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Mitochondria and reperfusion injury of the heart—A holey death but not beyond salvation

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Abstract The combination of calcium overload and oxidative stress opens a non-specific pore in the inner mitochondrial membrane known as the mitochondrial permeability transition pore (MPTP). This uncouples oxidative phosphorylation and compromises intracellular ATP levels eventually leading to necrotic cell death. In cardiac ischemia and reperfusion, as during treatment of a coronary thrombosis or cardiac surgery, the extent of MPTP opening determines the amount of irreversible damage (infarct size). Furthermore, cardioprotection can be achieved by inhibiting MPTP opening either directly with cyclosporin A analogues, or indirectly by reducing oxidative stress. The detailed molecular mechanism of the MPTP remains uncertain. Knockout studies have confirmed important regulatory roles for cyclophilin-D (CyP-D) and the adenine nucleotide translocase (ANT) but not the voltage dependent anion channel. Our own studies have implicated a calcium-triggered conformational change of the mitochondrial phosphate carrier that is facilitated by CyP-D and modulated by the conformation of the ANT.

Keywords Mitochondrial permeability transition pore · Ischemia · Cardioprotection · Oxidative stress · Calcium

Abbreviations

ANT	adenine nucleotide translocase
BKA	bongkrekic acid
CAT	carboxyatractyloside
CsA	cyclosporin A
CyP	cyclophilin
DOG	2-deoxyglucose
IMM	inner mitochondrial membrane
IP	ischemic preconditioning
MPTP	mitochondrial permeability transition pore
NEM	N-ethylmaleimide
PAO	phenylarsine oxide
PDBR	peripheral benzodiazepine receptor
PiC	mitochondrial phosphate carrier
PPIase	peptidyl-prolyl cis-trans isomerase
ROS	reactive oxygen species
SfA	sanglifehrin A
VDAC	voltage dependent anion channel

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Introduction

In most cells the major role of mitochondria is to provide ATP by oxidative phosphorylation. This involves the generation of a membrane potential and pH gradient, together referred to as the proton motive force (pmf) which requires the permeability barrier of the inner mitochondrial membrane to be maintained. Should this barrier be breached, the resulting uncontrolled proton entry into the mitochondria dissipates the pmf and prevents oxidative phosphorylation. Thus it comes as something of surprise that mammalian mitochondria contain a latent non-specific pore within their inner membrane, known as the mitochondrial permeability transition pore (MPTP). This is normally held in the totally closed state but under some pathological conditions it can open. When this occurs not only does this prevent ATP synthesis but the ATP synthesis machinery actually goes into reverse and catalyses the hydrolysis of ATP produced by glycolysis or "healthy" mitochondria. This results in a rapid

decline in cellular ATP concentrations and unless something is done to reverse this process, the cell will eventually die by necrosis (see Halestrap et al. 1998; Halestrap et al. 2004). Thus opening of the MPTP causes mitochondria to undergo a Jekyll to Hyde like transition that converts them from ATP providers to agents of cell death (Halestrap 2005). This is illustrated schematically in Fig. 1.

In this brief review I will summarise what is known about the mechanism of the MPTP before describing its role in reperfusion injury of the heart, such as occurs during treatment of a coronary thrombosis or in heart surgery, and how inhibiting the MPTP is an effective means of cardioprotection.

General characteristics of the MPTP

It has been known for more than 40 years that when energised mitochondria take up large amounts of calcium in the presence of phosphate they undergo massive swelling and become uncoupled. This is accompanied by a large decrease in light scattering and was originally thought to reflect a non-specific permeabilisation of the mitochondrial inner membrane through activation of Ca-sensitive phospholipases (see Gunter and Pfeiffer 1990). However, it was subsequently demonstrated that this increase in permeability involved the opening of a non-specific pore in the inner membrane that was permeable to any molecule less than 1.5 kDa (Haworth and Hunter 1979; Crompton et al. 1987). The swelling occurs because opening of the MPTP allows small molecular weight solutes to move freely across the inner membrane whilst proteins are left behind and exert a colloidal osmotic pressure (see Bernardi 1999; Halestrap et al. 2004). The primary trigger for opening of the MPTP in isolated mitochondria is an increase in matrix [Ca^{2+}]. Thus, in vitro, the pore can be opened by calcium addition and then rapidly closed again by calcium chelation (Haworth and Hunter 1979; Crompton et al. 1987). However, there are many factors that can change the matrix [Ca^{2+}] required to open the MPTP, and several of these may play a role in mediating MPTP opening during ischemia and reperfusion as discussed further below ("The role of the MPTP in reperfusion injury"). In particular, sensitivity to matrix [Ca^{2+}] is enhanced by oxidative stress, increased [Pi] and membrane depolarisation, whilst desensitisation is induced by increased matrix [ATP], [ADP], [Mg^{2+}] and [H^{+}] (i.e. low pH) (see Halestrap et al. 2002). Indeed MPTP opening can occur at resting matrix [Ca^{2+}] if one of these other factors is changed sufficiently. This is most clearly demonstrated with oxidative stress (Halestrap et al. 1997) which is thought to be especially important in MPTP opening during ischemia / reperfusion of the heart where opening correlates better with changes in oxidative stress than [Ca^{2+}] (Kim et al. 2006; Juhaszova et al. 2008). A detailed list of regulators of the MPTP may be found elsewhere (see Gunter and Pfeiffer 1990; Halestrap 2009).

