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Review

The role of the mitochondrial permeability transition pore in heart disease

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ABSTRACT

Like Dr. Jeckyll and Mr. Hyde, mitochondria possess two distinct persona. Under normal physiological conditions they synthesise ATP to meet the energy needs of the beating heart. Here calcium acts as a signal to balance the rate of ATP production with ATP demand. However, when the heart is overloaded with calcium, especially when this is accompanied by oxidative stress, mitochondria embrace their darker side, and induce necrotic cell death of the myocytes. This happens acutely in reperfusion injury and chronically in congestive heart failure. Here calcium overload, adenine nucleotide depletion and oxidative stress combine forces to induce the opening of a non-specific pore in the mitochondrial membrane, known as the mitochondrial permeability transition pore (mPTP). The molecular nature of the mPTP remains controversial but current evidence implicates a matrix protein, cyclophilin-D (CyP-D) and two inner membrane proteins, the adenine nucleotide translocase (ANT) and the phosphate carrier (PiC). Inhibition of mPTP opening can be achieved with inhibitors of each component, but targeting CyP-D with cyclosporin A (CsA) and its non-immunosuppressive analogues is the best described. In animal models, inhibition of mPTP opening by either CsA or genetic ablation of CyP-D provides strong protection from both reperfusion injury and congestive heart failure. This confirms the mPTP as a promising drug target in human cardiovascular disease. Indeed, the first clinical trials have shown CsA treatment improves recovery after treatment of a coronary thrombosis with angioplasty.

1. Introduction

In the healthy heart changes in mitochondrial calcium concentrations ($[Ca^{2+}]_m$) play a critical role in regulating oxidative phosphorylation and enable the supply of ATP to match ATP demand in response to increased work load. This involves the operation of specific calcium transport pathways [1,2] that relay the beat by beat increases in cytosolic $[Ca^{2+}]$ into the mitochondrial matrix [3,4], where they activate mitochondrial dehydrogenases [5–7] to provide an increased supply of NADH to fuel the respiratory chain. When the heart works harder, the greater frequency and magnitude of the calcium transients produces greater activation of the dehydrogenases thus stimulating ATP production [8]. Additional Ca^{2+}-dependent mechanisms may also be involved including stimulation of the proton translocating ATPase and activation of the respiratory chain by increases in matrix volume [9–11]. Taken together, these mechanisms provide an explanation of how the ATP and creatine phosphate concentrations of the heart are maintained as workload increases [8,11]. However, there are pathological conditions in which cytosolic $[Ca^{2+}]$ becomes excessively high. This can lead to mitochondrial calcium overload with an increased risk of damage to the mitochondria through the opening of the mitochondrial permeability transition pore (mPTP).

In this review we will first introduce the mPTP, describing the consequences of its opening and what is known of its molecular mechanism and regulation (Section 2 'The mitochondrial permeability transition pore'). In Section 3 'mPTP opening is a critical factor in reperfusion injury and heart failure' we will describe how calcium overload and oxidative stress occur during ischaemia (loss of blood flow) and subsequent reperfusion, and how this can lead to mPTP opening and damage to the heart (reperfusion injury). This has major clinical implications in cardiac surgery or following a coronary thrombosis (a blood clot in the coronary arteries supplying the heart with blood that leads to a heart attack) and subsequent treatment (reperfusion). In Section 4 'Inhibition of the mPTP protects hearts against ischaemia reperfusion injury' we review how inhibition of the mPTP can protect the heart from such reperfusion injury and thus offers an effective therapeutic strategy for cardioprotection. Finally, in Section 5 'Heart failure in cardiac hypertrophy may be ameliorated by inhibition of the mPTP' we describe how dysregulation of calcium

Abbreviations: ANT, Adenine nucleotide translocase; BKA, bongkrekic acid; CAT, carboxyatractyloside; CsA, cyclosporin A; CyP, cyclophilin; GSK3, glycogen synthase kinase 3; IP, ischaemic pre-conditioning; $mitoK_{ATP}$, mitochondrial ATP-dependent potassium channels; mPTP, mitochondrial permeability transition pore; PAO, phenylarsine oxide; PiC, mitochondrial phosphate carrier; PKC, protein kinase C; PPIase, peptidyl-prolyl *cis–trans* isomerase; ROS, reactive oxygen species; SfA, sanglifehrin A; VDAC, voltage activated anion channel

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handling occurs in congestive heart failure leading to cardiac hypertrophy (enlarged heart) that can develop into chronic hypertension (high blood pressure). The mPTP has also been implicated in the development of this disease and here too its inhibition may offer a promising approach to reducing disease progression.

2. The mitochondrial permeability transition pore

The mPTP is a non-selective pore, permeable to any molecule of less than 1.5 kDa, that opens in the mitochondrial inner membrane under conditions of very high (pathological) $[Ca^{2+}]_m$. However, an increase in $[Ca^{2+}]_m$ alone is relatively ineffective at triggering pore opening, but the sensitivity to $[Ca^{2+}]_m$ can be greatly enhanced by adenine nucleotide depletion, high [Pi] and most importantly, oxidative stress [12–15]. Such conditions prevail during ischaemia and reperfusion and initiate mPTP opening under such conditions as will be described below (Section 3 'mPTP opening is a critical factor in reperfusion injury and heart failure'). There are two consequences of mPTP opening that can play an important role in cell death. The first of these is uncoupling which occurs because the increased permeability to protons leads to dissipation of the two components of the proton motive force (pmf), the pH gradient and the membrane potential. In the absence of a pmf, mitochondria cannot synthesise ATP via oxidative phosphorylation. Indeed the ATPase goes into reverse and starts to breakdown the ATP produced by glycolysis and any remaining competent mitochondria. As a result, myocytes in which a significant number of mitochondria have undergone the mPTP cannot maintain their ATP levels and the resulting disruption of metabolism and ionic homeostasis leads to necrotic cell death [13,14,16].

The second consequence of mPTP opening, mitochondrial swelling, occurs because the increased permeability of the inner mitochondrial membrane to small molecules causes equilibration of low molecular weight osmolytes whilst retaining proteins in their respective compartments. Since the matrix protein concentration is very high, it exerts a colloidal osmotic pressure leading to swelling of the matrix compartment. Swelling can occur without compromising the integrity of inner membrane because the cristae unfold, but as the matrix expands it exerts pressure on the outer membrane that eventually ruptures. This will cause release of cytochrome *c* and other pro-apoptotic proteins and has the potential to lead to apoptotic cell death [17–21]. However, only if the pores close again sufficiently to maintain ATP levels will apoptotic death predominate over necrotic cell death [13]. Fig. 1 provides an overview of the consequences of mPTP opening and key features of the proposed molecular mechanism described in Section 2.1 'The molecular composition of the mPTP' below.

2.1. The molecular composition of the mPTP

The phenomenon of the permeability transition was first recognised more than 40 years ago as the massive swelling of mitochondria that accompanies calcium overload. It was originally thought to represent a non-specific permeabilisation of the mitochondrial inner membrane through activation of Ca-sensitive phospholipases [22–24]. However, pioneering studies by Haworth and Hunter [25,26] and later by Crompton et al. [27] demonstrated that the pore was likely to be a unique molecular identity with a molecular cut-off of about 1.5 kDa. Its opening is triggered by calcium and can be rapidly closed again by calcium chelation. Work from this laboratory and that of many others have sought to elucidate the molecular mechanism of the mPTP although its exact composition remains uncertain [12,14,15,28]. However, the most recent data provides the strongest evidence for an involvement of three proteins: a mitochondrial peptidyl-prolyl *cis–trans* isomerase known as cyclophilin-D, the adenine nucleotide translocase (ANT) and the mitochondrial phosphate carrier (PiC) [28]. Since these components act as potential targets for mPTP and hence cardioprotection, evidence for their involvement will be summarised briefly.

