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The Mitochondrial Permeability Transition – A Pore Way for the Heart to Die

A. P. Halestrap

In reperfusion injury the mitochondria change from being the major ATP producers that sustain heart cell function to instruments of cell death. The molecular basis of this transition is the opening of a non-specific pore in the inner mitochondrial membrane, known as the permeability transition pore (MPTP). The MPTP is thought to be formed by a Ca-triggered conformational change of the adenine nucleotide translocase (ANT) that is facilitated by the binding of matrix cyclophilin-D (CyP-D). The process is greatly enhanced by oxidative stress and adenine nucleotide deprivation, conditions associated with reperfusion after a period of ischaemia. Opening of the MPTP causes swelling and uncoupling of mitochondria which, unrestrained, leads to necrosis. We have developed a technique to demonstrate directly that the MPTP opens during reperfusion of the ischaemic heart, but not during ischaemia itself. Recovery of hearts correlates with subsequent closure of the pore, and agents that prevent opening or enhance closure such as cyclosporin A, free radical scavengers (eg pyruvate and propofol) and low pH protect from injury. Mg^{2+} is another inhibitor of the MPTP (competitive with Ca^{2+}) and this may be one aspect of the protective effect of Mg^{2+}. No data are available on whether ischaemic preconditioning exerts its protective effects by inhibiting pore opening. In addition to its role in necrosis, transient MPTP opening may be involved in apoptosis through the release of cytochrome c and other pro-apoptotic molecules. These then activate the caspase that sets apoptosis in motion. However, only if subsequent MPTP closure occurs will ATP levels be maintained, ensuring that cell death continues down an apoptotic rather than a necrotic pathway. *J Clin Basic Cardiol 2002; 5: 29–41.*

Key words: mitochondrial permeability transition pore, reperfusion injury, adenine nucleotide translocase, cyclophilin-D, calcium overload, oxidative stress

The primary function of mitochondria in the healthy cardiac myocyte is the provision of ATP to support normal cell function, since the ATP demands of the beating heart are far greater than can be met by glycolysis. However, in recent years it has become apparent that mitochondria may also play a critical role in the induction of both necrotic and apoptotic cell death. It is perhaps not surprising that disruption of mitochondrial function and loss of ATP production will lead to necrotic cell death, but it might not be expected that mitochondria should posses a specific, but latent mechanism, to cause such mitochondrial dysfunction.

The Mitochondrial Permeability Transition

Under normal physiological conditions, the mitochondrial inner membrane is impermeable to all but a few selected metabolites and ions. However, under conditions of stress, a non-specific pore known as the mitochondrial permeability transition pore (MPTP) can open in the mitochondrial inner membrane [1–4] as illustrated in Figure 1.

Role in Necrotic Cell Death

When the MPTP opens, the permeability barrier of the inner membrane becomes disrupted and mitochondria become uncoupled and unable to synthesise ATP. Compounding the problem further, as the proton-translocating ATPase reverses direction the mitochondria now actively hydrolyse ATP synthesised by glycolysis. Under such conditions, intracellular ATP concentrations rapidly decline, leading to the disruption of ionic and metabolic homeostasis and activation of degradative enzymes such as phospholipases, nucleases and proteases. Unless reversal of pore opening occurs, these changes will cause hypercontracture and irreversible damage to the cell resulting in necrotic death. The release of intracellular proteins that accompanies necrosis then attracts neutrophils causing a secondary inflammatory response with yet further damage to the heart. Thus it is hardly surprising that the MPTP is kept firmly closed under normal physiological conditions and is only activated under pathological conditions. The key factor leading to the opening of the MPTP is mitochondrial calcium overload (ie when mitochondrial matrix $[Ca^{2+}]$ is greatly increased), especially when this is accompanied by oxidative stress (the production of reactive oxygen species), adenine nucleotide depletion, inorganic phosphate accumulation and mitochondrial depolarisation [1, 2]. These conditions are exactly those that the heart experiences in response to reperfusion after a period of ischaemia (reperfusion injury). Indeed there is increasing evidence that opening of the MPTP may be critical in the transition from reversible to irreversible cell injury in response to a range of insults of which reperfusion injury is one [1, 2].

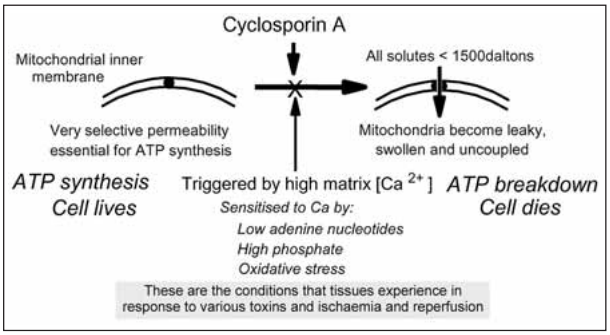

Figure 1. The essential features of the permeability transition and its potential role in necrotic cell death

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Role in Apoptotic Cell Death

It is now known that mitochondria release factors from the intermembrane space that induce apoptosis. The first of these to be identified, and probably the most important, is cytochrome c. The sequence of events that is thought to occur down-stream of cytochrome c release is illustrated in Figure 2 and involves a cascade of specific proteases, known as caspases. An inactive form of caspase-9 (pro-caspase-9) is complexed with its activating enzyme, Apaf-1 (apoptosis activating factor-1). Once released, cytochrome c activates Apaf-1 in the presence of deoxy-ATP (dATP) which proteolytically activates pro-caspase-9 to caspase-9 which then activates caspase-3. Caspase-3 can activate a range of other enzymes involved in the cascade of events responsible for the changes in cytoskeletal, plasma membrane and nuclear morphology that culminates in apoptotic cell death. More recently it has been shown that mitochondria may release other apoptosis inducing factors in addition to cytochrome c. These include Smac (also known as DIABLO) which eliminates the activity of endogenous caspase inhibitors and AIF (apoptosis inducing factor) which migrates to the nucleus and induces changes in chromatin structure [5–11]. The release of these factors further commits the cell to apoptosis. One mechanism responsible for the release of these pro-apoptotic proteins, particularly in response to chemical toxins and oxidative stresses, is opening of the MPTP [1, 2, 4]. This leads to massive mitochondrial swelling and rupture of the outer mitochondrial membrane. Indeed, it has been known for many years that such swelling causes cytochrome c release [12], although its significance for apoptosis was not recognised until much later.

However, there is a problem associated with invoking the MPTP in apoptotic cell death. As noted above, one consequence of pore opening is mitochondrial uncoupling and tissue ATP depletion. Yet apoptosis is an ATP-dependent process and thus if ATP levels were to fall the cell would ultimately die by necrosis, even if release of mitochondrial factors has already initiated the apoptotic cascade. Thus it may be that the extent to which mitochondria within a cell undergo the permeability transition provides the cell with a sensing device that determines whether death is to be by necrosis or apoptosis [1, 3, 13]. Prolonged opening of the MPTP, such as accompanies a major insult, will inevitably lead to necrosis because levels of ATP cannot be maintained. However, if only a few mitochondria experience MPTP opening, or the opening is transient, this may lead to sufficient swelling to rupture the outer membrane and release cytochrome c, whilst enabling ATP synthesis to be maintained. This would enable cells experiencing a relatively milder insult to die in a controlled and non-inflammatory manner by apoptosis in which the cell body shrinks and forms apoptotic bodies that are engulfed by macrophages. This contrasts markedly with necrosis in which the cell and its organelles swell, rupturing the plasma membrane and spewing out the intracellular contents into the surrounding medium. The resulting invasion by neutrophils causes an inflammatory response and secondary damage to the tissue. By analogy, apoptosis can be likened to an insurance company "writing off" a car involved in an accident, when the cost of repairing damage is greater than the cost of replacement. In contrast, necrosis is more like a catastrophic accident in which the car is smashed beyond repair. Thus mitochondria are acting both to assess the extent of the damage and to initiate the final fate of the cell (see also Fig. 6).