The molecular mechanism of the MPTP

The facilitating role of cyclophilin D in MPTP opening

A key observation in the elucidation of the molecular mechanism of the MPTP was the demonstration in 1988 by Crompton and colleagues that the immunosuppressant drug cyclosporin A (CsA) inhibits the permeability transition at sub-micromolar concentrations (Crompton et al. 1988). It was known that CsA binds to a small cytosolic protein, cyclophilin A, to induce its immunosuppressant activity through inhibition of the calcium-dependent protein phosphatase, calcineurin (Schreiber and Crabtree 1992). However, we demonstrated that the drug FK506, an immunosuppressant that inhibits calcineurin independently of CyP-A, did not inhibit the MPTP implying that calcineurin was not involved in the calcium-triggering of MPTP opening (Kay et al. 1990). Rather we showed that CsA acts by inhibiting the activity of a matrix peptidyl-prolyl cis-trans isomerase (PPIase) that we subsequently identified as a mitochondrial isoform of CyP-A cyclophilin D (CyP-D) (Halestrap and

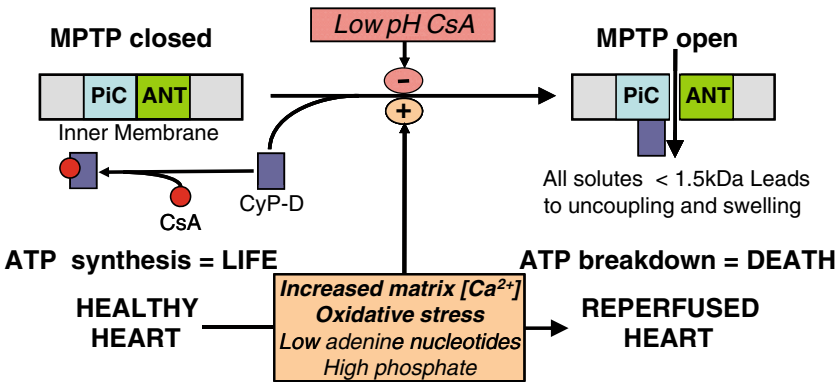

Fig. 1 Scheme to illustrate the nature of the mitochondrial permeability transition pore and the consequences of its opening

Davidson 1990; Connern and Halestrap 1992). CyP-D is encoded by the nuclear gene *PPIF* and is synthesised with a targeting sequence that is cleaved following translocation into the matrix (Johnson et al. 1999). A range of CsA analogues, including non-immunosuppressant analogues such as 6-methyl-ala-CsA, 4-methyl-val-CsA, N-methyl-4-isoleucine-CsA (NIM811) and D-3-MeAla-4-EtVal-CsA (Debio-025) also inhibit MPTP opening with a potency that matches their inhibition of the PPIase activity of CyP-D (Griffiths and Halestrap 1991; Griffiths and Halestrap 1995; Waldmeier et al. 2002), and this is also the case for the unrelated MPTP inhibitor, sanglifehrin A (SfA) (Clarke et al. 2002). Confirmation of the critical role of CyP-D in MPTP opening came when it was shown that MPTP opening in liver mitochondria isolated from CyP-D knockout mice required much higher calcium loading than did those from wild-type mice but was insensitive to CsA (Basso et al. 2005; Nakagawa et al. 2005; Baines et al. 2005). It is important to note that the absence of CyP-D or the presence of CsA and SfA only desensitises the MPTP to [Ca^{2+}] and does not totally abolish MPTP opening if the stimulus is large enough (Halestrap et al. 1997; Clarke et al. 2002; Basso et al. 2005).

