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Mitochondrial Permeability Transition Pore Opening as an Endpoint to Initiate Cell Death and as a Putative Target for Cardioprotection

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Key Words
Mitochondria • Permeability transition pore • Heart • Ischemia/reperfusion • Post-infarction remodeling • Cardioprotection

Abstract
In recent years, mitochondria have been recognized as regulators of cell death via both apoptosis and necrosis in addition to their essential role for cell survival. Cellular dysfunctions induced by intra- or extracellular insults converge on mitochondria and induce a sudden increase in permeability of the inner mitochondrial membrane, the so-called mitochondrial permeability transition. The mitochondrial permeability transition is caused by the opening of permeability transition pores (PTP) in the inner mitochondrial membrane with subsequent loss of ionic homeostasis, matrix swelling and outer membrane rupture. The detailed molecular mechanisms underlying the PTP-induced cellular dysfunction during cardiac pathology such as ischemia/reperfusion or post-infarction remodeling remain to be elucidated. However, a growing body of evidence supports the concept that pharmacological inhibition of the PTP is an effective and promising strategy for the protection of the heart against ischemia/reperfusion injury and for attenuation of the remodeling process which contributes to heart failure. This review summarizes and discusses current data on i) the structure and function of the PTP, ii) possible mechanisms and consequences of PTP opening and iii) the inhibition of PTP opening as a therapeutic approach for treatment of heart disease.

Introduction

Besides oxidative phosphorylation and ATP synthesis, mitochondria play a crucial role in the regulation of Ca^{2+}- and reactive oxygen species (ROS)-mediated processes in cardiac cells. In order to maintain cell survival, energy metabolism and ion homeostasis are tightly coupled through dynamic feedback mechanisms between the mitochondria and cytoplasm. However, external insults such as myocardial ischemia and reperfusion can increase intracellular Ca^{2+} concentrations and enhance ROS generation within the cytoplasm and

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mitochondria. The cellular Ca^{2+} overload that accompanies ischemia and subsequent reperfusion may be explained, at least in part, by reversal activation of the Na^+/Ca^{2+} exchanger (NCX). This occurs because ischemia causes metabolic acidosis that in turn activates the Na^+/ H^+ exchanger-1 (NHE-1) leading to high cytosolic [Na^+]. Under such conditions of Ca^{2+}overload, especially when accompanied by oxidative stress and ATP depletion, mitochondria undergo a permeability transition that is associated with the formation of a non-specific permeability transition pore (PTP) in the inner mitochondrial membrane [*reviewed in* 1-9]. During a mild stress, transient opening of the PTP and/or irreversible pore opening can occur in highly Ca^{2+}-sensitive mitochondrial population, leading to the release of pro-apoptotic proteins from intermembrane space and the induction of apoptosis. However, the majority of mitochondria must remain competent for ATP synthesis, otherwise the energy requiring processes of apoptosis could not occur [3, 4, 10]. This is what is thought to happen during a severe insult such as a long period of ischemia followed by reperfusion. Under this condition, massive swelling and mitochondrial membrane depolarization induces further ROS production and ATP hydrolysis and even though caspase activation will occur as a result of the release of pro-apoptotic proteins from the intermembrane space, cell death will occur via necrosis rather than apoptosis. Thus, the extent of PTP opening probably determines the balance between apoptosis and necrosis following an ischemic insult. In fact, both cell death pathways may occur during heterogeneous damage to the same tissue such as in the case of myocardial infarction, where necrosis occurs in the core whilst apoptosis is mostly observed in the border zone where the insult is less severe [11].

The critical role proposed for PTP opening in cell death suggests that inhibition of the PTP may represent an effective therapeutic approach in protecting the heart against various cardiac pathologies. Indeed, in recent years, pharmacological agents that inhibit PTP opening directly (e.g. cyclosporine A) and indirectly (e.g. ROS scavengers, NHE-1 inhibitors and Ca^{2+} antagonists) as well as ischemic preconditioning (IPC) have been shown to inhibit PTP opening in the ischemic/reperfused heart which was associated with a greater post-ischemic recovery of cardiac hemodynamic function and reduced necrotic damage as assessed by intracellular enzyme release and infarct size [1, 2, 6]. However, a detailed understanding of the molecular mechanisms underlying metabolic regulation of the pore formation during both physiological and pathological conditions is still lacking. The purpose of this review are threefold: first to summarize current knowledge of the structure and function of the PTP and factors involved in its regulation in terms of pore opening; second to describe how PTP opening acts as the end-effectors of mitochondrial dysfunction to trigger both apoptosis and necrosis; and third, to discuss PTP opening as target for therapeutic interventions that protect the heart against various forms of injuries, especially ischemia/reperfusion and post-infarction remodeling.