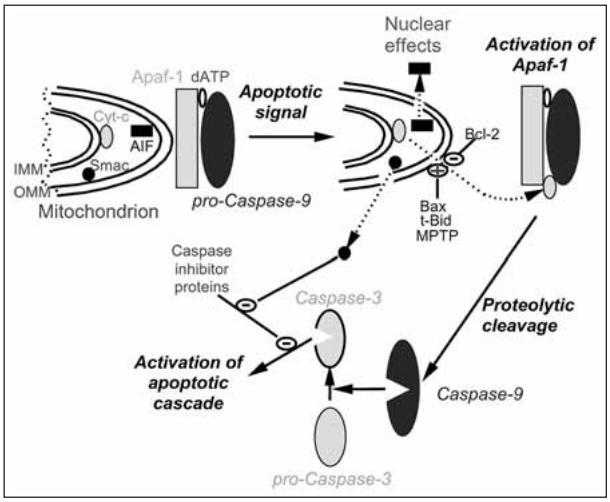

Figure 2. The release of mitochondrial proteins plays a central role in the initiation of apoptosis. An apoptotic signal leads to the release of pro-apoptotic proteins located within the intermembrane space between the inner and outer mitochondrial membranes (IMM and OMM) into the cytosol. The pathway for release may be OMM rupture following opening of the MPTP and mitochondrial swelling, or through recruitment of specific pore-forming proteins to the outer membrane such as Bax and t-Bid. Release is inhibited by Bcl-2. The released cytochrome binds to Apaf-1 that proteolytically activates caspase 9 which in turn activates caspase 3 and so initiates the apoptotic cascade. Caspases are inhibited by endogenous inhibitors which are themselves inactivated by Smac (or DIABLO) that is also released along with apoptosis inducing factor (AIF). This migrates to the nucleus where it causes chromatin condensation and DNA fragmentation. Further details are given in the text.

Although the emphasis of this article will be on the role of the MPTP in cell death, it is important to recognise that there are other mechanisms for the induction of apoptosis involving mitochondria that are independent of the MPTP. In this case, more specific changes in the permeability of the mitochondrial outer membrane are induced by pro-apoptotic members of the Bcl-2 oncogene family that lead to the release of cytochrome c [10, 14]. Bcl-2 is a 26 kDa protein that associates with the outer mitochondrial and endoplasmic reticulum membranes, and when over-expressed, blocks apoptosis induced by a wide range of stimuli. In contrast, there are proteins that share sequence homology to Bcl-2, such as Bax and Bid stimulate rather than inhibit apoptosis. In response to some apoptotic stimulus such as tumour necrosis factor, these pro-apoptotic proteins translocate to the mitochondria where they incorporate into the outer membrane and somehow induce an increase in its permeability to cytochrome c [15, 16]. However, the intention of this brief review is to summarise what is known about the molecular mechanism and regulation of the MPTP and its role in reperfusion injury of the heart. Emphasis will be placed on how studies of the molecular structure of the MPTP may lead to effective protective strategies in the clinical setting. The relevance of the MPTP to any consideration of the role of magnesium in heart function may not be immediately apparent, but Mg^{2+} acts as an inhibitor of pore opening (see below) and thus may provide some explanation of the protective effects of magnesium against ischaemia/reperfusion injury.

The Molecular Mechanism of the MPTP

Our current understanding of the molecular mechanism of the MPTP is illustrated in Figure 3. However, it is instructive to summarise the experimental evidence on which the model is based. The phenomenon of the mitochondrial permeability transition (MPT) was first described several decades ago,

and was initially thought to be a result of non-specific damage to the inner membrane by phospholipases [17]. However, elegant experiments by Haworth and Hunter and later in Martin Crompton's laboratory implied the opening of a non-specific pore that transports any molecule of < 1500 daltons [18–23]. The extensive swelling of the mitochondria is the result of the equilibration of all small solutes across the membrane leaving a colloidal osmotic pressure exerted by the matrix proteins. The trigger for opening is a rise in matrix [Ca^{2+}], but the concentration required is very dependent on the prevailing conditions. Several factors greatly enhance the sensitivity of the pore to [Ca^{2+}] of which the most potent and relevant to the cellular setting are oxidative stress, adenine nucleotide depletion, increased inorganic phosphate concentrations and mitochondrial depolarisation. The pore is also sensitive to the ligand induced conformational changes of the adenine nucleotide translocase (ANT), an integral inner membrane protein responsible for ATP and ADP translocation across the membrane. It is strongly inhibited when the matrix pH falls below pH 7.0 [1, 2, 24]. The mechanism responsible for these effects will be discussed further below.

The Role of Cyclophilin D

A major breakthrough in elucidating the molecular mechanism of the MPTP came in 1988 when Crompton and colleagues demonstrated that pore opening could be inhibited specifically by sub-micromolar concentrations of the immunosuppressive drug, cyclosporin A (CsA) [25]. This discovery provided the impetus for our own research into the molecular mechanism of the MPTP and led us to investigate the role of a matrix CsA binding proteins, cyclophilin (CyP) in pore opening. Cyclophilins exhibit peptidyl-prolyl cis-trans isomerase (PPIase) activity, that is able to catalyse changes in the conformation of proline peptide bonds in proteins. We were able to demonstrate that the matrix contained such a CsA-sensitive PPIase [26] and the $K_{0.5}$ values of several CsA analogues for the inhibition of this activity correlated with their potency as inhibitors of pore opening [26–28]. This led us to propose in 1990 that the MPTP was formed from an interaction between the adenine nucleotide translocase (ANT) and mitochondrial CyP [26] as discussed further below.

Subsequently we were able to purify, clone and sequence the CsA-sensitive mitochondrial PPIase [29, 30] and hence confirm that it was a unique cyclophilin, the rat equivalent of

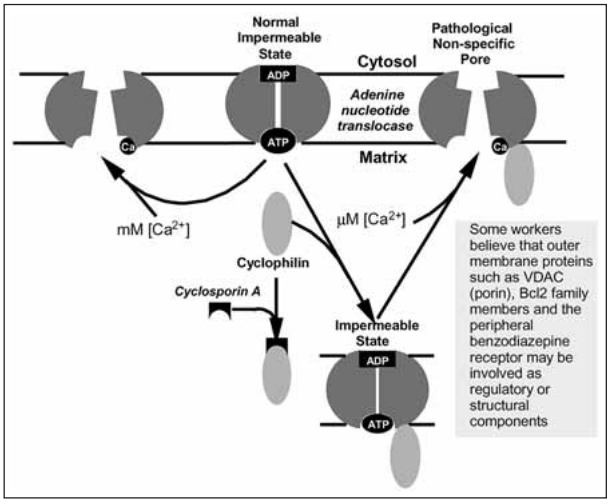

Figure 3. The proposed mechanism of the mitochondrial permeability transition pore. The probable sites of action of known effectors of pore opening are shown in Table 1.

the human CyP-3 gene product [31] and distinct from the cytosolic cyclophylin A. Now known as cyclophilin D (CyP-D), this nuclear encoded protein has a mitochondrial targeting presequence that is cleaved after translocation of the protein into the matrix. Cleavage occurs at one of two points leading to mature proteins of about 17.6 kDa (minor product) and 18.6 kDa (major product) [29]. Subsequently, Crompton and colleagues also purified and sequenced mitochondrial CyP-D and confirmed that the protein can be imported into the mitochondria before cleavage of its presequence [32, 33]. Northern blots demonstrate that mRNA for CyP-D is present in rat muscle, heart, liver, kidney and brain and is of identical size (1.5 kb) in all tissues. This makes it unlikely that there are differently spliced tissue-specific isoforms [30].