The pore forming component(s) of the MPTP

The data summarised above confirm that MPTP opening is likely to involve the PPIase activity of CyP-D facilitating a calcium-triggered conformational change of an integral membrane protein. More controversial is the identity of this protein. Indeed, it has even been suggested that there may not be a unique protein involved but that the pore may be formed by aggregation of different mis-folded integral membrane proteins that have been damaged by oxidant and other stresses (He and Lemasters 2002). However, this model does not readily explain many of the defined characteristics of the MPTP (see Halestrap 2009).

The adenine nucleotide translocase In 1990 we proposed that the adenine nucleotide translocase (ANT) underwent a conformational change to form the MPTP (Halestrap and Davidson 1990) and there is a considerable body of evidence to support such a role which is reviewed in detail elsewhere (see Halestrap and Brenner 2003). In outline, several laboratories including our own, have shown that MPTP opening is enhanced by adenine nucleotide depletion and inhibited by addition of ATP, ADP and their deoxyribose analogues, but not by AMP, GTP or GDP that do not bind to the ANT. Adenine nucleotides were shown to reduce the sensitivity of MPTP opening to [Ca^{2+}] and this effect is overcome by oxidative stress which was found to cross-link two matrix facing thiol groups on the ANT and prevent adenine nucleotide binding (Halestrap et al. 1997; McStay et al. 2002). MPTP opening is also modulated by other specific ligands of the ANT. Thus carboxyatractyloside (CAT) which induces the 'c' conformation of the ANT activates MPTP opening and attenuates the inhibition by adenine nucleotides whilst bongkrekic acid (BKA) induces the 'm' conformation of the ANT and inhibits pore opening (Halestrap et al. 1997). Furthermore, CyP-D was shown to bind to the ANT and this binding was increased by oxidative stress and prevented by CsA but not its inactive analogue, CsH (Woodfield et al. 1998).

We have proposed that the inhibition of MPTP opening by high membrane potential (Bernardi 1992) may also involve the ANT which undergoes a potential-dependent conformational change during the electrogenic translocation cycle (Halestrap et al. 1997). In contrast, we suggest that the inhibitory effect of low pH on MPTP opening is probably mediated by protons competing for Ca^{2+} at the calcium trigger site (Halestrap 1991). Although the identity of this site has not been established, there are several glutamate and aspartate residues on the inner surface of the ANT that might be involved (Halestrap and Brenner 2003). However, the published three dimensional structure of the ANT in its CAT-bound form does not suggest an obvious calcium binding site (Pebay-Peyroula et al. 2003). What does emerge from this structure is that the ANT has a large channel on the cytosolic surface that penetrates deep into the membrane and is blocked by a constriction provided by three helices. If these helices were to re-arrange through a calcium-induced conformational change a pore could be formed. In this context, Brustovetsky and Klingenberg have used electrophysiological techniques to demonstrate that the reconstituted bovine ANT can produce a non-specific channel at very high (mM) [Ca^{2+}] (Brustovetsky and Klingenberg 1996). In subsequent studies they showed that a similar calcium-activated pore could be formed by reconstituted ANT from *Neurospora crassa* and that a cyclophilin from *N crassa* modulated the voltage gating of this pore. Furthermore, the opening probability of these channels was increased by oxidative stress (Brustovetsky et al. 2002). However, there is no evidence that intact *N crassa* mitochondria undergo a calcium-dependent CsA-inhibitable permeability transition and, if they behave like *Saccharomyces cerevisiae* mitochondria, it is unlikely that they do (Manon et al. 1998).

Despite the evidence summarised above it is now know that the ANT cannot be essential for the formation of a CsA-inhibitable MPTP since mouse liver mitochondria in which ANT1 and ANT2 have been genetically ablated can still exhibit a CsA-sensitive permeability transition (Kokoszka et al. 2004). However, the sensitivity of the MPTP to [Ca^{2+}] in these mitochondria was reduced, and ADP, CAT and BKA no longer modulated MPTP opening. These data could be interpreted in two ways. First, it is possible that the ANT never forms the transmembrane

component of the MPTP but rather plays a purely regulatory role. Second, it might be that in the absence of the ANT other less well expressed members of the mitochondrial carrier family, which share a common structure with the ANT (Palmieri 2004), can take its place. Which ever of these explanations is correct our own recent studies have implicated the mitochondrial phosphate carrier (PiC) in MPTP formation (Leung et al. 2008).