PTP: structure, function and regulation

PTP Structure

Electron transport through respiratory chain complexes generates an electrochemical gradient across the mitochondrial inner membrane comprising both a membrane potential ($\Delta\Psi_m$) and H^+ gradient. These gradients, collectively known as the proton motive force (pmf), are used to drive ATP synthesis by the F_1,F_0-ATPase. In order to maintain the pmf it is essential that the inner membrane of mitochondria remains impermeable or selectively permeable (via carriers and channels) to metabolites and ions under normal aerobic conditions. However, in response to stress, the permeability of the mitochondrial membrane may increase, with the formation of a voltage-dependent non-specific pore in the inner membrane known as the mitochondrial PTP. The open state of the PTP possesses a channel ~3 nm wide in diameter thus allowing diffusion of all molecules with molecular masses less than 1.5 kDa [1, 2, 5]. The exact molecular composition of the PTP remains uncertain although both the adenine nucleotide translocase (ANT) in the inner membrane and cyclophilin D (CyP-D) in the matrix are widely believed to be key structural components [1, 7, 9] (Fig. 1). The voltage-dependent anion channel (VDAC, also known as porin) in the outer membrane has also been proposed to play a critical role in PTP opening although the recent demonstration that mitochondria from VDAC1 knockout mice possess a normal PTP has questioned this proposal [12]. Purified molecules of VDAC reconstituted in planar bilayers or proteoliposomes form channels and the electro-physiological properties of these channels have been shown to be similar to those of the PTPs [13, 14]. Furthermore, the channels formed by VDAC are modulated by the same factors that regulate PTP activity [15-17]. The benzodiazepine receptor, hexokinase and