2.1.1. A role for cyclophilin-D (CyP-D)

In 1988 Crompton et al. reported that cyclosporin A acts as a potent inhibitor of mPTP opening [29]. Subsequent work in this laboratory demonstrated that inhibition of pore opening by CsA and its analogues was mediated by inhibition of a matrix peptidyl-prolyl *cis–trans* isomerase (PPIase) that was later purified and showed to be cyclophilin-D (CyP-D) [30–32]. The potency of a range of CsA analogues as inhibitors of the mPTP matched their potency as inhibitors of the PPIase activity of CyP-D [33] and a similar correlation was found with an unrelated drug, sanglifehrin A (SfA) [34]. CyP-D is an 18 kDa matrix protein that is encoded by the nuclear gene *PPIF* and is synthesised with a targeting sequence that is cleaved following translocation into the matrix [31,35]. Final proof of its role in mPTP opening was provided recently using CyP-D knockout mice. Mitochondria from these mice were found to be highly resistant to calcium-induced mPTP opening and behaved the same as mitochondria from wild-type mice treated with CsA [36–39]. The inhibitory effect of CsA and SfA on mPTP opening is associated with a reduction in the calcium sensitivity of pore opening rather than a total blockade. Thus, in the presence of CsA or SfA [34,40], or in mitochondria from CyP-D knockout mice [36], pore opening can still be induced at high calcium loading. These data imply that mPTP opening involves a conformational change in a membrane protein that is triggered by calcium and facilitated by (rather than totally dependent on) CyP-D.

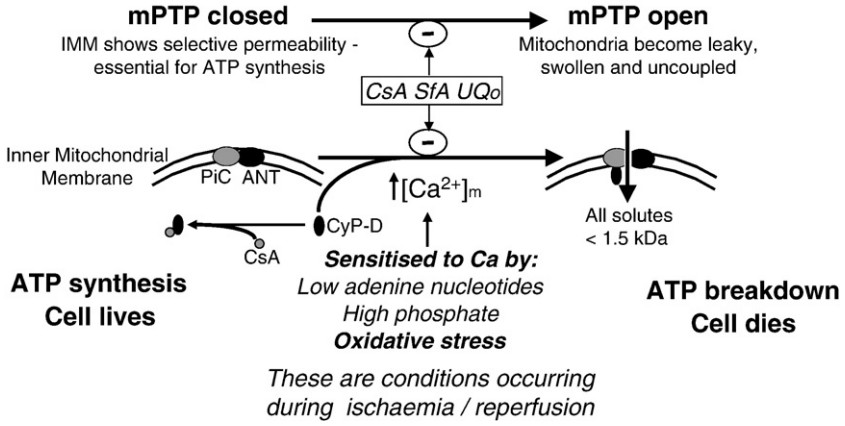

Fig. 1. An overview of the proposed mechanism of the mitochondrial permeability transition pore and the consequences of its opening. Further details are given in Section 2 'The mitochondrial permeability transition pore' of the text.

The identity of this membrane protein will be considered below, but it should be noted that He and Lemasters [41] have suggested that there may not be a single unique membrane protein involved. Rather, they propose that the mPTP forms as a result of the aggregation of misfolded integral membrane proteins that have been damaged by oxidant and other stresses. In their model, CyP-D is suggested to block conductance through these protein aggregates, but when protein clusters exceed the CyP-D available, unregulated pore opening occurs that is stimulated by calcium and inhibited by CsA binding to CyP-D. The apparent involvement of the ANT and PiC in pore formation (see below) could be explained by the abundance of these proteins in the inner mitochondrial membrane and their susceptibility to oxidative damage. However, the ability of different ligands of the ANT and PiC to either activate or inhibit mPTP opening (see below) is not readily reconcilable with this model.

2.1.2. A role for the adenine nucleotide translocase (ANT)

Work from several laboratories, including our own, has demonstrated that opening of the mPTP can be inhibited by ATP, ADP and bongkrekic acid (BKA), an inhibitor of the ANT, all of which decreased the sensitivity of pore opening to [Ca^{2+}] [26,30,42]. Conversely, activation is achieved by adenine nucleotide depletion or another inhibitor of the ANT, carboxyatractyloside (CAT) that sensitise the pore to [Ca^{2+}] [40]. Since CAT and BKA trap the ANT in opposite conformations [43] these data implicate the ANT in the formation or regulation of the mPTP. Furthermore, the specificity for inhibition of the mPTP by nucleotides matches their ability to be transported by the ANT [40]. In the light of these data we proposed [30,33] and subsequently refined [24,44] a model for the mPTP in which CyP-D binds to the ANT and then, when triggered by calcium, causes it to undergo a conformational change to induce pore formation. Since its original proposal additional evidence has accumulated in support of this model. Thus the ANT was found to bind to a glutathione-CyP-D column [45,46] and binding was increased by oxidative stress [45,47,48]. Oxidative stress was shown to further sensitise the mPTP to [Ca^{2+}] by antagonising adenine nucleotide binding to the ANT through cross-linking of two thiol groups on the matrix surface of the ANT close to the adenine nucleotide binding site [48]. Furthermore, Brustovetsky and Klingenberg demonstrated that the purified and reconstituted adenine nucleotide translocase of *Neurospora crassa* forms non-specific channels at high calcium concentrations [49] and that the opening probability of these channels is increased at high membrane potential by oxidative stress and the presence of cyclophilin [50].

Despite this extensive evidence for an important role for the ANT in mPTP formation, an *essential* role has been ruled out since mouse liver mitochondria lacking ANT1 and ANT2 still exhibit a CsA-sensitive permeability transition [51]. However, the mPTP in these mitochondria is insensitive to ligands of the ANT and requires much higher [Ca^{2+}] to open. Although there are some criticisms that can be leveled against these studies [28,52] they do imply that another protein must play an important role in mPTP formation. Recent studies from this laboratory have suggested that the mitochondrial phosphate carrier (PiC) may be that protein [28,53].

2.1.3. A role for the mitochondrial phosphate carrier

Phosphate has been recognised as an activator of the permeability transition for more than 20 years and our recent studies have suggested that this effect may involve the PiC [53]. We had previously shown that phenylarsine oxide (PAO) could mimic the activation of the mPTP by oxidative stress and that this correlated with modification of two matrix cysteine groups on the ANT [24,40]. We were also able to show that immobilised PAO was able to pull down the ANT from detergent-solubilised inner mitochondrial membranes and that this could be prevented by pre-treatment of the mitochondria with CAT or BKA [40,53]. However, PAO could still activate mPTP opening in CAT- or BKA-treated mitochondria which is not consistent with this effect being purely mediated through PAO-binding to the ANT [53]. Thus we investigated whether other inner membrane proteins from CAT-treated mitochondria bound to a PAO-affinity matrix. We identified four such proteins, one of which was the PiC [53]. Its binding was unaffected by CAT- or BKA-treatment but could be abolished by treatment with the ubiquinone analogues UQo and Ro 68-3400 that had previously been identified as potent inhibitors of the mPTP [54]. Co-immunoprecipitation and GST-CyP-D pulldown experiments confirmed that CyP-D binds to the PiC in a CsA-sensitive fashion and that binding is increased by oxidative stress and to a lesser extent CAT that sensitise mPTP opening to [Ca^{2+}] [53]. Furthermore we were able to demonstrate a close correlation between the ability of both ubiquinone analogues and N-ethylmaleimide to inhibit mPTP opening and phosphate transport into mitochondria [53]. Additional support for an important role for the PiC in mPTP opening comes from the recent observation that phosphate is required for inhibition of mPTP opening by CsA or CyP-D knockdown [55]. There is also circumstantial evidence to support such a role. Thus the knockdown of the PiC in HeLa cells reduces their sensitivity to apoptosis induced by staurosporine [56], conditions under which the mPTP mediates apoptosis [57], whilst PiC over-expression can induce apoptosis [56].