The mitochondrial phosphate carrier We have demonstrated that oxidative stress and the vicinal thiol reagent phenylarsine oxide can activate MPTP opening even in the present of CAT which prevents the cross-linking of matrix thiol groups on the ANT and inhibits the binding of the ANT to a phenylarsine oxide (PAO) affinity column. These data imply that PAO must have an additional activating site independent of the ANT and led us to analyse what additional membrane proteins from CAT-treated mitochondria bound to the PAO column. Four such proteins were identified by mass spectrometry, one of which was the PiC (Leung et al. 2008) and its binding was prevented by pre-treatment of the mitochondria with the ubiquinone analogues Qo and Ro 68-3400 that had previously been identified as potent MPTP inhibitors (Walter et al. 2002). Furthermore, pre-treatment of mitochondria with these inhibitors was shown to inhibit mitochondrial phosphate transport and also prevent PAO activation of MPTP opening. These data all pointed towards the PiC being an important component of the MPTP and further evidence for this was obtained using co-immunoprecipitation and GST-CyP-D pull-down experiments that demonstrated binding of CyP-D to the PiC. This binding was prevented by CsA but was increased by oxidative stress and to a lesser extent by CAT (Leung et al. 2008). The recent report that phosphate is required for inhibition of MPTP opening by CsA or CyP-D knockdown (Basso et al. 2008) is also consistent with a critical role of the PiC in the formation of the MPTP. So too is the observation that knockdown of the PiC in HeLa cells reduces their sensitivity to apoptosis induced by staurosporine (Alcala et al. 2008), a process which involves opening of the MPTP (Tafani et al. 2001). Conversely, PiC over-expression can induce apoptosis (Alcala et al. 2008).

Other proteins Several other proteins have been proposed to be either structural or regulatory components of the MPTP. These include the voltage dependent anion channel (VDAC, also known as porin), members of the Bcl-2 family and the peripheral benzodiazepine receptor (PDBR). Although these proteins are in the outer mitochondrial membrane whilst the MPTP is associated with the inner membrane, it has been suggested that interactions between inner and outer membranes at the "contact sites" may be important (Crompton 2000; Brdiczka et al. 2006). Initially the evidence appeared strongest for an important role of VDAC. Thus Crompton and colleagues demonstrated that VDAC present in detergent solubilised heart mitochondria bound to GST-CyP-D together with the ANT (Crompton et al. 1998), although our own data did not confirm this (Woodfield et al. 1998). Furthermore, it was reported that ubiquinone analogues such as UQ_0 and Ro 68-3400 inhibit MPTP opening by binding to VDAC1 (Cesura et al. 2003). However, this was later disproved since identical results were obtained in mitochondria lacking VDAC1 (Krauskopf et al. 2006), and mitochondria lacking all isoforms of VDAC still exhibit normal pore opening (Baines et al. 2007).

Reconciling the data with a molecular model

Although the exact molecular mechanism of the MPTP remains to be elucidated, we have proposed a working model that we believe to be consistent with the data. This has been discussed in detail elsewhere (Leung and Halestrap 2008; Halestrap 2009) and is only summarised here. We suggest that the ANT and PiC may interact in the inner mitochondrial membrane for which there is some direct evidence within the 'ATP synthasome' (Ko et al. 2003; Chen et al. 2004). This interaction may be greater when the ANT is in the 'c' conformation and when either or both the ANT and PiC have been modified by oxidative stress. An increase in matrix $[Ca^{2+}]$ will trigger a conformational change in one or both of the ANT and PiC that is facilitated by the PPIase activity of CyP-D. This may produce a pore by wedging open the translocation gate in a single ANT or PiC monomer or by creating a channel between the subunits of PiC/ANT heterodimer. The conformation changes responsible for MPTP opening may be enhanced by Pi binding to the PiC but inhibited by adenine nucleotides binding to the ANT. The latter effect is prevented by oxidative stress which also enhances CyP-D binding. CsA inhibits the MPTP by preventing CyP-D binding to the PiC/ANT complex, whilst the inhibition by SfA can occur without dissociation of CyP-D (Halestrap et al. 2002). Oxidative stress and PAO may activate MPTP opening by inhibiting adenine nucleotide binding to the ANT and by enhancing CyP-D binding to the PiC. By contrast, ubiquinone analogues and NEM may inhibit MPTP opening by enhancing the 'm' conformation of the ANT and by binding to the PiC to inhibit its pore-forming conformational change. Of course, it remains entirely possible that another key component of the MPTP remains to be identified and our model must be regarded as no more than a reasonable speculation that informs future experiments. Only when appropriate knockdown and experiments have been performed and a functional MPTP is generated out of purified, reconstituted proteins will the true identity of the MPTP be known.