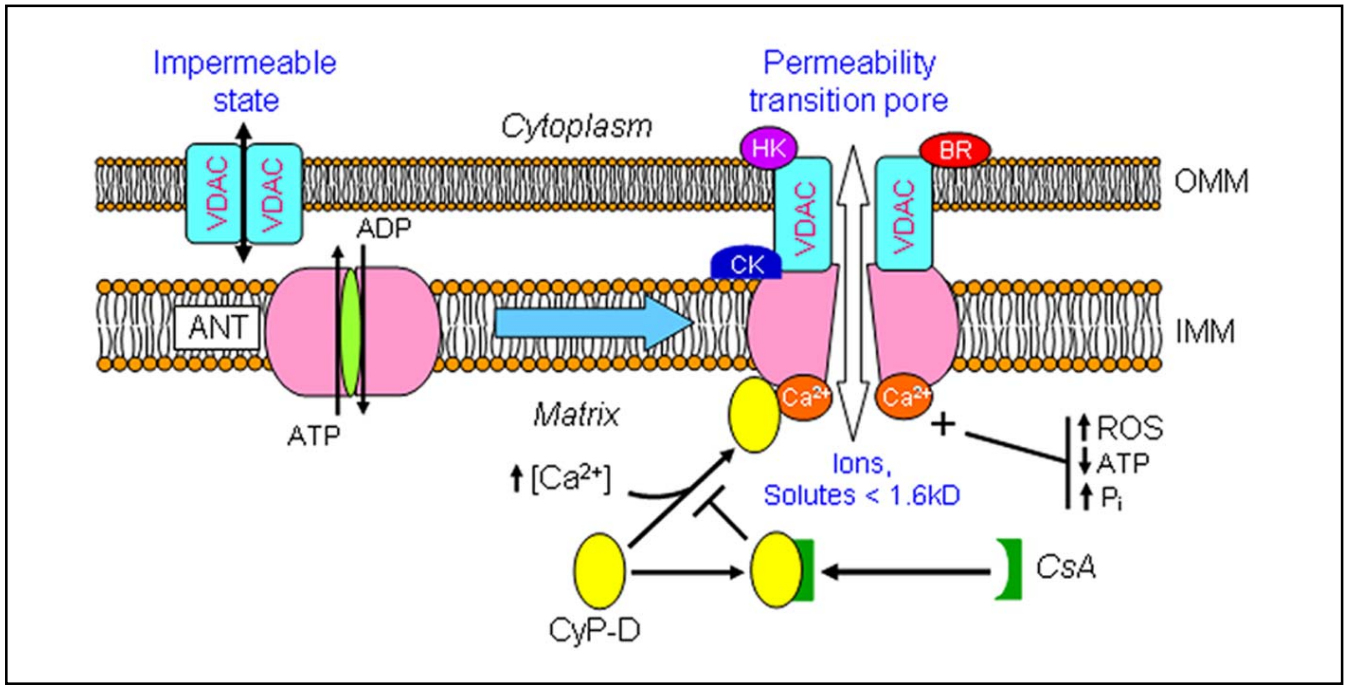

Fig. 1. The commonly held mechanism of mitochondrial permeability transition pore (PTP) formation. As described in the text, the core components of the mitochondrial PTP are thought to be cyclophilin D (CyP-D), the adenine nucleotide translocase (ANT) and the voltage-dependent anion channel (VDAC - also known as porin), although the role of the latter two has been questioned. In addition, benzodiazepine receptor (BR), creatinekinase (CK), hexokinase (HK) and Bcl-2 family members have been suggested to play a regulatory role although the evidence for this is very modest. IMM, inner mitochondrial membrane, OMM, outer mitochondrial membrane, ROS, reactive oxygen species, CsA, cyclosporine A.

creatine kinase have also been proposed to be involved in pore formation although it is still debated whether these proteins are structural components or rather that they play a regulatory role [1, 3, 7].

CyP-D is a nuclear-encoded mitochondrial isoform of cyclophilin and enters mitochondria using a mitochondrial targeting sequence [18, 19]. CyP-D exhibits peptidyl-prolyl *cis-trans* isomerase (PPIase) activity, and its role in pore formation was initially proposed because the immunosuppressant cyclosporine A (CsA) acts as a potent inhibitor of the PTP with the same affinity as it inhibits the PPIase activity of the protein [18, 19]. Aside from inhibition of PTP opening, CsA induces Ca^{2+}-calmodulin-dependent inhibition of the cytosolic phosphatase, calcineurin [20]. CsA forms a high-affinity complex with its ubiquitous cytosolic binding partner (immunophilins), cyclophilin (CyP-A) [21]. The CsA-cyclophilin complex binds with the catalytic subunit of calcineurin to inhibit its phosphatase activity and thus, prevents dephosphorylation of NFAT and its translocation into the nucleus [22]. However, numerous studies suggests that the effect of CsA to inhibit PTP opening does not include calcineurin since other cyclosporine derivatives , such as the imunosupressants sanglifehrin A (SfA) and 4-methylvaline-CsA as well as non-immunosupressants NIM 811 and UNIL025 were found to exhibit similar properties in terms of blocking pore opening but were devoid of calcineurin inhibitory activity [23, 24-27]. On the other hand, the immunosuppressant FK506 inhibits calcineurin, but does not inhibit PTP opening and does not prevent mitochondrial dysfunction [28, 29] CsA prevents CyP-D binding to the ANT, whereas SfA inhibits the PPIase activity of CyP-D but does not prevent its binding to the ANT [23]. It has been proposed that CyP-D facilitates a conformational change in the ANT that is triggered by calcium and that this creates a channel. Both CsA and SfA inhibit the PTP by preventing this conformational change [23, 30]. The contribution of CyP-D to PTP formation was recently confirmed using mice in which the *Ppif* gene encoding CyP-D was knocked out [31-34]. Mitochondria from the heart, liver and brain of these "knockout" mice required much greater uptake of calcium to induce PTP opening which was insensitive to CsA. Indeed, calcium sensitivity of mitochondria in terms of PTP opening was identical to mitochondria from control mice treated with CsA.

