delay in diastolic relaxation and hence reduced pumping efficiency [265,266]. The perturbation of normal Ca\(^{2+}\) handling may also be important for the development of cardiac hypertrophy since a similar phenotype can be induced in transgenic mice with enhanced sarcolemmal L-type Ca\(^{2+}\) channel activity [268]. When the hearts of these mice were subject to acute stimulation of β-adrenergic receptors to enhance Ca\(^{2+}\) influx further, progressive myocyte necrosis was observed leading to pump dysfunction and premature death [268]. When mitochondria were isolated from hypertrophic hearts, they showed impaired respiratory chain activity and increased ROS production, suggesting that the dysregulation of Ca\(^{2+}\) handling in cardiac hypertrophy might lead to mitochondrial MPTP opening. This would provide an explanation for the observed death of myocytes in this hypertrophic model and, in support of this, it was shown that such cardiac dysfunction was absent from mice also lacking CyP-D [268]. Thus it seems possible that pharmacological targeting of the MPTP may be beneficial for preventing or slowing the progression of congestive heart failure.

Similarly, genetic or pharmacological knockdown of CyP-D protects mice from some muscular dystrophies associated with Ca\(^{2+}\) overload [269–272]. This implies that chronic MPTP opening may also be important in the necrotic damage associated with these pathologies and suggests a possible therapeutic intervention in patients with these diseases [273].

Conclusions

It is becoming increasingly recognized that, in addition to their ‘normal’ role in supplying the ATP requirements of the cell, mitochondria have a dark side: they can commit the cell to a death sentence. A major player in this transition between their two persona is the MPTP whose opening is triggered under stressed conditions that are associated with Ca\(^{2+}\) overload, oxidative stress and adenine nucleotide depletion. These are exactly the conditions occurring during reperfusion following a period such as may occur acutely following open heart surgery and in the treatment of an MI or stroke or chronically in congestive heart failure. It is now clear that inhibition of MPTP opening is a promising drug target for the treatment of cardiovascular disease. Indeed, the first clinical trials have confirmed that CsA treatment improves recovery of patients undergoing PCI (percutaneous coronary intervention) treatment following a coronary thrombosis [169]. However, CyP-D is not an ideal target for two reasons. First, even when the PPIase activity of CyP-D is fully inhibited, MPTP opening can still be induced with increased Ca\(^{2+}\) load or oxidative stress [60,77]. Secondly, even CsA analogues such as Debio-25 that do not inhibit calcineurin bind to other cyclophilins and inhibit their PPIase activity which may lead to possible undesirable side effects [274]. The development of CsA analogues that are specifically targeted to mitochondria [76] may circumvent this problem, but there is still considerable scope for the development of better drugs that target another component of the MPTP without interfering with the normal function of the mitochondria. If they could be designed with sufficient specificity, such drugs could even be used prophylactically in those patients at risk of MI or stroke. However, a major limitation in the development of such drugs is our lack of knowledge of the true molecular identity of the MPTP which work in my laboratory continues to address.

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