The role of the MPTP in reperfusion injury

During a myocardial infarction or cardiac surgery the heart, or portions of it, may become totally deprived of blood (ischemia). Subsequent restoration of the blood flow, which is essential to salvage the heart, may exacerbate the damage that has occurred during the ischemic phase and this phenomenon is known as reperfusion injury. The conditions that occur during reperfusion are exactly those that favour MPTP opening and there is now a large body of evidence that implicates MPTP opening as the critical event in mediating this damage as will be summarised below and is represented schematically in Fig. 2. The reader is directed elsewhere for a more detailed account (see (Halestrap et al. 2004; DiLisa and Bernardi 2006; Yellon and Hausenloy 2007; Halestrap and Pasdois 2009)).

During the ischemic period there is a profound drop in ATP/ADP ratio and an increase in [Pi] that stimulates glycolysis causing a build up of lactic acid and a drop in intracellular pH (pH_i). This activates the Na^+/H^+ antiporter loading the cell with sodium which cannot be pumped out by the Na^+/K^+ ATPase because of the low ATP levels. This leads to reversal of the Na^+/Ca^{2+} antiporter which loads the cell (including the mitochondria) with calcium. Upon reperfusion the replenished oxygen supply together with a highly reduced respiratory chain leads to the formation of reactive oxygen species (ROS—comprising superoxide, hydrogen peroxide and hydroxyl radical). Additional ROS may come from other sources such as NADPH oxidase and xanthine oxidase. Although conditions might appear optimal for MPTP opening, the low pH_i should prevents this until the loss of lactic acid and the operation of pH regulatory pumps enables the pH to return to normal. At this point it would be predicted that the MPTP would open and experimental data confirms this.

Confirming that MPTP opening occurs during reperfusion of the ischemic heart

In order to demonstrate MPTP opening within the intact perfused heart we developed the "Hot-DOG" technique in which the heart is perfused with tracer [^{3}H]-2-deoxyglucose (DOG) that is metabolised to DOG-6-phosphate (DOG-6P) (Griffiths and Halestrap 1995). [^{3}H]-DOG-6P remains in the cytosol unless the MPTP opens at which point it can enter the mitochondria. Mitochondria are then rapidly isolated in the presence of EGTA to close the MPTP and entrap the [^{3}H]-DOG-6P in the matrix. Measurement of the matrix [^{3}H] content relative to the citrate synthase activity enables the extent of pore opening to be determined (Griffiths and