The observations in mitochondria isolated from "knockout" mice are consistent with data from studies using purified ANT reconstituted into artificial phospholipid membranes. At high [Ca^{2+}], the ANT was found to produce a non-specific channel without CyP-D, whilst its presence enhanced the pore sensitivity to [Ca^{2+}] [35-38]. These studies confirmed the conclusions of earlier work that had implicated the ANT in PTP formation [39]. For example, the ability of adenine nucleotides to inhibit PTP opening matches their affinity for transport by the ANT and carboxyatractyloside and bongkekic acid, specific ligands of the ANT that induce different conformations of the protein, act as activators and inhibitors of the PTP, respectively [38, 39]. Furthermore, the ability of oxidative stress to sensitize PTP opening to calcium can be explained by oxidation of critical thiol groups on the ANT [39, 40]. However, despite the strength of this evidence, the results obtained from studies in mice whose liver ANT1 and ANT2 have been knocked out suggest that ANT may not be an essential component of the PTP [41]. Liver mitochondria from these mice still possessed CsA-sensitive PTP opening activity although with a greatly reduced sensitivity to [Ca^{2+}] and no sensitivity to adenine nucleotides or ligands of the ANT leading the authors to conclude that ANT is not an essential structural component of the PTP, but that it does have a role in regulating pore opening [41]. However, this interpretation may not be warranted particularly because others have shown that mouse liver contains a novel ANT isoform, ANT4 which was not knocked out in the ANT1/ANT2 knockout mice [42]. This may explain the ability of these mice to survive in view of the fact that ANT is essential for hepatic metabolism [43]. Nevertheless, the ability to show a PTP in the mitochondria that was insensitive to ligands of the ANT implies that other inner membrane proteins are capable of producing CsA-sensitive pores. This may reflect the involvement of other members of the mitochondrial carrier family [43] or an alternative mechanism involving the interaction of CyP-D with unfolded membrane proteins [44]. Further investigations using purified components reconstituted into proteoliposomes are required to demonstrate the precise role of ANT in the pore structure.

Factors that modulate PTP opening

Numerous metabolic factors can change the sensitivity of PTP to Ca^{2+} inducing its opening or closure [1, 5, 9, 45] (Fig. 1). For example, adenine nucleotide depletion and high phosphate concentrations in the matrix enhance PTP opening, probably by preventing adenine nucleotide binding to the ANT [1]. The specificity and potency of nucleotides as inhibitors of the PTP match their ability to be translocated by the ANT [46]. Specific ligands of the ANT such as carboxyatractyloside (CAT) and bongkrekic acid (BKA) affect the conformational state of the ANT by decreasing and increasing matrix adenine nucleotide binding affinity, respectively. Oxidative stress has also been shown to sensitize the PTP to Ca^{2+} by inhibition of adenine nucleotide binding as well as by increasing CyP-D binding to the ANT [15, 46, 47]. In studies investigating the effects of oxidative stress and thiol reagents on PTP opening, Cys^{160} in ANT was identified as a critical thiol group responsible for preventing the inhibitory effect of adenine nucleotides because of its cross-linking with Cys^{257}. Therefore, besides explaining the effects of oxidative stress, these studies provide further evidence for the involvement of the ANT in PTP formation [40]. High [Ca^{2+}] in the matrix favors pore opening whereas Mn^{2+} and Sr^{2+} inhibit pore opening by blocking Ca^{2+} binding [38, 48]. Protons also compete for the same binding site and thus PTP opening is inhibited by lowering the matrix pH below 7.0. [49-51]. Glutamate, aspartate [38] and histidyl [52] residues on the matrix side of the ANT may be involved in this proton-induced inhibition of the pore opening although the precise mechanism remains to be elucidated.

Nitric oxide (NO) exhibits a dual effect on the PTP opening. At high concentrations, NO has been shown to sensitize PTP opening, whereas at physiological concentrations it may inhibit [53]. In addition, it has been proposed that NO may affect the PTP directly by activation of cGMP-dependent protein kinase (PKG) [54], interaction with VDAC via its nitration [55] as well as indirectly through regulation of Ca^{2+} and ROS levels in mitochondria.

Experimental approaches for measurement of PTP opening

Various experimental approaches ranging from whole heart preparations to structural and functional characterization of individual components have been applied in studying the PTP structure, function and consequences of its opening. All of these methods have their own weaknesses and strengths. However together they provide a comprehensive approach to elucidate the role of the PTP under both normal physiological and pathological conditions.