The role of CyP-D in opening of the MPTP is likely to involve its binding to a membrane protein and induction of a conformational change that is triggered by Ca^{2+}. Thus, one mechanism by which the MPTP might be sensitised to [Ca^{2+}] would be through an increased binding of CyP-D to its target protein. We provided evidence in support of this by demonstrating that oxidative stress induced with t-butyl hydroperoxide (which produces reactive oxygen species [ROS]) or with diamide (which depletes mitochondria of reduced glutathione required to remove ROS) both increased CyP-D binding to the inner mitochondrial membrane in parallel with increasing the sensitivity of pore opening to [Ca^{2+}]. Phenylarsine oxide (PAO), which modifies vicinal thiol groups on proteins had a similar effect [34, 35]. In order to demonstrate these effects, the mitochondrial membranes had to be prepared in iso-osmotic potassium isothiocyanate (KSCN) medium to stabilise the complex between CyP-D and its membrane target protein. Stabilisation could also be achieved by the addition of low concentrations of guanidinium hydrochloride, implying that it is the chaotropic properties of KSCN that are responsible for its stabilising effects [35]. Chaotropes de-stabilise hydrophobic interactions and stabilise hydrophilic interactions involved in maintaining the three dimensional structure of proteins. This suggests that CyP-D forms a complex with the target protein, inducing a conformational change that exposes more of the protein surface to the aqueous medium. Such an effect might be predicted for the formation of a channel. Another factor that enhances both CyP-D binding and MPTP opening in response to [Ca^{2+}] is an increase in matrix volume [35]. Although the mechanism behind this effect is unknown, unfolding of the inner membrane cristae leading to greater exposure of the membrane target protein is a possible explanation.

In all cases, binding of CyP-D was almost totally prevented by CsA, suggesting that when CyP-D binds CsA it is unable to bind to the target protein. In contrast, we have been unable to show significant effects on CyP-D binding of several other known modulators of the MPTP including matrix [Ca^{2+}], [ADP], pH or membrane potential [34–36]. This implies that these effectors are acting at another site that regulates the sensitivity of the MPTP towards [Ca^{2+}] probably on the target membrane protein as will be discussed further below. However, Bernardi and colleagues have demonstrated an inhibitory effect of low pH on CyP-D binding to sub-mitochondrial particles. The effect was blocked by the histidine reagent diethylpyrocarbonate which also blocks the inhibitory effect of pH on the MPTP [37, 38]. These experiments were performed in low ionic strength media where a large number of other matrix proteins also remained bound to the membrane at low pH, and thus it is possible that the effect of pH was on non-specific binding of CyP-D to charged groups on the phospholipids or membrane proteins. Crompton and colleagues [39, 40] used similar conditions

when labelling the membrane bound CyP-D with photoactivatable CsA derivatives. Their data suggested that Ca^{2+} might enhance and ADP diminish CyP-D binding under such conditions, but our own experiments failed to demonstrate such effects [35, 41].

Despite the evidence described above, there is now a body of data to suggest that CyP-D binding may not be essential for pore opening, but may rather sensitise the process to $[Ca^{2+}]$. Thus Novgorodov et al. [42] and Crompton and Andreeva [43], using different techniques, have shown that at high matrix $[Ca^{2+}]$ inhibition of pore opening by CsA is overcome. We have confirmed this in both heart mitochondria [27] and liver mitochondria [34–36]. Yet under the same conditions, CsA is able to prevent almost totally the binding of CyP-D to the inner mitochondrial membrane [34, 36]. In addition, studies on the megachannel of patched clamped mitochondria, which it has been suggested may represent an electrophysiological manifestation of the MPTP, have shown that the inhibitory effect of CsA is overcome at higher $[Ca^{2+}]$ [44, 45]. However, other data cast doubt on whether the megachannel and the MPT pore are the same molecular entity [46].

The Role of the Adenine Nucleotide Translocase in the Mitochondrial Permeability Transition

An involvement of the ANT in pore opening was first proposed by Hunter and Haworth [19] and more convincing evidence provided by LeQuoc and LeQuoc [47] and ourselves [26, 36]. The ANT can be stabilised in one of two conformations (known as "c" and "m" to designate cytosolic and matrix facing substrate binding sites) that are thought to intercovert during the translocation cycle. Any reagent such as carboxyatractyloside (CAT) that stabilises the "c" conformation of the ANT, was found to stimulate the MPT, whilst any reagent such as bongkrekic acid (BKA) that stabilises the "m" conformation of the ANT, inhibited the MPT. Furthermore, matrix ADP is an important modulator of pore opening that acts by decreasing the sensitivity of the calcium trigger site to $[Ca^{2+}]$. There are two ADP binding sites with K_i values of about 1 and 25 μM. The high affinity site is blocked by the inhibitor CAT and therefore thought to be associated with the ANT [18, 19, 36, 42, 48, 49]. We tested the ability of a range of nucleotides to inhibit the MPT, and found that only ATP and deoxy-ADP inhibit with $K_{0.5}$ values 500 and 20 times greater than ADP respectively. This correlates with their affinity for the matrix binding site of the ANT [36]. Adenine nucleotide binding is antagonised by oxidative stress induced by t-butyl hydroperoxide (TBH) or diamide and also by thiol reagents such as PAO, a powerful activator of the MPT [36, 50]. PAO has the greatest effect of the reagents tested, raising the $K_{0.5}$ for ADP inhibition of the MPT to > 500 μM [36]. We have shown that this effect is accompanied by covalent modification of the ANT [36] which may explain why PAO is a more potent stimulus of the MPT than diamide or TBH, and yet has a smaller effect on CyP-D binding [35].

Bernardi and colleagues have provided strong evidence that the MPTP is voltage-regulated, being activated as the membrane potential becomes less negative [51–56]. We have suggested that the membrane potential is sensed by the ANT itself through an effect on adenine nucleotide binding. The ANT catalyses the electrogenic exchange of ATP^{4-} for ADP^{3-} with a mechanism that may well involve a potential driven conformational change that alters the affinity of the ANT for adenine nucleotides on either side of the membrane [57, 58]. In support of this hypothesis, we have demonstrated that in mitochondria depleted of adenine nucleotides by exchanging matrix ADP with pyrophosphate, not only is the MPT much more sensitive to $[Ca^{2+}]$, but it is also no longer voltage sensitive [26, 28]. Oxidative stress shifts the voltage dependence of the MPT, allowing the pore to open at more negative potentials. Two distinct thiol groups have been implicated in this effect [59–61]. One is sensitive to oxidation of glutathione, for example by TBH or diamide, and is protected by both monobromobimane and N-ethylmaleimide. The other responds to the redox state of matrix NAD(P), and is protected by N-ethylmaleimide but not monobromobimane. The former reagent attacks specific cysteine groups on proteins whilst the latter covalently attacks reduced glutathione and prevents it from becoming oxidised. Oxidised glutathione can catalyse disulphide bond formation between adjacent thiol groups on proteins perhaps through the mediation of thioredoxin or lipoamide [62, 63]. Thus it seems probable that two critical cysteine groups on the ANT might account for these effects. One cysteine is probably attacked by oxidised glutathione in response to oxidative stress before forming a disulphide link with an adjacent cysteine. Indeed, the ANT is known to have three cysteine residues that show differential reactivity to various thiol reagents and oxidising agents in a conformation dependent manner [64–66]. In particular cysteine 56 (Cys^{56}) of the ANT is especially sensitive to N-ethylmaleimide whilst eosine maleimide specifically attacks cysteine 159 (Cys^{159}) [64]. These cysteines may well represent the thiol groups that regulate both CyP-D binding and the inhibitory effects of ADP and membrane potential on the MPT [36]. In support of this, Cys^{159} is located within the adenine nucleotide binding site [67], and treatment with eosine maleimide inhibits ANT activity and also abolishes the ability of ADP to inhibit the opening of the MPTP [36]. Furthermore, the ANT binds specifically to a phenylarsine oxide column [36] and this binding is prevented by pre-treatment with eosinemaleimide [unpublished data of Gavin McStay and Andrew Halestrap].