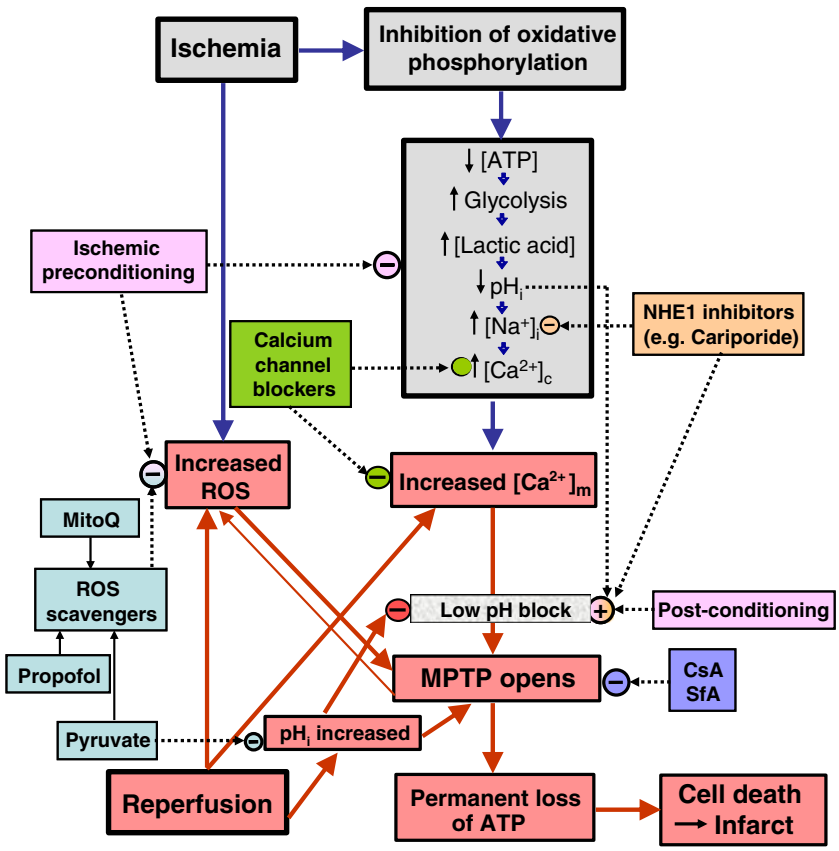

Fig. 2 Scheme to illustrate the involvement of MPTP opening in reperfusion injury and how various cardioprotective agents may prevent this. *Blue arrows* represent changes that occur primarily in ischemia, *red arrows* depict changes that occur primarily during reperfusion whilst *dotted black arrows* indicate the locus of action of cardioprotective agents

Halestrap 1995). Using this method we confirmed that MPTP opening is not detected during ischemia but does occur after 2–3 min reperfusion when the pH has returned to normal (see (Halestrap et al. 2004)). However, this technique has two drawbacks. First, should mitochondria swell to the point of rupturing their inner membrane any entrapped 2-DOG-6P will be released. This can be largely corrected for since such mitochondria will also release most of their citrate synthase and recovery of mitochondrial citrate synthase is measured. The second problem is that if the MPTP opens and then subsequently closes the 2-DOG-6P will remain entrapped even though the mitochondria are resealed and competent for oxidative phosphorylation. To determine how many mitochondria undergo such resealing we slightly modified the "Hot DOG" technique by loading hearts with [^{3}H]-DOG following a period of ischemia and reperfusion (i.e. "post-loading" as opposed to "pre-loading"). The mitochondrial [^{3}H]-DOG content now provides an estimate of how many mitochondria remain "open" as hearts recover during reperfusion. By comparison of this value with that obtained with DOG pre-loading we were able to assess the percentage of those mitochondria that were "open" at the start of reperfusion that subsequently "close". This was found to be about 50% and the hemodynamic recovery of the heart correlated with this value (Kerr et al. 1999).

Further confirmation that MPTP opening plays a critical role in reperfusion injury was provided by the ability of SfA and CsA analogues to protect the heart from reperfusion injury the heart (Griffiths and Halestrap 1993; Clarke et al. 2002; Hausenloy et al. 2003) and similar protection was seen in hearts from CyP-D knockout mice (Nakagawa et al. 2005; Baines et al. 2005). These data imply that inhibition of MPTP opening is a prime target for protecting the heart from reperfusion injury and this is indeed the case.

The MPTP as a target for cardioprotection

Inhibition of MPTP opening is now known to be a critical target for cardioprotection and a wide variety of cardioprotective protocols have now been shown to reduce MPTP opening during reperfusion. These include drugs that directly inhibit the MPTP such as CsA and those that act indirectly through decreasing oxidative stress, calcium overload or pH_i during reperfusion. Below I give a brief account of those cardioprotective strategies for which strong evidence for MPTP inhibition is available. For a fuller account the reader is directed elsewhere (see Halestrap et al. 2004; Halestrap and Pasdois 2009).

Targeting Cyclophilin D Many studies have now confirmed our original observation that cardioprotection can be afforded by inhibiting the PPIase activity of CyP-D with CsA or SfA (Griffiths and Halestrap 1993; Clarke et al. 2002) and these studies have been extended to include non-immunosuppressive analogues of CsA such as 6-MeAla-CsA, 4-methyl-val-CsA, *N*-methyl-4-isoleucine-CsA (NIM811) and D-3-MeAla-4-EtVal-CsA (Debio-025) (Griffiths and Halestrap 1995; Hausenloy et al. 2003; Argaud et al. 2005a). Importantly, CsA and SfA were shown to reduce the infarct size of isolated rat hearts in which a coronary artery is occluded and then re-opened to mimic the clinical treatment of a coronary thrombosis (Hausenloy et al. 2002; Hausenloy et al. 2003). These studies were later extended to an *in vivo* mouse model that allowed 24 h and 30 days reperfusion following 25 min regional ischemia. Here Debio-025 given at reperfusion was found to give substantial protection (Gomez et al. 2007). There is also some evidence for a role of the MPTP in the development of decompensated heart failure associated with cardiac hypertrophy since, in a mouse model of this disease state, mice in which CyP-D had been genetically ablated showed significant protection (Nakayama et al. 2007).