2.1.4. Other proteins

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 [13,58,59]). However, the evidence for their involvement has been largely circumstantial [24,28]. Of these proteins the one for which the evidence was considered strongest is VDAC. Thus Crompton et al. reported that GST-CyP-D pulled down both VDAC and ANT from solubilised heart mitochondria [46], although in our own experiments using solubilised liver mitochondrial membranes only VDAC was bound [45]. Evidence was also presented implicating VDAC1 in the binding of ubiquinone analogues that inhibit the mPTP such as UQ_0 and Ro 68-3400 [54,60,61]. However, it was later demonstrated that mitochondria lacking VDAC1 were still inhibited by these compounds [62] and that mitochondria lacking all three isoforms of VDAC showed normal mPTP opening [63]. These data eliminate VDAC as an essential component of the mPTP.

2.2. A proposed model for the mPTP

The data summarised above led us to propose a model for the mPTP that is summarised in Fig. 1. It is suggested that the some of the ANT and PiC may form a heterodimer in the inner mitochondrial membrane for which there is some evidence from electron microscopy studies of “the ATP synthasome” [64,65]. When triggered by elevated matrix [Ca^{2+}] [25,27,40], one or both of these membrane transporters will undergo a conformation change facilitated by the peptidyl-prolyl *cis–trans* isomerase activity of CyP-D that is inhibited by the binding of adenine nucleotides to the ANT and activated by oxidative stress. It should be noted that the triggering by calcium does not necessarily require an increase in matrix [Ca^{2+}] but may be the consequence of an increase in oxidative stress that sensitises the mPTP to the prevailing [Ca^{2+}] [29,40]. Indeed, this is likely to be the critical mechanism that operates during reperfusion [66] as will be discussed further in Section 3.2 ‘Conditions during ischaemia and reperfusion favour mPTP opening’ [67]. Interestingly, Krämer et al. have demonstrated that the PiC of yeast mitochondria can be converted into a non-specific anion channel by dithiol cross-linking between two Cys^{28} residues within trans-membrane helix 1 to form a PiC dimer [68] and there is an equivalent cysteine residue (Cys^{27}) in mammalian PiC. This could be the site of action of $HgCl_2$ that converts the mammalian PiC into a unidirectional transporter with

reduced specificity [69]. This cysteine might also be responsible for the observed binding of the PiC to the PAO-column that is CAT-insensitive. If so, then it is likely that the PiC forms the pore of the mPTP whilst the conformation of the associated ANT plays more of a regulatory role [53]. When interacting with the ANT in the "c" conformation or with no adenine nucleotide bound it is proposed that the PiC is more sensitive to the conformational change that induces pore formation. By contrast, when associated with the ANT in the "m" conformation the PiC would undergo the conformation change less readily. This could explain the ability to demonstrate pore opening in mitochondria containing no ANT1 or ANT2, but with reduced sensitivity to calcium and no sensitivity to ligands of the ANT [51]. Oxidative stress and PAO may activate mPTP opening both by inhibiting adenine nucleotide binding to the ANT and by enhancing CyP-D binding to the PiC. Ubiquinone analogues and NEM are proposed to inhibit mPTP opening by both enhancing the "m" conformation of the ANT and by binding to the PiC to inhibit its conformational change into a pore.

3. mPTP opening is a critical factor in reperfusion injury and heart failure

3.1. Ischaemia and reperfusion injury defined (reviewed in [16,70–73])

During a myocardial infarction or cardiac surgery the heart, or portions of it, may become totally deprived of blood (ischaemia). A myocardial infarction is caused by a clot forming in one of the coronary arteries supplying the left ventricle with oxygen and respiratory fuels. This is known as a coronary thrombosis and produces an area of ischaemia that corresponds to that part of the ventricle exclusively supplied by the blocked artery. Ischaemia also occurs during surgical procedures such as in valve replacement and some coronary artery bypass graft operations where blood flow through the coronary arteries must be prevented. In both cases, if the blood flow is absent for long enough, the ischaemic tissue will die of necrosis and so if the heart is to be salvaged, the blood flow must be restored as soon as possible. However, the very act of reperfusion can exacerbate the damage to the heart that occurred during ischaemia. This phenomenon is called "reperfusion injury" and is characterised by an area of cell death, known as the infarct, the size of which reflects both the area supplied by the coronary artery(ies) whose blood flow was blocked and the time of ischaemia. The majority of the cell death is necrotic and is accompanied by release of intracellular enzyme such as lactate dehydrogenase and Troponin I that together with infarct size, can be used as an indicator of the extent of damage. Necrotic cell death attracts neutrophils to the damaged area producing an inflammatory response that exacerbates damage to the heart. Around the periphery of the infarct there may be a smaller area of apoptotic cell death that is not inflammatory [74,75]. In order to optimise the recovery of the heart following a coronary thrombosis or cardiac surgery it is important to minimise reperfusion injury, and for this to be achieved it is important to understand the cause(s) of the damage. As we describe below (Section 3.2 'Conditions during ischaemia and reperfusion favour mPTP opening'), the conditions that prevail during the initial stages of reperfusion are exactly those required for mPTP opening suggesting that this may be a critical factor in determining the extent of damage and recovery. Subsequent sections confirm that this is the case.

3.2. Conditions during ischaemia and reperfusion favour mPTP opening

During ischaemia and reperfusion the metabolism and ionic homeostasis of the heart are profoundly perturbed in a way that greatly favours mPTP opening as described below. These changes are reviewed elsewhere [16,70–73] but are summarised below and schematically in Fig. 2.