Isolated and perfused hearts

At present, two methods are used to measure PTP opening in the heart. The first method, devised by Halestrap et al [1, 6], determines PTP opening by measuring the mitochondrial entrapment of a radioactive molecule. Briefly, isolated Langendorff-perfused hearts are loaded with [^{3}H]-2-deoxyglucose (^{3}H-DOG), that accumulates within the cytoplasm as ^{3}H-DOG-6-phosphate (^{3}H-DOG-P). ^{3}H-DOG-P can only enter the mitochondria if the PTP is open and the extent of pore opening can be determined by rapid isolation of mitochondria in the presence of EGTA. This reseals the pores in mitochondria during their isolation ensuring that they retain the ^{3}H-DOG-P within their matrix. Results are normalized by taking into account the yield of mitochondria (citrate synthase activity), the initial loading of the heart with ^{3}H-DOG and the heart weight to provide a quantitative analysis of the extent of PTP opening [24, 56]. To investigate the contributions of pathological conditions, such as ischemia/reperfusion to pore opening, the heart can be loaded with ^{3}H-DOG either before ischemia (DOG-preloading) or during reperfusion after ischemia (DOG-postloading). DOG-preloading allows the extent of pore opening that occurs during the initial phase of ischemia/reperfusion but it does not take into account whether or not some of the pores close again. However, DOG-postloading only detects those mitochondria that retain open pores. Thus, a comparison of the two sets of data provides an estimate of those mitochondria whose pores have opened and then closed again as reperfusion continues. It must be noted that any negative effect of the isolation procedure inducing additional pore opening will not be measured in this method because ^{3}H-DOG-P will be released upon pore opening during isolation. However, the DOG technique does have some limitations, the most significant of which is its inability to measure PTP opening in cells that have progressed down the necrotic pathway to the point of plasma membrane rupture. In these cells the mitochondrial inner membrane will also rupture and all of the ^{3}H-DOG entrapped in both the cytoplasm and mitochondria will be lost during isolation procedure and so will not be detected. This will lead to an underestimation of pore opening. Thus, in situations associated with extensive necrotic damage the mitochondrial DOG-entrapment technique is less useful [1, 46]. Despite these drawbacks, the ^{3}H-DOG-entrapment technique is the only method currently available for the direct measurement of PTP opening and subsequent closure in the perfused heart.

The second method, devised by DiLisa et al [57] determines the extent of PTP opening indirectly by measuring the loss of the mitochondrial NAD^+ and its release into the perfusate during reperfusion of the heart. Addition of CsA was shown to inhibit this PTP-induced NAD^+ release [2, 57]. Since ischemia/reperfusion damages the plasma membrane and increases its permeability, both cytoplasmic and mitochondrial NAD^+ will be released from the cells. Thus, the amount of the NAD^+ in the perfusate is proportional to PTP opening and can be used as a surrogate measurement of pore opening. However, NAD^+ can also be released from fragile and damaged mitochondria during the isolation procedure. Errors introduced in this way represent a significant disadvantage of this method, although normalization of the results to citrate synthase activity in isolated mitochondria may help to avoid the problem.

Isolated myocytes

At about the same time as the DOG technique was developed for measurement of PTP opening in the perfused heart, a fluorescence methodology was introduced to measure PTP opening in single hepatocytes during injury produced by t-butylhydroperoxide (t-BuOOH) [58]. Cultured hepatocytes are first loaded with the fluorescence probes, calcein and tetramethyl-rhodamine methyl ester (TMRM). After addition of t-BuOOH, mitochondria quickly fill with calcein, indicating the onset of mitochondrial permeability transition, which is accompanied by mitochondrial depolarization detected as a decrease in mitochondrial TMRM fluorescence. This method was subsequently modified by others who developed conditions under which calcein loaded both the cytosol and the mitochondria, but with the cytosolic signal silenced by quenching with Co^{2+}, leaving only the mitochondrial calcein fluorescence [59]. PTP inducers caused a rapid, though limited, decrease in mitochondrial calcein fluorescence in hepatocytes and MH1C1 cells co-loaded with Co^{2+} and calcein, which was attenuated by CsA [59]. This method has been recently applied to isolated cardiac myocytes [60-62].