CyP-D Binds to the ANT to Form the Calcium-Dependent MPTP

The data described above strongly support a critical role for the ANT in the formation of the MPTP, as we originally proposed in 1990 [26], but falls short of proof. In order to confirm that CyP-D does bind specifically to the ANT under conditions favouring opening of the MPTP we over-expressed CyP-D as a fusion protein with glutathione-S-transferase (GST) that enabled us to make a CyP-D affinity column. When solubilised inner mitochondrial membranes were passed over this column and weakly binding proteins washed off, only one protein remained bound and this was the ANT. Binding was inhibited by pre-treatment of the GST-CyP-D with CsA and enhanced when the inner membranes were prepared from mitochondria subjected to oxidative stress with diamide [41]. This effect also appears to involve oxidation of thiol groups on the ANT and our most recent data [Samantha Clarke and Gavin McStay unpublished data] suggests that thiol cross-linking between Cys^{159} and Cys^{56} is responsible. This would explain why oxidative stress is such a potent activator of the pore since by modifying both Cys^{159} and Cys^{56} it affects both adenine nucleotide and CyP-D binding. In view of the peptidyl-prolyl cis-trans isomerase activity of CyP-D, a proline residue close to Cys^{56} on a matrix loop of the ANT such as Pro^{61} would seem a likely binding site as proposed in our original model [26]. This proline is absent in the three ANT isoforms found in yeast mitochondria, which do not exhibit a CsA-sensitive MPTP [36].

We have sought to address whether CyP-D bound to the ANT represents the minimal configuration of the MPTP by

reconstituting the purified ANT into proteoliposomes in the presence and absence of CyP-D, and determining pore opening with a continuous spectrophotometric assay [3]. Previous studies had shown that the reconstituted ANT can form non-specific pores when exposed to high (millimolar) concentrations of calcium [68, 69], but we were able to show that in the presence of added purified CyP-D, micromolar concentrations of Ca^{2+} could induce pore opening that was abolished by CsA. This is consistent with several studies suggesting that CyP-D binding may not be essential for the opening of the MPT, but rather sensitize the process to [Ca^{2+}]. Thus at high matrix [Ca^{2+}] inhibition of pore opening by CsA is overcome in both heart mitochondria and liver mitochondria [28, 34–36]. Yet under the same conditions, CsA is able to prevent almost totally the binding of CyP-D to the inner mitochondrial membrane [34, 36]. Thus our data strongly support our original hypothesis that only the ANT and CyP-D are required for pore formation, although this does not rule out the possibility that other proteins may play a regulatory role.

Other Possible Components of the MPTP

Several other proteins have been proposed to be components of the MPTP, including the voltage activated anion channel (VDAC, also known as porin) and the peripheral benzodiazepine receptor (see [1, 2, 4, 14]). VDAC is a major component of the outer mitochondrial membrane and is the protein responsible for allowing permeation of metabolites and ions through the membrane bilayer. The peripheral benzodiazepine receptor is an outer membrane protein of unknown function that has a high affinity for benzodiazepines. The possibility that these proteins might be involved was originally proposed by Zoratti and Szabo [45] because they had been shown to copurify with the ANT as a complex under some conditions [70]. The same proteins are also thought to interact at contact sites, points of intimate contact between the inner and outer mitochondrial membranes. Since that time the groups of Kroemer and Brdizka have suggested that other proteins associated with the contact sites, such hexokinase, creatine kinase and the anti- and pro-apoptic proteins BCl-2 and BAX may also be involved in the regulation of the MPTP [71–74]. Indeed fractions of detergent solubilised proteins containing these components, along with CyP-D, could be reconstituted into proteoliposomes to form a calcium activated, CsA-sensitive pore [71, 74]. However, which of the components was essential for pore formation could not be established by such techniques. The evidence is perhaps most compelling for VDAC since Crompton and colleagues, using a GST-CyP-D affinity column in a similar manner to us, but with heart mitochondria solubilised with different detergents, found that both VDAC and the ANT bound tightly (even in the presence of CsA). Furthermore, they showed that when eluted with glutathione, these components could be reconstituted into proteoliposomes to form a calcium activated pore that was inhibited by CsA [75]. The question remains, what is the minimum configuration of the MPTP; is VDAC an essential component or rather a regulatory component? Our own data described above would suggest the latter but further data are required to answer this question unequivocally. This is important if the MPTP is to become a future pharmaceutical target, for example in the protection of the heart from reperfusion injury.

A Model of the MPTP That Explains the Mode of Action of Different Modulators

In Figure 3 we summarise our current understanding of the mechanism of the MPTP, which represents a development of our original model [26], and is becoming widely accepted [1, 2, 4]. We propose that CyP-D binds to the ANT on Pro^{61} and that this binding is greatly enhanced when Cys^{56} is cross-linked (eg by oxidative stress) to Cys^{159}. Yeast mitochondria lack both these residues and do not possess a CsA-sensitive MPTP [36, 76]. We suggest that Ca^{2+} binds to a site on the ANT to trigger the conformational change required to induce pore formation probably involving a cis-trans isomerisation of the peptide bond adjacent to Pro^{61}. Binding of adenine nucleotides to the substrate binding site of the ANT greatly reduces the sensitivity of the pore to [Ca^{2+}]. It seems probable that this conformational change is facilitated rather than totally dependent on CyP-D, since at high (mM) [Ca^{2+}] pore formation can be induced in proteoliposome containing the purified, reconstituted ANT in the absence of CyP-D [68]. Furthermore, at high calcium concentrations or upon adenine nucleotide depletion, the pore becomes insensitive to CsA [28, 36].

This model is able to provide a plausible explanation of the effects of most known regulators of the pore that may act to

Table 1. Proposed sites of action of known effectors of the mitochondrial permeability transition. Further details may be found in the text.

Effect via change in cyclophilin-D binding[1]	Effect via change in nucleotide binding[1]	Direct effect on Ca^{2+} binding	Unknown mode of action
Activatory	**Activatory**	**Activatory**	**Activatory**
Vicinal thiol reagents (eg PAO)	Thiol reagents attacking Cys^{159} of ANT (eg PAO and eosine maleimide)	High pH	Some ubiquinone analogues (eg decyl-ubiquinone, ubiquinone 10)
Oxidative stress to cross-link Cys^{56} with Cys^{159} of the ANT (eg TBH and Diamide)	Oxidative stress to cross-link Cys^{56} with Cys^{159} of the ANT (eg TBH and Diamide)		
Increased matrix volume	"C" Conformation of ANT		
Chaotropic agents	Adenine nucleotide depletion		
	High matrix [Pi] and [PPi]		
Inhibitory	**Inhibitory**	**Inhibitory**	**Inhibitory**
CsA and some analogues	Membrane potential	Low pH	Some ubiquinone analogues (eg 2,5-dihydroxy-6-undecyl-1, 4-benzoquinone)
eg Cyclosporin G, [MeAla^6]-CsA and 4-methyl-val-CsA	"M" Conformation of ANT	Mg^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}	Trifluoperazine (may work via membrane surface charge)

[1] Note that both CyP-D binding and ADP binding exert their effects through changes in the sensitivity of the MPT to [Ca^{2+}]

modulate binding of adenine nucleotides, calcium or CyP-D to their respective sites as summarised in Table 1. Thus any intervention that reduces this adenine nucleotide binding enhances pore opening. Factors acting this way include adenine nucleotide depletion, matrix phosphate (competes for the nucleotide binding site), and the conformational state of the adenine nucleotide translocase (ANT). The latter can be influenced by depolarisation (decreases the affinity of the matrix binding site for ADP) and specific ligands of the ANT such as carboxyatractyloside (decrease matrix ADP binding affinity) and bongkrekic acid (increases matrix ADP binding affinity). Furthermore, modification of a specific thiol group on the ANT (Cys^{159}) either by oxidative stress or thiol reagents such as eosinemaleimide or phenylarsine oxide, also decreases adenine nucleotide binding and can account for the ability of these agents to activate the pore [77]. Factors that enhance cyclophilin binding and hence increase the sensitivity of the MPTP to [Ca^{2+}] include oxidative stress (Cys^{56}-Cys^{159} cross-linking), chaotropic agents and increased matrix volume. In contrast, CsA has the opposite effect by preventing CyP-D binding. Low pH and Mg^{2+} are suggested to compete directly with Ca^{2+} at the trigger site and in view of the emphasis of this volume on Mg^{2+} will be considered in more detail below.