3.2.1. Ischaemia

During ischaemia mitochondria in the myocytes that are deprived of oxygen can no longer make ATP by oxidative phosphorylation and this leads to a rapid fall in tissue [ATP] with a concomitant rise in [ADP], [AMP] and [Pi]. These changes stimulate glycolysis through activation of phosphofructokinase but this is unable to provide sufficient ATP to fuel the beating heart and contraction rapidly ceases. Glycolysis continues to provide some ATP, but as lactic acid accumulates the intracellular pH drops progressively inhibiting phosphofructokinase and activating the Na^+/H^+ antiporter. The Na^+ that enters via this route would normally be pumped out again by the Na^+/K^+ ATPase but the greatly reduced [ATP] inhibits this efflux leading to a progressive rise in intracellular $[Na^+]$. This in turn causes $[Ca^{2+}]$ to rise because the Na^+/Ca^{2+} antiporter that usually pumps Ca^{2+} out of the cell, is inhibited or reversed. In addition to the impaired ionic homeostasis, the decrease in [ATP] leads to a large increase in [AMP] through the action of adenylate kinase, and some of this AMP is converted into adenosine and then inosine and xanthine through a purine degradation pathway. These nucleosides leak out of the cell (and may have vasodilator effects through purinergic receptors) and lead to a gradual depletion of total adenine nucleotide. This may contribute to the reversible impairment of haemodynamic function (known as stunning) seen following a short period of ischaemia. Perhaps more seriously, if sufficient residual oxygen is available, xanthine may be further oxidised by xanthine oxidase. This enzyme produces superoxide that can be further metabolised to other damaging reactive oxygen species (ROS), particularly hydrogen peroxide by superoxide dismutase and hydroxyl radicals through the Fenton reaction [76]. Heart cells actually contain xanthine dehydrogenase that is converted into xanthine oxidase during ischaemia/reperfusion by the oxidation of enzyme thiols and Ca^{2+}-activated proteases. Since the formation xanthine oxidase activity during ischaemia is very species dependent and does not correlate with reperfusion injury [16,77,78] its importance is unclear. However, this does not rule out a key role for ROS formation in ischaemia since there are additional potential sources of ROS. These include the respiratory chain, nitric oxide synthase and NADPH oxidase [76,79]. Indeed, direct measurement of ROS using dihydroethidium fluorescence in the perfused heart shows that there is a significant increase in ROS as ischaemia progresses and that this is a good indicator of the extent of damage that will occur on reperfusion [80,81]. It is well established that ROS production can increase under conditions of very low oxygen, in response to the highly reduced state of the mitochondrial NADH that favours superoxide production [82]. This increase in ROS is reflected in an increased protein carbonylation of mitochondria isolated at the end of ischaemia [83] that correlates with an increased calcium-sensitivity of the mPTP opening [83–85].

In isolated myocytes fluorescent measurements of $[Ca^{2+}]_m$ suggest that the amount of damage on reoxygenation also correlates with $[Ca^{2+}]_m$ at the end of reperfusion [86,87] and similar rises in $[Ca^{2+}]_m$ can be observed in the whole ischaemic heart [88]. Furthermore, attenuation of mitochondrial calcium overload with ruthenium red can protect hearts from reperfusion injury [89]. Since increased $[Ca^{2+}]_m$ triggers opening of the mPTP under conditions of oxidative stress, adenine nucleotide depletion and elevated [Pi], the changes in the intracellular milieu during ischaemia might be predicted to open the mPTP. However, this appears not to be the case since opening of the mPTP is strongly inhibited at pH values below 7, probably by protons competing with Ca^{2+} at the trigger site [25,90]. Thus at the low pH after prolonged ischaemia (<6.5) the mPTP is likely to remain closed and direct measurements confirm this (see Section 3.3 'Demonstration that mPTP opening occurs during reperfusion but not ischaemia' below). Nevertheless, the depletion of ATP and elevated $[Ca^{2+}]$ that occurs in ischaemia will lead to a gradual decline in cellular integrity as degradative enzymes such phospholipases (PLA_2) [91] and calcium-

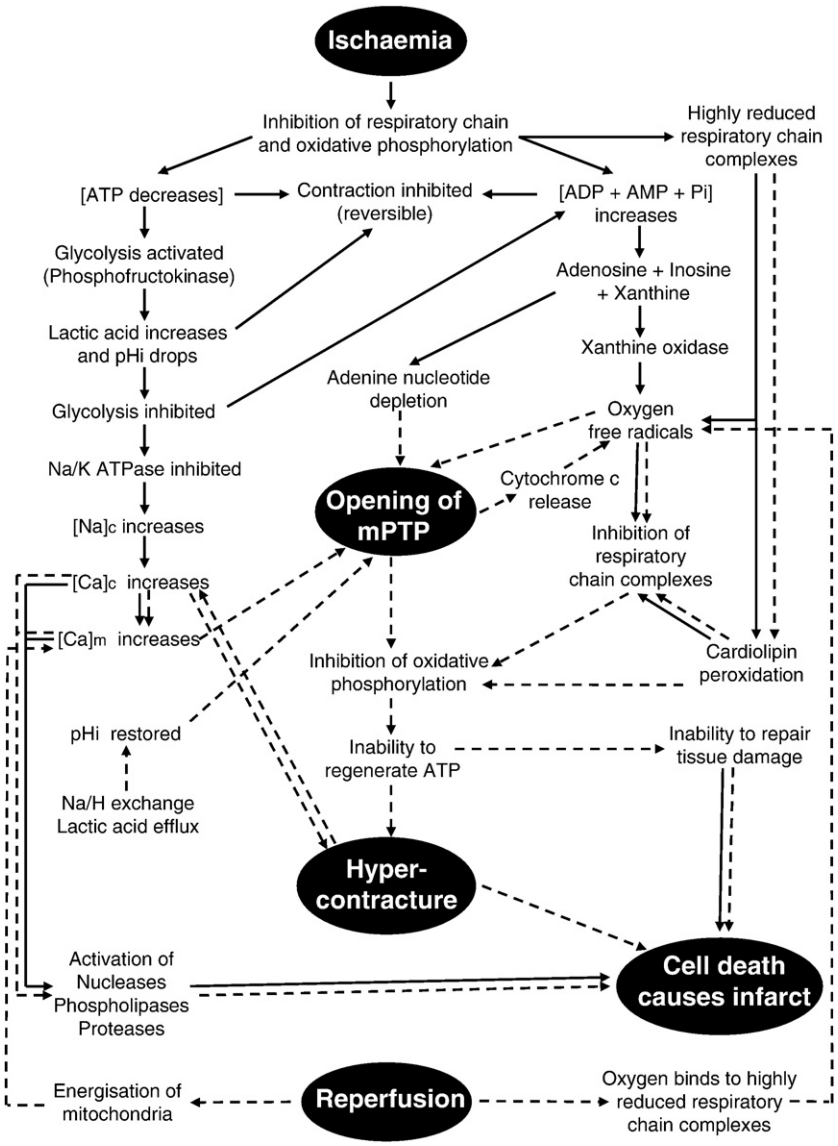

Fig. 2. Schematic representation of the effects of ischaemia and reperfusion on heart cells that lead to mPTP opening and the development of an infarct. Changes occurring primarily during ischaemia are indicated with solid lines and those during reperfusion with a dashed line. Where both lines are shown changes are initiated in ischaemia and continued or accentuated in reperfusion. Not shown on the diagram is the possible translocation of Bax to the mitochondria during ischaemia that may provide an additional means of cytochrome *c* release. Note that where the effects of reperfusion are not sufficient to induce substantial mPTP opening the heart will initially show impaired contraction (stunning) as a result of the free radical damage and adenine nucleotide loss, but will eventually recover fully. Further details are given in Section 3 'mPTP opening is a critical factor in reperfusion injury and heart failure' of the text.

activated proteases (calpains) [92] are activated at the same time as ATP-dependent repair processes are inhibited by the lack of ATP.

There may also be some BAX translocation to the mitochondria leading to permeabilisation of the outer membrane and cytochrome *c* release [93]. This has the potential to initiate apoptosis but only if ATP levels are maintained sufficiently to drive this energy dependent form of cell death [13]. If the tissue remains ischaemic for prolonged periods, damage to the cell will lead to rupture of the sarcolemma and necrotic cell death. However, shorter periods of ischaemia are tolerated and the heart recovers on reperfusion. It may show impaired function initially (stunning) but given sufficient time it will recover [70,71,94–96]. A major factor that determines the extent of damage upon reperfusion of the ischaemic heart appears to be the extent to which the mPTP opens at reperfusion as described below.