Targeting intracellular pH and calcium overload Low pH (< 7.0) is a potent inhibitor of MPTP opening (Halestrap 1991) and inhibition of the Na^+/H^+ exchanger with amiloride derivatives such as cariporide is known to protect the heart from reperfusion injury. Not only do these drugs decrease pH_i during reperfusion but they also reduce sodium and thus calcium overload during ischemia which may add to their cardioprotective effect (Avkiran et al. 2001; Mentzer et al. 2003). The "hot-DOG" technique has confirmed that the protection offered by cariporide is accompanied by inhibition of MPTP opening *in situ* (Porcelli et al. 2009). Reducing cytosolic and mitochondrial calcium overload with verapamil and ruthenium red, antagonists of plasma membrane or mitochondrial calcium channels respectively, is also cardioprotective (see Halestrap and Pasdois 2009).

Targeting oxidative stress Antioxidants are also known to be cardioprotective (see Halestrap et al. 2004) and mitochondrial-targeted ubiquinone antioxidants are especially effective in this regard (Adlam et al. 2005). The anaesthetic propofol is also known to be a good antioxidant that leads to less MPTP opening during reperfusion and provides effective protection from ischemia reperfusion injury in the rat heart under both Langendorff and working heart perfusion protocols (Javadov et al. 2000). Furthermore, propofol has been shown to be protective an *in vivo* pig model of cardiopulmonary bypass with warm blood cardioplegia that closely matches current clinical practice (Lim et al. 2005). Pyruvate has been shown to be an especially potent cardioprotective agent which reduces MPTP opening during reperfusion. In Langendorff-perfused rat hearts the presence of 10 mM pyruvate during

30 min ischemia followed by reperfusion enables almost complete hemodynamic recovery of rat hearts and this is accompanied by a 50% reduction in initial MPTP opening and almost total resealing as reperfusion progresses (Kerr et al. 1999). It may be especially effective because not only does it act as a free radical scavenger but it also maintains a low intracellular pH and is an excellent respiratory substrate to replenish ATP during reperfusion (Kerr et al. 1999). Reduction in oxidative stress also appears to be critical in the inhibition of MPTP opening that accompanies cardioprotection induced by ischemic preconditioning (Javadov et al. 2003), temperature preconditioning (Khaliulin et al. 2007) and urocortin (Townsend et al. 2007) which are discussed further below.

Preconditioning and post-conditioning Preconditioning refers to a treatment applied to the heart before ischemia that offers protection during reperfusion. Ischemic preconditioning (IP) is one of the most effective protective regimes and involves exposing the heart to several short ischemic episodes, followed by recovery, before the prolonged ischemia. There are two phases of protection; an immediate effect and a "second window" that occurs 24–48 h later (Yellon and Downey 2003). The latter 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, perhaps through a mechanism activated by free radicals and stress-activated protein kinases (Yellon and Downey 2003; Das and Maulik 2006). Preconditioning can also be induced pharmacologically by a wide range of agents (see (Yellon and Downey 2003)) and by temperature preconditioning (TP) in which hearts are subject to several brief 2 min cycles of 26°C hypothermia, much like the brief ischemic episode of IP (Khaliulin et al. 2007). Another cardioprotective regime is known as "post-conditioning" in which the heart is subject to very brief (10 s) intermittent ischemic periods during the first few minutes of reperfusion (VintenJohansen et al. 2005). The exact mechanisms involved in preconditioning and post-conditioning are still hotly debated but in both cases there is strong evidence that MPTP inhibition is involved (see Lim et al. 2007; Halestrap et al. 2007). We have used the "Hot-DOG" technique to demonstrate directly that IP not only reduces the opening of the MPTP during the early phase of reperfusion but also increased subsequent pore closure (Javadov et al. 2003). Several other studies have shown that mitochondria isolated from IP, TP and post-conditioned hearts following reperfusion have a reduced sensitivity to calcium-induced MPTP opening compared to those from control hearts subject to the same ischemia and reperfusion (Javadov et al. 2003; Argaud et al. 2004; Argaud et al. 2005b; Khaliulin et al. 2004b; Khaliulin et al. 2007; Clarke et al. 2008).