The modes of action of two other potent inhibitors of the MPTP are not so clear. Trifluoperazine is a potent inhibitor of the MPT under energised but not de-energised conditions [36]. It was originally thought to act indirectly through inhibition of phospholipase A_2, preventing the accumulation of free fatty acids. Fatty acids are known to inhibit the activity of the ANT and also to stimulate the MPT. However, inhibition by trifluoperazine occurs even without changes in free fatty acid accumulation and is now thought to be mediated by an effect on surface membrane charge that changes the voltage sensitivity of the MPTP [78]. More recently, it has been shown that ubiquinone analogues can act either as activators or inhibitors of the MPTP [79–81]. Earlier work had shown that the probability of pore opening varied according to the rate of electron transfer through Complex 1 of the respiratory chain respiratory [82]. These data led Fontaine and colleagues to suggest that components of Complex 1 may be involved in the formation and/or regulation of the MPTP [80, 81]. However, the recent observation that the uncoupling proteins UCP1, UC2 and UCP3 require oxidised ubiquinone to function suggest an alternative mechanism [83, 84]. Since the UCPs are close relatives of ANT it would seem quite likely that a ubiquinone binding site may also exist on the ANT.

Regulation of the MPTP by Mg^{2+} and pH

Activation of the MPTP by matrix calcium is totally selective for Ca^{2+} and pore opening is strongly inhibited by other divalent cations such as Sr^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+} [18, 85, 86]. The kinetics of inhibition are not well defined, in part because of difficulties in controlling the divalent cation concentration within the matrix and also because of the presence of a second divalent cation site on the external surface of the inner membrane with a K_i for Mg^{2+} of about 0.3 mM [87]. At a free [Ca^{2+}] of 50 μM, 1 mM [Mg^{2+}] inhibits opening of the MPTP by about 90 %. Inhibition has a strong competitive element and also increases the Hill coefficient for activation by Ca^{2+} [18]. An additional complicating factor that cannot be ignored when considering the effects of Mg^{2+} on the MPTP is that ATP binds magnesium tightly (Kd $\sim 10^{-4}$ mol×l^{-1} at pH 7.0) and the MgATP complex does not bind to the ANT. Thus, energisation of mitochondria, which in its own right protects against MPTP opening, will also decrease free [ATP] and [Mg^{2+}] that inhibit pore opening and the overall effect will be hard to predict.

Low pH (< 7.0) is another potent inhibitor of the MPTP which also appears to act by direct competition with Ca^{2+} for its binding at the trigger site [18, 85, 88]. Bernardi and colleagues have presented data to show that the effect of low pH involves a specific histidine residue and that this may modulate CyP-D binding to the inner mitochondrial membrane [37, 38]. However, in our experiments we did not detect such an effect of low pH on CyP-D binding to either inner membranes [35] or to the purified ANT [41].

The Role of the Mitochondrial Permeability Transition in Reperfusion Injury of the Heart

During myocardial ischaemia the lack of oxygen leads to inhibition of oxidative phosphorylation and hence a decrease in tissue ATP concentrations with a concomitant rise in ADP, AMP and Pi concentrations. These changes will activate glycolysis, but this is insufficient for the heart to continue beating, although initially sufficient ATP may be produced to maintain basic cellular functions such as ionic homeostasis. Indeed, the heart can survive a short period of ischaemia and then recover fully upon reperfusion, although its performance may be temporarily impaired (stunning). However, with prolonged ischaemia the accumulation of lactic acid within the cell causes intracellular acidification and inhibition of glycolysis. At this point ionic homeostasis can no longer be maintained, intracellular concentrations of sodium and calcium begin to rise and contracture follows. If the period of ischaemia is sufficiently prolonged, the heart becomes irreversibly damaged. Hence it is important to restore the blood flow as soon as possible, yet the very process of reperfusion may exacerbate the damage induced by ischaemia itself, a phenomenon known as reperfusion injury. This is associated with an increase in the release of intracellular enzymes and changes in cell morphology including the appearance of swollen and dysfunctional mitochondria. These observations suggest that the MPTP may be opening during reperfusion and there is now increasing evidence that this is indeed the case and may be a critical factor in the transition from reversible to irreversible reperfusion injury [1, 2, 89].

Evidence that the MPTP Opens During Reperfusion of the Ischaemic Heart

Circumstantial Evidence

The conditions that occur during reperfusion are exactly those that induce pore opening (see [1, 2, 89]). In outline, during ischaemia, the intracellular pH (pH_i) decreases rapidly as glycolysis is stimulated and leads to an accumulation of lactic acid. Activation of the Na^+/H^+ antiporter occurs as the cell attempts to restore the pH_i,, but in the process it becomes loaded with sodium. This cannot be pumped out of the cell because the Na/K ATPase is inhibited by the lack of ATP. Consequently, the activity of the Na^+/Ca^{2+} antiporter, that usually pumps Ca^{2+} out of the cell, is reduced or reversed and the cell becomes loaded with calcium. In addition to this loss of ionic homeostasis, there is a net loss of adenine nucleotides from the cell by progressive degradation of ADP to AMP, adenosine, inosine and xanthine through a purine degradation pathway. This may contribute to the stunning of hearts that recover upon reperfusion but also, in conjunction with the increased phosphate concentrations, will sensitise MPTP opening to [Ca^{2+}] as outlined above. Nevertheless, even with the increased [Ca^{2+}] that accompanies ischaemia, these factors alone will not induce pore opening since pH_i remains low and this inhibits MPTP opening. Free [Mg^{2+}]

may also be increased in ischaemia as a result of the hydrolysis of ATP to ADP and AMP, and this may have an additional inhibitory effect on the MPTP although measurements of mitochondrial [Mg^{2+}] have not been reported under these conditions. However, upon reperfusion pH_i is restored as lactic acid leaves the cell and the Na^+/H^+ antiporter becomes active again, and ADP/AMP will be rephosphorylated to ATP leading to a decrease in [Mg^{2+}]. Furthermore, the renewed supply of oxygen leads to oxidative stress as oxygen interacts with the reduced respiratory chain complexes to produce a burst of oxygen free radicals. This oxidative stress may be further enhanced by the accumulation of hydrogen peroxide formed through the action of xanthine oxidase whose substrate, xanthine, accumulates during ischaemia. In addition, the provision of oxygen reawakens respiration, energising the mitochondria and enabling them to take up the calcium that has accumulated in the cell during ischaemia. A range of factors are now in place that are capable of inducing pore opening.

Direct Evidence

In order to demonstrate directly that the MPTP only opens during reperfusion and not during ischaemia, three distinct approaches have been taken. First, fluorescence microscopy of isolated cardiac myocytes subjected to simulated ischaemia and reperfusion has been used. In these studies the mitochondrial membrane potential was determined with fluorescent dyes such as tetramethylrhodamine (red fluorescence) as a surrogate indicator of MPTP [90]. Such dyes accumulate within the mitochondria in response to the membrane potential and are released upon depolarisation when the MPTP opens. In the confocal microscope this is observed as a loss of fluorescence of individual mitochondria, whilst the fluorescence of the whole cell increases because the dye accumulated within mitochondria is quenched. Confirmation that any depolarisation is caused by opening of the MPTP is usually provided by the ability of CsA, sometimes supplemented with trifluoperazine, to inhibit the process [1, 91]. A more sophisticated approach is simultaneously to determine the distribution of another fluorescent dye, calcein (green fluorescence), that can only cross the inner mitochondrial membrane when the pore opens. Depending on the method of dye loading, it can either be entrapped within the matrix and then released upon pore opening or be excluded from the mitochondria, but enter when the pore opens [92, 93]. To date, the use of this more refined technique has not been applied to cardiac myocytes although it has been used successfully in isolated hepatocytes subjected to oxidative stress whose necrotic cell death is accompanied by pore opening. Both cell death and pore opening could be inhibited by CsA, especially in the presence of trifluoperazine [91, 94].