3.2.2. Reperfusion

At reperfusion additional factors come into play on top of the increased [Ca^{2+}], [Pi], oxidative stress and adenine nucleotide depletion that the myocytes experience during ischaemia. These are an additional burst of ROS production, further calcium overload of the mitochondria and the return of the intracellular pH from the acidic values present in ischaemia. The return of oxygen allows a resumption of respiration and hence mitochondrial energisation and ATP production. The increased mitochondrial membrane potential stimulates the uptake into the matrix of any Ca^{2+} that has accumulated in the cytosol during ischaemia [86,88,97] whilst the presence of oxygen induces an additional burst of ROS production [66,76,80,81]. The source of the ROS is debated but involves molecular oxygen interacting with a reduced electron carrier to produce superoxide. The rate of such superoxide production is very sensitive to [O_2] [82] which explains the burst of ROS seen upon reoxygenation as the returning oxygen meets a highly reduced respiratory chain. Some ROS may come from the activity of xanthine oxidase but probably the majority are formed by the mitochondrial respiratory chain. A major site of superoxide production is complex 1 which is stimulated by a high NADH/NAD ratio such as may occur at

the end of ischaemia and is thought to be mediated by reduced FMN. Additional matrix superoxide production may occur at the CoQ binding site on complex 1 that receives electrons from the N2 FeS centre, at succinate dehydrogenase (complex 2) and complex 3 [82,98,99]. There are also pathways for ROS elimination including superoxide dismutase (SOD) to convert $O_2.^-$ into H_2O_2 which is then removed by glutathione peroxidase or catalase [79,99]. However, at reperfusion these defence mechanisms may become overwhelmed leading to oxidative stress as ROS accumulate. In addition to their direct effects on the mPTP, ROS have additional effects that indirectly influence mitochondrial function and mPTP opening. Thus ROS can oxidise iron sulphur proteins in complex 1, complex 3 and aconitase, and they can also cause thiol oxidation and inhibition of the ATPase and adenine nucleotide translocase. In addition, they lead to oxidation of glutathione that may then form mixed disulphides with proteins, including inner membrane proteins, that modulate their activity [100]. Such protein modification is also thought to have inhibitory effects on ion pumps and therefore exacerbate the effects of ATP deprivation on ionic homeostasis [101–103]. Another effect of ROS is to cause peroxidation of the unsaturated fatty acid components of the phospholipids, and especially cardiolipin of the inner mitochondrial membrane, which leads to further inhibition of respiratory chain activity [104,105]. Furthermore, lipid peroxidation causes the release of reactive aldehydes such as 4-hydroxynonenal that can modify membrane proteins including the ANT [106] and might explain the cardioprotective effects of over-expressing mitochondrial aldehyde dehydrogenase [107].

An additional player during reperfusion is nitric oxide (NO). This may increase during ischaemia and can have either a protective or damaging role during reperfusion, depending on its concentration and the prevailing conditions [76,108,109]. At low concentrations it can exert a protective role that is mediated through a signalling pathway involving cyclic GMP formation as discussed below (Section 4.4 'Preconditioning and post-conditioning protect heart from reperfusion injury by inhibiting the mPTP'). NO will also inhibit cytochrome oxidase competitively with respect to oxygen [110] and this could exert a protective role by reducing the mitochondrial membrane potential, leading to less mitochondrial calcium accumulation. However, inhibition of cytochrome oxidase will also cause redox centres of the respiratory chain to become more reduced and this has the potential to enhance superoxide production [82]. Not only can this induce oxidative stress in its own right, but the superoxide may also react with NO to produce peroxynitrite that exerts its own effects on mitochondria. Peroxynitrite modifies mitochondrial proteins, including the ANT and components of the respiratory chain leading to an inhibition of oxidative phosphorylation and activation of the mPTP [111–115].

Although the increased ROS, peroxynitrite and $[Ca^{2+}]$ at reperfusion have the potential to cause mPTP opening on their own they are insufficient if the intracellular pH remains low [90,116]. However, reperfusion also allows the intracellular pH to return to normal over a period of 2–3 min as lactic acid leaves the cell and other pH regulatory transporters are active such as the Na^+/H^+ antiporter and bicarbonate-linked pH regulatory transporters [117,118]. It is at this point that the pore has been shown to open (see Section 3.3 'Demonstration that mPTP opening occurs during reperfusion but not ischaemia'), and this corresponds with the peak release of intracellular lactate dehydrogenase and the heart going into a hypercontracted state [34,116]. The initial damage in the first few minutes of reperfusion may be relatively modest, but the size of the infarct increases over several hours of reperfusion [95,96] and protection from reperfusion injury can be mediated by pharmacological intervention within the first 15 min of reperfusion [73]. This suggests that it may not only be the initial opening of the mPTP that is important for reperfusion injury but in addition a progressive opening that is transmitted from mitochondria to mitochondria and myocyte to myocyte. Such a phenomenon could be explained by "ROS-induced ROS release" whereby opening of the mPTP itself produces ROS that leads to a cascade of further mPTP opening [85,119,120], although others have proposed an alternative mechanism involving the activation of an inner membrane anion channel by ROS-mediated glutathione oxidation [121–123].

The cause of mPTP induced ROS production is not certain but may involve outer membrane rupture leading to cytochrome *c* release. This may directly affect ROS production since oxidised intermembrane cytochrome *c* has the potential to be reduced by superoxide, thus regenerating oxygen and reduced cytochrome *c* that can be re-oxidised by complex 4 [124]. Loss of cytochrome *c* may prevent this superoxide scavenging and hence lead to ROS accumulation. Alternatively, cytochrome *c* release may cause caspase activation and subsequent cleavage of the p75 subunit of complex 1 to enhance superoxide production at this site [125,126]. Even if the mPTP opens initially, but then closes again, as we have shown can occur during reperfusion [116], myocytes may still be susceptible to death by apoptosis if mitochondria swell sufficiently to rupture the outer membrane, and release cytochrome *c* and other apoptotic factors [13,19,127]. In reperfusion injury, apoptotic death may only be relevant to the cells surrounding the necrotic core of the infarct [74,75], but in the failing hypertrophic heart apoptosis may be more significant [128], although more recent data suggests that here too the majority of death may be necrotic [129] (and see Section 5 'Heart failure in cardiac hypertrophy may be ameliorated by inhibition of the mPTP'). Overall, it is thought that the combined effects of ROS, elevated $[Ca^{2+}]$ and possibly peroxynitrite play a decisive role in the transition from reversible to irreversible reperfusion injury, and that it is the opening of the mPTP that represents the critical point in committing the myocyte to cell death [13,15,16,73,130].

3.3. Demonstration that mPTP opening occurs during reperfusion but not ischaemia

In order to demonstrate directly that the predicted mPTP opening does occur at reperfusion, techniques have been developed that can measure mPTP opening *in situ*. In isolated cardiac myocytes subjected to simulated ischaemia and reperfusion, mPTP opening can be monitored using fluorescence microscopy to measure the mitochondrial membrane potential with a fluorescent dye such as tetramethylrhodamine methyl ester (TMRM) as a surrogate indicator of mPTP opening [131,132]. Confirmation that any depolarisation observed is due to mPTP opening is achieved by demonstrating the ability of CsA, sometimes supplemented with trifluoperazine, to inhibit the process [133]. 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 [134,135]. This technique has been applied successfully to cardiac myocytes subject to oxidative stress and simulated ischaemia/reperfusion where the data confirmed that the mPTP remains closed during ischaemia but opens upon reperfusion as the pH returns to normal. The data also suggested that the most critical factor influencing the extent of mPTP opening is the degree of oxidative stress rather than calcium overload [136–140]. Indeed there is increasing evidence that it is the increase of ROS during ischaemia that primes the mPTP for opening at reperfusion [81,85,141–143].