Although there is general consensus that the protection provided by all these "conditioning" protocols involves inhibition of MPTP opening as the end effector, the mechanisms responsible for this inhibition remain unclear. We and others have shown that preconditioning by IP (Clarke et al. 2008), TP (Khaliulin et al. 2007), urocortin (Townsend et al. 2007) and apomorphine (Khaliulin et al. 2004a) all lead to less oxidative stress developing during ischemia and reperfusion. The mitochondria isolated from these hearts show less protein carbonylation (a surrogate marker of oxidative stress) and are less sensitive to calcium-induced MPTP opening. Interestingly, when mitochondria were isolated immediately after the preconditioning protocol but before ischemia, they showed no differences in the sensitivity of MPTP opening to $[Ca^{2+}]$. This implies that the IP signalling pathway does not act directly on the MPTP through a mechanism such as phosphorylation of MPTP components, but rather develops during the ischemic period perhaps by decreasing the oxidative stress experienced by the heart (Halestrap et al. 2007).

The signalling pathways involved in mediating post-conditioning and the numerous pre-conditioning stimuli, and how these interact to bring about inhibition MPTP opening, remain uncertain and the reader is referred elsewhere for a detailed review of this literature (see Halestrap et al. 2007; Hausenloy and Yellon 2007; Downey et al. 2007; Murphy and Steenbergen 2008). However, there is general agreement that activation of protein kinase Cε (PKCε) by ROS and/or other factors (e.g. adenosine, bradykinin, noradrenaline) released during the preconditioning protocol is important as may be nitric oxide and protein kinase G activation. The Akt / glycogen synthase kinase 3β (GSK3β) pathway appears to be involved, especially during reperfusion, whilst the role of mitochondrial ATP-dependent and calcium activated potassium channels remains controversial (see Garlid et al. 2003; Hanley and Daut 2005; Ardehali and O'Rourke 2005; Halestrap et al. 2007). Our own data, reviewed in (Halestrap et al. 2007), can be summarised as follows. We find no evidence that the IP-mediated inhibition of MPTP opening at reperfusion involves translocation of protein kinases to the mitochondria or direct phosphorylation of mitochondrial proteins. Rather we propose that the unifying factor in preconditioning is a reduction in the oxidative stress experienced by mitochondria during prolonged ischemia and reperfusion. This causes less oxidation of the critical thiol groups on the MPTP responsible for sensitising MPTP opening to $[Ca^{2+}]$ (Halestrap et al. 1997; McStay et al. 2002). We suggest that this protection occurs in two phases. First, there is less oxidative stress during ischemia causing less MPTP opening during the initial phase of reperfusion. Second, protection continues during reperfusion by the prevention of a cascade of MPTP-

induced ROS production (Zorov et al. 2006) followed by further MPTP opening. This latter phase of protection may involve an increase in ROS removal or reduction in mitochondrial ROS production caused by Akt-mediated GSK3-β inhibition. In this context it is significant that pharmacological inhibition of GSK3-β during reperfusion has been shown to be cardioprotective (Gomez et al. 2008)

Conclusion

It is now clear that the MPTP plays a critical role in reperfusion injury of the heart and that pharmacological inhibition of MPTP opening provides a promising target for protecting the heart following a coronary thrombosis or cardiac surgery. Indeed the first small-scale clinical trials have confirmed that CsA treatment of patients undergoing percutaneous coronary intervention (PCI) for the treatment of coronary thrombosis show improved recovery (Piot et al. 2008). However, drugs that target CyP-D are not ideal because MPTP opening can still occur if the stimulus is increased. In addition, cyclophilins have other roles in the cell and their inhibition might have adverse effects (see Halestrap and Pasdois 2009). Drugs that target another component of the MPTP without interfering with the normal function of the mitochondria would be ideal, but achieving this is hampered by our uncertainty over the molecular identity of the MPTP. If the ANT and the PiC are the only components, other than CyP-D, then successful development of such a drug would seem unlikely. However, if there are additional components that remain to be identified prospects might be better. If direct inhibition of the MPTP proves difficult then targeting those factors that cause the MPTP to open may be the best strategy. Since our own data suggest that preconditioning protocols protect by reducing mitochondrial oxidative stress (Halestrap and Pasdois 2009) mitochondrial-targeted ROS scavengers (see Murphy and Smith 2007) look especially promising for clinical use. Indeed, it is already known that these are very effective at protecting the perfused rat heart against reperfusion injury (Adlam et al. 2005).

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