The perfused heart is not accessible to confocal microscopy and other techniques must be applied to estimate the extent of pore opening. One such method, devised by DiLisa and colleagues, is to determine the loss of mitochondrial NAD^+ that accompanies reperfusion as a surrogate indicator of pore opening [95]. Inhibition by CsA is taken as verification that this loss is through the MPTP. A more direct approach, devised in this laboratory, is to measure the mitochondrial entrapment of a radioactive marker, [^{3}H]-2-deoxyglucose (3H-DOG). In this technique Langendorff perfused hearts are first loaded with 3H-DOG which accumulates within the cytosol as ^{3}H-DOG-6-phosphate (3H-DOG-6P) but can only enter the mitochondria when the MPTP opens [28]. The extent to which the 3H-DOG enters the mitochondria can be determined by their rapid isolation in the presence of EGTA to reseal the pores and so entrap the 3H-DOG within them. Measurement of the ^{3}H content of the mitochondria gives a quantitative value for the extent of pore opening, provided that suitable controls and corrections are performed to account for variations in mitochondrial recovery and loading of the heart with 3H-DOG. As illustrated in Figure 4, using this technique we have been able to confirm that the MPTP remains closed during ischaemia but opens upon reoxygenation with a time course that reflects the return of pH_i from its ischaemic value of < 6.5 (inhibitory to the MPTP [88]) to pre-ischaemic values [28, 77].

A puzzling observation was made during these experiments; reperfusion after short periods of ischaemia led to total recovery of LVDP and ATP/ADP ratio [96], yet using the 3H-DOG technique, we demonstrated that some opening of the MPTP could be detected [28, 77]. One explanation for this paradox would be that opening of the MPTP was transient, and rapidly followed by resealing. This would allow total recovery of mitochondrial function and heart performance. Unfortunately, if resealing of the MPTP does occur, the entrapped 3H-DOG will remain within the mitochondria and thus closure will not be detected. However, it is possible to detect closure by using a modification of the 3H-DOG technique, in which hearts are loaded with [^{3}H]-DOG following a period of reperfusion sufficient to allow maximum functional recovery of the heart (post-loading), as opposed to loading during the pre-ischaemic phase (preloading). This is illustrated in Figure 5. If closure of pores does occur during reperfusion, mitochondrial DOG entrapment following post-loading should be less than with pre-loading, and we have confirmed that this is the case. After 40 min ischaemia post-loading gives about 50 % less mitochondrial DOG entrapment than observed with preloading. Indeed, the extent of subsequent MPTP closure correlated with the functional recovery of the heart [97]. Thus it would seem that if the insult caused by ischaemia/reperfusion is not too great, mitochondria may undergo a transient permeability transition, followed by closure of the pores and entrapment of the DOG. The closure probably occurs as a result of the loss of matrix [Ca^{2+}] through the open pores and its subsequent removal from the cytosol. However, this will only occur if enough "healthy" mitochondria (without open pores) remain in the cell to accumulate the Ca^{2+} released by those with open pores. These healthy mitochondria will also provide sufficient ATP to maintain the ionic homeostasis of the cell.

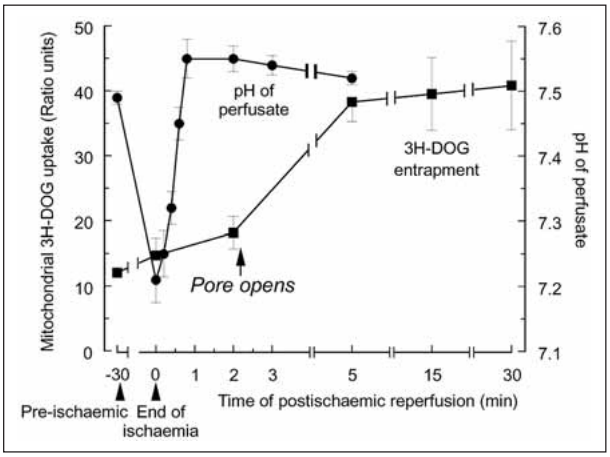

Figure 4. Time dependence of MPTP opening during reperfusion. The opening of the MPTP was detected using the DOG preloading technique. Hearts were subjected to 30 min global ischaemia before reperfusion for the time shown. Data are taken from [77]. Parallel data for the pH of the perfusate are taken from [97].

The balance between the number of "closed" and "open" mitochondria within any cell will be critical in determining whether a cell lives or dies. If there are too many "open" mitochondria, they will release more calcium and hydrolyse more ATP than the "closed" mitochondria can accommodate. In contrast, if there are sufficient "closed" mitochondria to meet the ATP requirements of the cell and also to accumulate released calcium without undergoing the permeability transition themselves, the "open" mitochondria will close again and the cell will recover.

Transient opening of the MPTP may have longer term implications for the heart, not apparent in the short-term experiments performed with the Langendorff perfused heart. As outlined in the Introduction, transient opening of the MPTP may not deplete ATP concentrations sufficiently to induce necrosis but does have the potential to release cytochrome c and other apoptotic factors from the mitochondria, and so induce apoptosis. It may be significant that the area around the necrotic core of an infarct shows a ring of apoptotic cell death [98, 99]. Thus the severity of the insult, strongest in core which suffers prolonged total ischaemia, necrosis is seen reflecting permanent MPTP opening and mitochondrial dysfunction whereas at the periphery where the insult is less, transient MPTP opening would cause apoptosis (Fig. 6).

The MPTP as a Target for Protecting Hearts From Reperfusion Injury

It would be predicted that if opening of the MPTP is a critical factor in the transition from reversible to irreversible reperfusion injury of the heart, inhibitors of pore opening would offer protection. There is increasing evidence that this is the case.

Cyclosporin A

Cyclosporin A would seem the most appropriate reagent to use in this context, since it acts to inhibit the MPTP with a K_i of about 5 nM [26, 27]. Crompton and colleagues were the first to demonstrate such protection using an isolated cardiac myocyte model of anoxia and re-oxygenation [100], and subsequently this has been confirmed by others [101, 102]. Interestingly, in such cells it has been shown that there is a correlation between mitochondrial [Ca^{2+}] content and subsequent cell death [103, 104]. In this laboratory, we were able to demonstrate protection by CsA in the Langendorff perfused heart model of reperfusion injury [77, 96]. Thus hearts were better able to re-establish a regular beat and left ventricular developed pressure after long periods of ischaemia when 0.2 μM CsA was present prior to ischaemia and then also in the reperfusion medium. This protective effect of CsA was accompanied by a return of the tissue ATP/ADP ratios and AMP levels to control values and lower end diastolic pressure (EDP) – an indicator of contracture whose elevation reflects elevated [Ca^{2+}]. Furthermore, we and others have confirmed that the protective effect is shared by other CsA analogues that also inhibit the MPTP such as cyclosporin G, [MeAla^6]-CsA and 4-methyl-val-CsA, but not by analogues such as cyclosporin H that are inactive against the MPTP [28, 95, 96]. However, it proved difficult to produce consistent results with CsA because the effect was extremely concentration dependent, being optimal at about 0.2 μM and declining again at higher concentrations [28, 95, 96]. This decline in protection probably reflects other sites of action of the drug such as binding to cytosolic cyclophilin A (CyP-A). CyP-A might be involved in repairing misfolded proteins, or act through inhibition of calcineurin-dependent enzymes by the CsA-CyP-A complex leading to disruption of normal cardiac function. The former possibility is supported by recent data of Crompton and colleagues who used antisense technology to knockout CyP-A in cardiac myocytes. These cardiac myocytes were more sensitive to oxidative stress than control myocytes, but exhibited greater protection by CsA [105].

Low Intracellular pH and Inhibition of Mitochondrial Calcium Overload

Another potent inhibitor of MPTP opening is low pH, and several groups have demonstrated that maintaining an acidic extracellular pH during reoxygenation after a period of an-

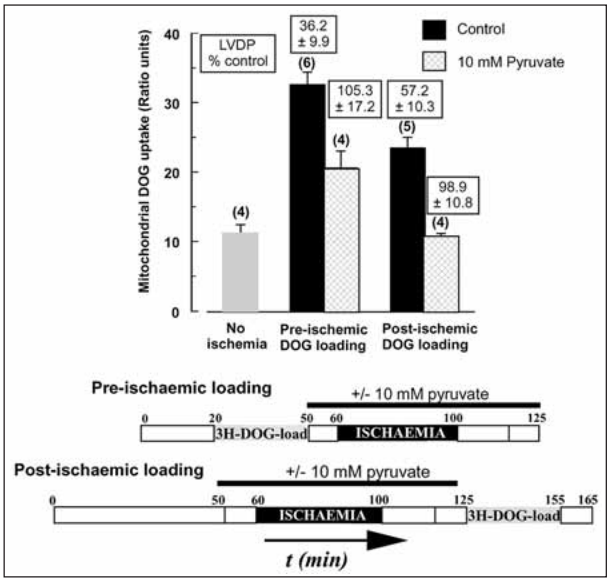

Figure 5. Pyruvate protects the heart against reperfusion injury through inhibiting MPTP opening and enhancing subsequent pore closure. Pore opening upon reperfusion was determined using the entrapment of pre-loaded 3H-DOG, whilst subsequent closure of the MPTP was detected by measuring the entrapment of 3H-DOG loaded after maximal recovery of the heart (post-loading). These protocols are illustrated in the Figure and further details are given in the text and reference [97] from where the data are taken.