In order to determine the extent of mPTP opening in the perfused heart subject to ischaemia/reperfusion, two alternative techniques have been developed. DiLisa et al. determined the loss of mitochondrial NAD^+ that accompanies reperfusion as a surrogate indicator of pore opening [144]. This was inhibited by CsA, confirming that it involved mPTP opening, although with this technique it is not possible eliminate the possibility that some of the CsA-sensitive NAD^+ loss represents disruption of mitochondria during their isolation rather

than *in situ* [34,145]. A more direct approach to determining mPTP opening *in situ*, devised in this laboratory, measures the mitochondrial entrapment of [^{3}H]-2-deoxyglucose (DOG) as an indicator of pore opening [145]. In this "Hot-DOG" technique, hearts are first loaded with [^{3}H]-DOG which is phosphorylated within the cytosol to the dead-end metabolite, DOG-6-phosphate. This can only enter the mitochondria when the mPTP opens. Thus when mitochondria are rapidly isolated in the presence of EGTA to reseal the mPTP, the amount of [^{3}H]-DOG entrapped within the mitochondria gives an indication of the extent of pore opening. However, if the pores open and then close again the [^{3}H]-DOG will remain entrapped. To determine the extent to which such resealing occurs, a comparison can be made between mitochondrial [^{3}H]-DOG entrapment of hearts loaded before (pre-loading) and after (post-loading) ischaemia/reperfusion [116]. Using the "Hot-DOG" technique we were able to confirm that the mPTP does not open during ischaemia, but rather occurs after the first 2–3 min of reperfusion when the intracellular pH normalises [145,146]. Some of the pores that open initially at the start of reperfusion where shown to close again as reperfusion progressed [116].

In this laboratory we have also used the "Hot-DOG" to confirm less mPTP opening is associated with cardioprotection induced by CsA and SfA [34], the free radical-scavenging anaesthetic propofol [147], pyruvate [116], urocortin [142] and ischaemic pre-conditioning [84], thus providing further evidence for the critical role of mPTP opening in reperfusion injury. Other laboratories have used the technique to demonstrate that protection induced by pharmacological blockade of the sodium/proton antiporter (NHE1) [148–150] or short-term physical training [151] is also associated with less mPTP opening.

4. Inhibition of the mPTP protects hearts against ischaemia reperfusion injury

Inhibition of mPTP opening *in situ* with resulting protection against reperfusion injury can be achieved using a variety of strategies. These are described below and summarised in Fig. 3.

4.1. Targeting CyP-D

CsA was initially shown to protect isolated cardiac myocytes from reoxygenation injury [152] and we subsequently showed protection from reperfusion injury in the Langendorff perfused heart [153]. Since then many laboratories have confirmed these data using both global and regional models of ischaemia and reperfusion with determination of injury using enzyme release, infarct size and haemodynamic function [154–156]. Furthermore, genetic ablation of CyP-D also provides potent protection against ischaemia reperfusion injury of the heart [37,38] and brain [39] with CyP-D knockout mice showing a substantial decrease in infarct size. Thus targeting the mPTP has proven potential as a pharmacological target for the reduction of reperfusion injury following stroke, coronary thrombosis and heart surgery. However, the use of CsA in cardioprotection is not ideal because of its tight binding to cytosolic cyclophilin-A and resulting inhibition of the calcium sensitive protein phosphatase, calcineurin,

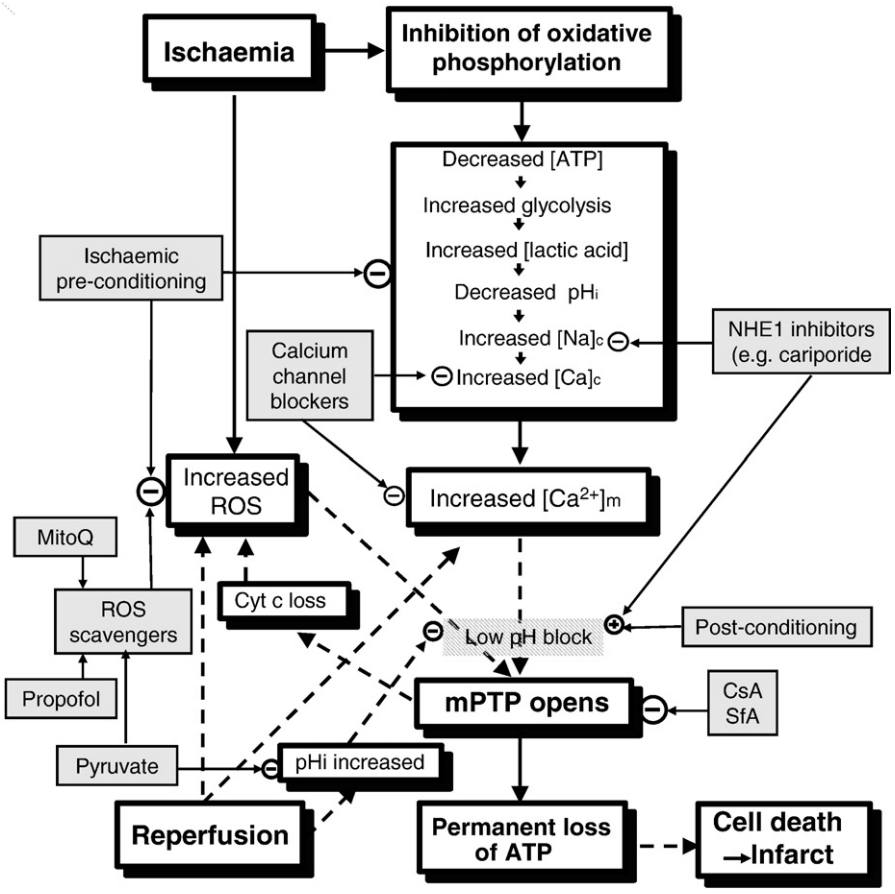

Fig. 3. Proposed sites of action of cardioprotective protocols Proposed sites of action of cardioprotective protocols. This scheme provides an outline of the major factors that sensitise the mPTP during ischaemia (solid lines) and lead to its opening at reperfusion as shown in Fig. 2. The proposed sites of action of cardioprotective agents or protocols described in Section 4 'Inhibition of the mPTP protects hearts against ischaemia reperfusion injury' are indicated with grey boxes and solid arrows. Circles containing + or − indicated an increase or decrease in the change of the parameter shown. No attempt is made to incorporate into the diagram the signalling pathways involved in pre-conditioning or post-conditioning. A brief discussion of these pathways is given in Section 4.4 'Pre-conditioning and post-conditioning protect heart from reperfusion injury by inhibiting the mPTP', but for a more detailed account the reader is referred elsewhere [205–207].

that has direct effects on heart function [157] and also undesirable immunosuppressive activity [158]. To circumvent these problems CsA analogues or SfA can be employed that are inactive against calcineurin but bind tightly to CyP-D, and inhibit mPTP opening. Such drugs are at least as cardioprotective as CsA [34,145,156,159] and importantly, are effective even when added only during early reperfusion. Thus CsA and SfA can reduce the infarct size of hearts in which a coronary artery is occluded and then re-opened to mimic the clinical treatment of a coronary thrombosis [154,155]. This has led us to propose that the initial opening of the mPTP is followed by "ROS-induced ROS release" (see above – Section 3.3 'Demonstration that mPTP opening occurs during reperfusion but not ischaemia') that may be responsible for the increase in infarct size with time [85,119,120]. Inflammation through neutrophil recruitment and activation may be an additional cause of this progressive increase in infarct size [73]. Recently, in an *in vivo* mouse model allowing 24 h and 30 days reperfusion following 25 min regional ischaemia, the CsA analogues Debio-025 given at reperfusion was found to give substantial protection [159]. This is encouraging for future clinical practice in the treatment of coronary thrombosis with thrombolysis or percutaneous coronary intervention (PCI). Indeed, protection by CsA against mPTP opening has been reported in human heart cells subject to simulated ischaemia/reperfusion injury [160] and the first small clinical trials do confirm improved recovery following CsA treatment of patients undergoing PCI treatment following a coronary thrombosis [161].

4.2. Targeting other components of the mPTP

Opening of the mPTP is not totally dependent on CyP-D and even when CyP-D is ablated genetically or fully inhibited with CsA the permeability transition can be induced with a higher calcium load or oxidative stress [36,40]. Thus targeting another component of the mPTP would be an attractive strategy for cardioprotection. The mPTP can be inhibited by targeting the ANT with BKA and this can protect cells from mPTP-induced cell death in some systems [13,44]. Clearly this is not an appropriate strategy for cardioprotection since the ANT is vital for the generation of cytosolic ATP via oxidative phosphorylation to energise the contractile cycle [44]. Targeting the phosphate carrier is also unlikely to be effective for similar reasons and we have found ubiquinone analogues to be damaging to the heart rather than cardioprotective [13]. Thus, at present, targeting CyP-D with non-immunosuppressive CsA derivatives is the best available strategy, but it remains possible that other targets that affect the mPTP may be found. However there are very effective indirect ways of inhibiting the mPTP that have shown potent cardioprotective effects.

4.3. Indirect strategies for inhibiting the mPTP

Cardioprotection is provided by free radical scavengers such as the anaesthetic propofol which can directly inhibit mPTP opening in isolated mitochondria [162,163] and mitochondria from propofol treated hearts [147]. Furthermore, we have demonstrated inhibition of the mPTP by propofol in the intact heart using the "Hot-DOG" technique [147] and confirmed that propofol is protective in an *in vivo* pig model of cardiopulmonary bypass with warm blood cardioplegia that closely matches current clinical practice [164]. Other anaesthetics such as isoflurane and desflurane have also been shown to offer protection that is associated with less ROS formation and calcium overload [165–169]. ROS-scavengers such as MitoQ that are specifically targeted to mitochondria through a positively-charged hydrophobic moiety [170] may be especially promising and have already been shown to protect against reperfusion injury in the Langendorff perfused rat heart model [171]. Inhibition of the Na^+/H^+ exchanger with amiloride derivatives such as cariporide is also known to protect the heart from reperfusion injury [172]. These drugs are assumed to act by preventing sodium and therefore calcium overload during ischaemia whilst maintaining low pH during reperfusion[173]. Here too the "Hot-DOG" technique has confirmed inhibition of mPTP opening *in situ* [148–150].

Pyruvate is one of the most protective agents against reperfusion injury which probably works by acting as a free radical scavenger that also maintains a low intracellular pH and is an excellent respiratory substrate to replenish ATP during reperfusion [116,174]. Again, direct measurement of mPTP opening with the "Hot-DOG" technique has confirmed that pyruvate reduces mPTP opening at reperfusion and also causes those pores that open initially to close again [116]. Protection of hearts from reperfusion injury can also be achieved by reducing cytosolic and mitochondrial calcium overload with antagonists of plasma membrane or mitochondrial calcium channels such as verapamil and ruthenium red [175–182]. It would seem probable that this involves inhibition of the mPTP although this has not been demonstrated directly. A similar mechanism probably applies to the protection seen with elevated extracellular $[Mg^{2+}]$ (>8 mM) during the reperfusion phase [183–185]. Protection from reperfusion injury by trimetazidine, an "anti-ischaemic" drug used for the treatment of heart failure has also been shown to reduce mPTP opening [186]. Although the mechanism is unclear it is likely to be secondary to its metabolic effect of switching the heart from oxidising fatty acids (which produce more ROS) to carbohydrates [187].

4.4. Pre-conditioning and post-conditioning protect heart from reperfusion injury by inhibiting the mPTP

A very effective way of protecting the heart from reperfusion injury is "*ischaemic pre-conditioning*" (IP) whereby the heart is subject to one or more brief ischaemic episodes with recovery before the prolonged period of ischaemia. This procedure induces two phases of protection; an immediate effect and a "second window" that occurs 24–48 h later [188,189]. The latter probably involves upregulation 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 [189,190]. Protection can also be induced when very brief (10 s) intermittent ischaemic periods are included within the first few minutes of reperfusion, a protocol known as "*post-conditioning*" [191,192] which has obvious clinical potential in the treatment of coronary thrombosis by angioplasty [193].

The exact mechanisms involved in first window of pre-conditioning and post-conditioning are still debated but in both cases there is strong evidence that mPTP inhibition is involved [85,194]. Direct and indirect techniques have been used to confirm this. 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 [84]. Indeed, there is a good correlation between reduced pore opening and cardioprotection. Indirect measurements have led to similar conclusions. Thus several other studies have shown that mitochondria isolated from IP hearts following ischaemia or reperfusion are less sensitive to calcium-induced mPTP opening than mitochondria from control hearts but that this effect is not seen immediately after the IP protocol [83,84,141,195,196]. These data suggest that the protective effect of IP on mitochondria somehow develops during the ischaemia and reperfusion phases.

Our own data have suggested that the mechanism for this involves the mitochondria from IP hearts experiencing less oxidative stress during ischaemia and reperfusion than those from control hearts [141,196]. Indeed we and others have shown that three other pre-conditioning protocols, treatment with urocortin [142], apomorphine [197] or exposure to several intermittent brief hypothermic episodes prior to index ischaemia (temperature pre-conditioning) [141] are also associated with mitochondria that develop less oxidative stress during ischaemia and reperfusion and are less

sensitive to calcium-induced mPTP opening. Other studies using isolated cardiac myocytes have revealed that IP desensitises the mitochondria to pore opening induced by oxidative stress [139,198] and experiments performed with two-photon microscopy in the perfused heart have yielded similar data [199]. Thus it seems probable that IP may increase the ability of mitochondria to scavenge ROS, perhaps coupled with a decrease in ROS production, although the mechanisms involved remain unclear [85]. Post-conditioning is also thought to involve desensitisation of the mPTP to calcium-induced opening since mitochondria isolated from such hearts during reperfusion are less sensitive to mPTP opening than those isolated from control hearts [200].

Taking all the data together, the emerging picture is that ischaemic pre-conditioning protects the heart by reducing oxidative stress during ischaemia and reperfusion and that this leads to less mPTP opening. There may also be additional effects of IP on mitochondrial calcium loading since IP has been reported to reduce the detrimental effects of ischaemia on sarcoplasmic Ca^{2+} release and uptake leading to decreased Ca^{2+} overload during ischaemia and reperfusion [201–204].

What is less clear is the signalling pathways involved in mediating the effects of pre-conditioning and post-conditioning. Both post-conditioning and the numerous pre-conditioning stimuli probably recruit common signalling pathways to inhibit mPTP opening, although there is no universal consensus as to what these pathways are or how they interact. However, there is strong evidence for the activation of protein kinase Cε (PKCε) by ROS and/or other factors (e.g. adenosine, bradykinin, noradrenaline) released during the pre-conditioning protocol. Others have implicated NO and protein kinase G, perhaps via activation of mitochondrial K_{ATP} (mitoK_{ATP}) channels, and the Akt/glycogen synthase kinase 3β (GSK3β) pathway and we have recently reviewed this evidence [85]. Reviews expressing alternative views can be found elsewhere [205–207].