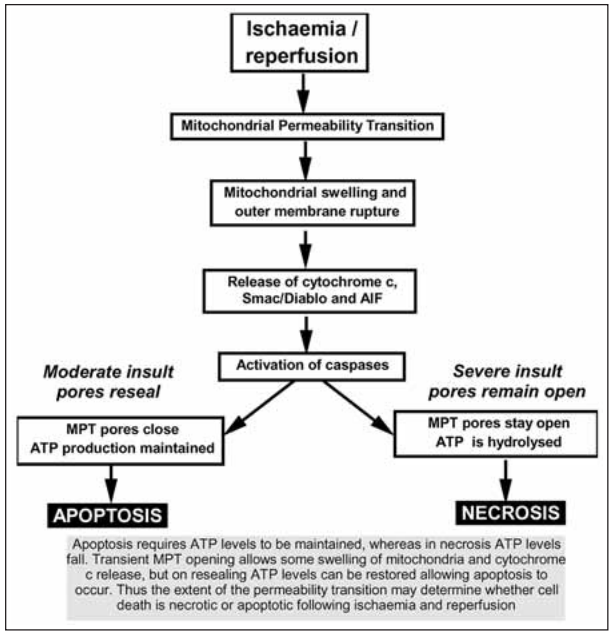

Figure 6. A scheme to illustrate how the extent and permanence of MPTP opening may determine whether heart cells die by apoptosis or necrosis.

oxia can protect cells from damage. Support for this view comes from the observation that during reperfusion of the heart the MPTP opens over the same period of time as the pH_i is restored from less than 6.5 to pre-ischaemic values (> 7.0) [77, 97, 106]. In contrast, acid pH during the ischaemic phase is detrimental, probably because it enhances Na^+/H^+ exchange, thus causing greater loading of the heart with Na^+ and Ca^{2+} [107]. An important protective regime that works in part through decreasing pH_i is the use of inhibitors of the Na^+/H^+ exchanger and these might also be working either through reducing the loading of the heart with Na^+ and Ca^{2+} during the ischaemic phase, or slowing the return of pH_i during reperfusion from its low ischaemic value to normal physiological values which favor pore opening [108–110]. Either mechanism would ultimately inhibit opening of the MPTP. So too would inhibition of mitochondrial calcium uptake. Ruthenium red, which blocks mitochondrial calcium uptake, or calcium antagonists that block plasma membrane Ca channels, do offer protection from mitochondrial Ca-overload and reperfusion injury in the perfused heart [89, 111, 112]. However, these protocols are unlikely to be of clinical use.

Free Radical Scavengers

It is well established that reducing oxidative stress through the use of free radical scavengers offers some protection against reperfusion injury (see [111, 113]). In addition to a direct effect on the MPTP, oxidative stress is known to inhibit plasma membrane ion pumps leading to perturbation of ionic homeostasis and calcium overload. Thus protection by free radical scavengers could involve prevention of MPTP opening through both direct and indirect effects. In most cases no measurements have been made of effects of free radical scavengers on MPTP opening, but we have performed such experiment with pyruvate [97] and the anaesthetic propofol [114], both of which have profound protective effects on the heart that are associated with diminished opening of the MPTP.

Pyruvate

It is well documented that pyruvate can protect hearts against ischaemia/reperfusion anoxia/reoxygenation injury [115–117] as it does other tissues such as gut, liver, kidney and brain [118–121]. This protective effect may in part be through its ability to act as a free radical scavenger, but in addition it is a good respiratory substrate that will support ATP production during reperfusion. In this respect pyruvate is superior to glucose since the latter requires ATP for activation before it can be metabolised to give a net production of ATP. As a good respiratory substrate, pyruvate will also generate a high mitochondrial membrane potential and increased mitochondrial $NADH/NAD^+$ and $NADPH/NADP^+$ ratios. The latter will provide further protection of the ANT from thiol group oxidation, over and above that provided by free radical scavenging properties of pyruvate. This effect, in conjunction with the higher membrane potential, might be expected to reduce opening of the MPTP or enhance resealing. We have confirmed this prediction experimentally using the 3H-DOG technique. When hearts were perfused with 10 mM pyruvate just before ischaemia and the pyruvate maintained during the ischaemic phase and throughout reperfusion, MPTP opening was diminished during the initial stages of reperfusion and allowed more extensive closure during later stages [97]. Indeed, in the presence of pyruvate hearts were found to recover 100 % of their LVDP after 40 min ischaemia, compared to only about 50 % in the absence of pyruvate. This was associated with 3H-DOG entrapment returning to pre-ischaemic values as opposed to a 50 % decrease in 3H-DOG entrapment in controls. These data, illustrated in Figure 5, provide further direct evidence that once opened, the MPTP can close again and allow hearts to recover fully during reperfusion, provided the initial insult is not too great. During the course of these experiments it became apparent that pyruvate also caused a greater accumulation of intracellular lactic acid during ischaemia and slowed the return of pH_i during reperfusion to normal values from the low values of ischaemia. There is also direct evidence from NMR studies that pyruvate causes a decrease in pH_i in a low-flow model of ischaemia [122]. In view of the inhibitory effect of low pH on the MPTP, this might provide yet another mechanism by which pyruvate can protect the heart against reperfusion injury.

Propofol

Propofol is an anaesthetic that is frequently used during cardiac surgery and in post-operative sedation [123]. There are reports that propofol can act as a free radical scavenger [124, 125] and also that at concentrations higher than used clinically, it may exert a direct inhibitory effect on MPTP [126]. In the Langendorff perfused heart propofol has been shown to protect against reperfusion injury [127, 128] and damage caused by hydrogen peroxide-induced oxidative stress [129]. It has been proposed that this protection is a consequence of both the free radical scavenging activity of propofol and its ability to inhibit plasma membrane calcium channels [125, 130]. Since these two effects would lead to a decrease in oxidative stress and cytosolic $[Ca^{2+}]$ it might be expected that Propofol would also reduce opening of the MPTP and we have used the 3H-DOG to confirm this [114]. In the Langendorff perfused rat heart we were able to demonstrate significant protection from reperfusion injury when propofol (11 μM) was added 10 min prior to ischaemia and maintained during reperfusion. This concentration is lower than that employed by others and is more typical of concentrations used in clinical anaesthesia [123]. The recovery of propofol-treated hearts after 30 min of ischaemia was significantly improved with the LVDP expressed as a percentage of the pre-ischaemic value increasing from 36 % in the absence of propofol to 70 % in its presence. This improvement was accompanied by a 25 % decrease in mitochondrial entrapment of preloaded $[^3H]$-DOG. Mitochondria from the propofol-treated hearts were also less sensitive to Ca^{2+} induced pore opening, although when the drug was added to isolated heart mitochondria at the same concentration, no inhibition of MPTP opening was observed [114]. Thus the protective effect of propofol on the MPTP may not be direct, but through other mechanisms such as its free radical scavenging properties causing a decrease in oxidative stress or inhibition of calcium channels leading to a reduction in calcium overload. We have also investigated the cardioprotective effect of propofol on the functional recovery of the working rat heart following cold cardioplegic ischaemic arrest, a model that is closer to the situation experienced in open heart surgery [114]. Both cardiac output and external cardiac work were significantly improved when hearts were perfused with media containing concentrations of propofol similar to those used clinically.