Our own data on pre-conditioning [141,142,196] can be summarised as follows. We find no evidence that the IP-mediated inhibition of mPTP opening at reperfusion involves translocation of protein kinases or connexin 43 to the mitochondria or direct phosphorylation of mitochondrial proteins. Rather our data suggest that the key factor causing inhibition of mPTP opening by different pre-conditioning stimuli is a reduction in the oxidative stress experienced by mitochondria during prolonged ischaemia and reperfusion. This causes less oxidation of the critical thiol groups on the mPTP responsible for sensitising mPTP opening to [Ca^{2+}] [40,48]. The mechanisms by which ROS levels are decreased in IP hearts during prolonged ischaemia and reperfusion remain to be elucidated but probably involve activation of protein kinase Cε (PKCε), either via receptor-mediated events or through transient increases in ROS during the IP protocol. Other signalling pathways may show cross-talk with this primary mechanism, but we can find no evidence for a role for mitoK_{ATP} channels [85]. Indeed, we are sceptical about the conclusions reached in many experiments performed with openers and blockers of this channel since they can have many non-specific effects on mitochondrial function [85,208,209]. Our own data and our review of the literature also lead us to conclude that protection by pre-conditioning occurs in two phases: first, prevention of mPTP opening at the initial phase of reperfusion; second, through a continuing protection during reperfusion by preventing a cascade of mPTP-induced ROS production with 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 [210].

A plausible mechanism for these effects is as follows. It is well established that mPTP opening causes mitochondrial swelling, rupture of the outer membrane [211] release of cytochrome *c* and caspase activation [20]. In addition, the pro-apoptotic factor Bax can translocate to mitochondria during ischaemia [93] and this, in conjunction with cleaved Bid (tBid), might cause cytochrome *c* release during prolonged ischaemia [212] despite there being no evidence of mPTP opening [145,199]. The resulting loss of cytochrome *c* will slow electron transfer out of complex 3 and thus potentially cause increased ROS production from either complex 3 or complex 1. Indeed such increased ROS production has been shown to occur follow cytochrome *c* loss during apoptosis [213]. Increased ROS production during ischaemia and reperfusion may also occur through caspase 3 mediated cleavage of the p75 component of complex 1[125,126]. Protection from the rise in ROS that accompanies such cytochrome *c* release could be mediated by survival kinase cascades (e.g. Akt and GSK3-β) in two ways. They might stimulate ROS removal as described above for the ischaemic phase or they could reduce the Bax-induced cytochrome *c* release. Indeed, it is well established that survival kinases can block apoptosis by inhibiting cytochrome *c* release [214]. This is brought about by Akt-mediated phosphorylation of the pro-apoptotic Bcl-2 family member Bad [215,216] and, via GSK3β phosphorylation and inactivation, the stabilisation of the anti-apoptotic Bcl-2 family member Mcl-1 [217].

5. Heart failure in cardiac hypertrophy may be ameliorated by inhibition of the mPTP

Congestive heart failure may develop following cardiac infarction or in chronic hypertension (high blood pressure) as the heart undergoes compensatory hypertrophy to restore some measure of cardiac output. However, over time this progresses into a decompensated state including dilation and decreased function. This is associated with a gradual death of normal cardiac myocytes with a proliferation of fibroblasts leading to a disorganised enlarged heart that fails to pump efficiently [218–220]. There is evidence that dysregulation of calcium handling may occur under such conditions, with a reduction in peak systolic [Ca^{2+}] but an elevation and prolongation in diastolic [Ca^{2+}]. This will result in reduced systolic contraction and a delay in diastolic relaxation [218,219]. The perturbation of normal calcium handling may also be a critical factor in the development of cardiac hypertrophy since it has been shown that a similar phenotype can be induced in transgenic mice with enhanced sarcolemmal L-type Ca^{2+} channel activity. When accompanied by acute stimulation of β-adrenergic receptors that led to further enhancement of Ca^{2+} influx, hearts of these mice showed progressive myocyte necrosis that caused pump dysfunction and premature death of the mice [129].

It has been proposed that such calcium overload might act via calcineurin mediated upregulation of gene expression to induce the hypertrophic phenotype. Indeed pharmacological or genetic down-regulation of calcineurin activity reduces hypertrophy in mouse models [220–222] whilst calcineurin over-expression induces hypertrophy [223]. Interestingly, the heart mitochondria from such mice showed impaired respiratory chain activity and increased ROS production. Taken together these data suggest that the dysregulation of calcium handling in cardiac hypertrophy might also lead to mitochondrial calcium overload and ROS production that together would favour enhanced mPTP opening and provide an explanation for the observed death of myocytes. In support of this hypothesis, it was shown recently that the development of progressive myocyte cell death and heart failure in the transgenic model described above was not apparent in mice lacking CyP-D [129]. Thus it seems possible that pharmacological targeting of the mPTP may be beneficial for preventing or slowing the progression of congestive heart failure. Indeed, such inhibition of mPTP opening may provide an additional mode of action of the "anti-ischaemic" drug trimetazidine, which is used for the treatment of heart failure and switches the fuel

preference of hearts from fatty acids to carbohydrates [187]. In support of this trimetazidine has been shown to reduce mPTP opening in a rabbit model of reperfusion injury [186]. Interestingly, the use of CyP-D knockout mice is also providing evidence for a critical role of the mPTP in other chronic diseases associated with skeletal muscle dysfunction. Thus in some muscular dystrophies changes in the disposition of the sarcolemma leads to calcium overload with resulting necrotic damage that is not seen in CyP-D knockout mice or following treatment with CsA and Debio-025 (a CsA analogue inactive against calcineurin) [224,225].

6. Conclusion

In recent years it has become apparent that mitochondria have two distinct persona, akin to Dr. Jeckyll and Mr. Hyde. In the former state they act to supply the voracious appetite of the beating heart for ATP, with increased matrix calcium providing the link between the greater ATP demand at higher work rates with higher rates of respiration and oxidative phosphorylation. However, when the heart is overloaded with calcium, especially when this is accompanied by oxidative stress, the mPTP opens and the mitochondria embrace their darker side, and precipitate death of the myocytes. This happens acutely in reperfusion injury and chronically in congestive heart failure. Inhibition of mPTP opening by either genetic or pharmacological inhibition of CyP-D provides protection from both pathologies in animal models, confirming the mPTP as a promising drug target in human cardiovascular disease. Indeed, the first clinical trials have shown promising results and confirm improved recovery following CsA treatment of patients undergoing PCI treatment following a coronary thrombosis [161]. However, CyP-D is not an ideal target because even when it is ablated genetically, or is fully inhibited with CsA, the permeability transition can be induced with a higher calcium load or oxidative stress [36,40]. There are also potential problems associated with CsA and its analogues, since even those such as Debio-25 that do not inhibit calcineurin do bind to other cyclophilins and inhibit their peptidyl-prolyl *cis–trans* isomerase activity [226]. Thus there remains a scope for the development of better drugs that target another component of the mPTP without interfering with the normal function of the mitochondria. These might have the potential to be used prophylactically in those patients at risk of ischaemic heart disease. For this to be achieved it will be important to establish the true molecular composition of the mPTP and the mechanism and regulation of its opening. This continues to be an on going focus of research in our laboratory.

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