Whatever the exact mechanisms involved, both pyruvate and propofol provide examples of reagents whose protection of the heart from reperfusion injury is accompanied by a decrease in MPTP opening *in vivo*. These data suggest that propofol and pyruvate may be a useful adjunct to the cardioplegic solutions used in cardiac surgery. We are currently using a pig model of warm-blood cardioplegic arrest that closely parallels the clinical setting and preliminary results are very promising with both propofol and pyruvate improving functional recovery of the heart, as well as maintaining higher tissue ATP levels whilst reducing troponin-I release.

Magnesium

Magnesium is well known to protect hearts from ischaemia and reperfusion injury and seems most effective when present at high levels (> 8 mM) during the reperfusion phase [131–134]. It is generally accepted that it exerts its protective effects on the heart by inhibiting L-type calcium channels and the Na^+/Ca^{2+} antiporter, thus decreasing calcium overload [131, 132]. The Na^+/Ca^{2+} antiporter is thought to operate in reverse during ischaemia and the initial phase of reperfusion when intracellular [Na^+] is high, thus acting as a major cause of calcium overload. Any factor that acts to decrease calcium overload is likely to protect against opening of the MPTP, but no direct measurements have yet been made. There are also data to suggest that the presence of supraphysiological [Mg^{2+}] prior to ischaemia exerts an antioxidant effect during reperfusion. This is reflected in a decrease in thiobarbituric acid reactive material and an increase in reduced glutathione concentrations [134]. The cause of this is unclear. It could be a secondary consequence of protecting the mitochondria from opening of the MPTP. Alternatively, there may be an explanation that is independent of the effects of Mg^{2+} on mitochondrial function. However, even if this were the case, the observed decrease in free radical production would protect the mitochondria from subsequent pore opening. Mg^{2+} might also exert a direct inhibitory effect on the MPTP as outlined above, but the available evidence suggests that the protective effect involves extracellular Mg^{2+}, since intracellular [Mg^{2+}] increases very little even at greatly increased [Mg^{2+}] in the perfusion medium [132, 135]. Further relevant discussion on the regulation and role of intracellular [Mg^{2+}] is given in the article by McGuigan et al. in this issue.

Preconditioning

Another protocol to protect hearts (and indeed other tissues) against reperfusion injury is to subject them to brief ischaemic periods with intervening recovery periods before the prolonged period of ischaemia is initiated. This phenomenon, known as ischaemic "preconditioning" (IPC), is associated with two phases of protection; an immediate effect and a "second window" that occurs 24–48 hours later [136–138]. The exact mechanisms involved in preconditioning are still debated but several processes have been implicated. The longer term effects are probably caused by stimulation of the transcription of specific genes, perhaps through a mechanism activated by free radicals and stress-activated protein kinases. The short term effects are thought to reflect release of mediators such as adenosine, bradykinin, endothelin-1, opiods and catecholamines occur during the brief ischaemic periods. These then act on their relevant receptors that are coupled via G proteins to the activation of phospholipase C, causing increased diacylglycerol production and stimulation of protein kinase C (PKC) [136–138]. Thus PKC inhibitors antagonise IPC, whilst adenosine agonists and PKC activators mimic the effect [138]. There is also strong evidence for an involvement of sulphonylurea-sensitive K_{ATP} channels, since K_{ATP} channel openers such as diazoxide can mimic IPC whilst blockers such as glibencamide inhibit [139–142]. Furthermore, PKC-dependent activation of plasma membrane K_{ATP} channels by IPC has been demonstrated [143].

It was originally proposed that opening of the plasma membrane K_{ATP} channel might hyperpolarize the cell and lead to a shorter action potential duration (APD) and calcium loading. However, data obtained with a range of K_{ATP} channel openers showed a poor correlation between their effect on APD and their protective effects [144]. Although this does not preclude these channels operating through other mechanisms (see [145]), in recent years the emphasis has shifted towards a role for the putative mitochondrial K_{ATP} channel in IPC [139, 140, 142, 144]. One reason for this is that diazoxide protects hearts at concentrations much lower than reported to open the plasma membrane K_{ATP} channel but similar to values reported for the mitochondrial K_{ATP} channel. Furthermore, protection was inhibited by 5-hydroxydecanoate (5-HD), supposedly a specific inhibitor of the mitochondrial K_{ATP} channel (but see below). On several counts these data are not totally convincing (see also [139]). First, there are reports that diazoxide can work at much lower concentrations on the plasma membrane K_{ATP} channel when ADP is present (as it will be in the cell) [141]. Second, 5-HD cannot be considered to be a specific mitochondrial K_{ATP} channel inhibitor; indeed the evidence for the existence of a specific mitochondrial K_{ATP} channel that is sulphonylurea and diazoxide sensitive is somewhat limited (see below). Third, opening of a mitochondrial K^+ channel would depolarize mitochondria (inhibiting oxidative phosphorylation) and induce K^+ cycling (effectively increasing ATP demand). Yet published data suggests the opposite to be the case [146] as might be predicted for a protective agent.

Nevertheless, Marban and colleagues have published data demonstrating that diazoxide can oxidize mitochondrial flavoproteins in cardiac myocytes and argue that this does reflect opening of a mitochondrial K_{ATP} channel causing mitochondrial depolarization and stimulation of the respiratory chain [147–150]. However, the interpretation of these data is open to question. First, the effect was observed only after a substantial time lag, which would not be predicted for the mechanism proposed. Second, to obtain the degree of mitochondrial depolarization observed would required extremely large fluxes of K^+ into mitochondria and no studies with isolated mitochondria under physiological conditions have demonstrated this [24, 144]. Third, inhibition of mitochondrial succinate oxidation by diazoxide has been reported [151], which, through inhibition of the citric acid cycle, might provide another explanation of the flavoprotein oxidation observed by Marban and colleagues. Thus the jury is still out on whether mitochondrial or plasma membrane K_{ATP} channels are involved in IPC, or whether both IPC and K_{ATP} channel openers work through other means. To date, there are no published data on the effects of ischaemic preconditioning on the MPTP, although it might be predicted that any protocol that improves the recovery of the heart would be associated with either less initial pore opening or greater subsequent closure. This needs to be tested directly using both pre-ischaemic and post-ischaemic loading with 3H-DOG as described above.

Conclusions

The opening of the MPTP converts the mitochondrion from an organelle that provides ATP to sustain heart function into an instrument of ATP hydrolysis and cell death. The swelling that accompanies pore opening can lead to outer membrane rupture and the release of pro-apoptotic proteins, including cytochrome c that activates the caspase 9/caspase 3 cascade and Smac/Diablo which eliminate the activity of endogenous caspase inhibitors [5–9]. This commits the cell to apoptosis. However, apoptosis is an ATP-dependent process and if the MPTP stays open for prolonged periods in a large percentage of the mitochondria the cell cannot maintain the supply of ATP required for apoptosis, and death will become necrotic. Only if subsequent MPTP closure occurs will ATP levels be maintained, ensuring that cell death continues down an apoptotic rather than a necrotic pathway.

This provides an explanation for the coexistence in injured tissues of both apoptotic and necrotic cells with some cells

showing characteristics of both apoptosis (DNA laddering and caspase activation) and necrosis (gross mitochondrial swelling and ATP depletion). Whatever the final pathway of cell death, pharmacological interventions that can inhibit MPTP opening, or enhance pore closure, are likely to be effective in protecting tissues from damage during insults such as ischaemia/reperfusion. This has clear implications for improving the outcome of open heart surgery and coronary thrombosis. The former requires the coronary flow to be stopped and subsequently restarted, and if the time of ischaemia is prolonged as a result of surgical complications the risk of reperfusion injury is substantial. Similarly, following a coronary thrombosis the blocked arteries must be cleared with clot busters or angioplasty and the window of opportunity for a successful outcome might be prolonged by the prior administration of appropriate inhibitors of the MPTP. Although CsA is effective at reducing reperfusion injury under some circumstances, it has not proved as useful as once hoped, because of its non-mitochondrial targets. However, the search is on for more specific agents and our studies using propofol and pyruvate to protect isolated perfused rat hearts from reperfusion injury demonstrate the potential of this approach. It remains to be seen whether the MPTP plays a primary or secondary role in the protective effects of magnesium.

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