Isolated mitochondria

Two methods are most commonly used to measure PTP opening in isolated mitochondria both of which involve the determination of the PTP susceptibility to added extramitochondrial Ca^{2+} that must first enter the mitochondria. One method is performed by monitoring the Ca^{2+}-induced decrease in light scattering that reflects the mitochondrial swelling accompanying by calcium-induced PTP [25, 31, 39, 41]. Briefly, Ca^{2+} is added directly

to the cuvette containing a mitochondrial suspension to induce swelling of the mitochondria and thereby, a decrease of absorbance measured at a wavelength such as 520 nm that is unaffected by the mitochondrial redox state. An alternative method was designed for the measurement of the Ca^{2+}-induced Ca^{2+} release from mitochondria that occurs due to PTP opening. Progressive additions of calcium are made to energized mitochondria and the extramitochondrial [Ca^{2+}] is monitored. Since the calcium is taken up by the mitochondria, the extramitochondrial [Ca^{2+}] is rapidly reduced after each addition until the PTP opens when mitochondria become denergised and can no longer take up more calcium but rather release what they have already accumulated. The calcium sensitivity of the PTP is expressed as the amount of added Ca^{2+} necessary to reach this point [63, 64]. Both methods have a number of drawbacks. For example, effects on PTP opening may simply reflect differences in mitochondrial membrane potential or calcium entry into the mitochondria. For this reason Halestrap's group frequently employs de-energised mitochondria in the presence of a calcium ionophore to eliminate this problem [1]. Another problem relates to the extrapolation of data obtained with isolated mitochondria to the situation *in situ*. Thus, any loss of the damaged and fragile mitochondria during isolation may result in an underestimate of pore opening.

PTP as a "mitochondrial megachannel" can also be studied electrophysiologically using patch-clamp technique [7, 65]. This technique is a reliable and direct method to measure PTP opening at the single-channel level in isolated mitochondria. However, the method has some limitations: it is laborious and results are difficult to interpret for whole cell or tissue sample [45].

PTP opening: causes and consequences

PTP opening leads to free bi-directional movement of low molecular weight molecules across the inner membrane, whilst proteins remain in the matrix. As a consequence, colloidal osmotic pressure increases and causes mitochondrial swelling. As the matrix expands, the inner membrane cristae unfold, keeping the membrane intact, but the outer membrane ruptures. This releases proteins, including apoptotic factors such as cytochrome c, from the intermembrane space into the cytoplasm, as will be discussed in section 5. In addition, after PTP opening, the inner membrane becomes permeable to protons resulting in the uncoupling of oxidative phosphorylation which in turn causes the reverse mode activation of ATPase leading to ATP hydrolysis rather than ATP synthesis. Reduction of intracellular ATP levels leads to the dysfunction of ATP-dependent processes and, through the resulting disruption of ionic homeostasis, activation of calcium-dependent degradative enzymes such as phospholipase, nucleases and proteases in the cell [1, 2, 5].

Ischemia and reperfusion

There is increasing evidence to suggest that PTP opening is crucial to the transition from reversible to irreversible reperfuson injury [*reviewed in* 1-5]. During ischemia, the absence of oxygen forces the heart to provide its ATP through glycolysis leading to the accumulation of lactic acid and a reduction in pH_i that in turn inhibits glycolysis causing further inhibition of ATP synthesis. The decrease in pH_i stimulates NHE-1 activity which represents a major mechanism for H^+ extrusion and pH_i regulation under both normal physiological and pathological conditions, especially during ischemia/reperfusion [66, 67]. As the cell attempts to restore the pH_i through NHE-1 activation, [Na^+] in the cytoplasm increases and this increase is accentuated as a result of the inhibition of the Na^+/K^+ ATPase caused by a lack of ATP. The elevation of cytoplasmatic [Na^+] then results in either reduced extrusion of Ca^{2+} through the Na^+/Ca^{2+} exchanger or the activation of reverse mode Na^+/Ca^{2+} exchanger. Either effect on the Na^+/Ca^{2+} exchanger plus the inhibition of the sarcoplasmic reticular Ca^{2+}-ATPase due to ATP depletion will result in Ca^{2+} overload in the cytoplasm and increased [Ca^{2+}] in mitochondria [68, 69]. As mentioned above, adenine nucleotide depletion and high inorganic phosphate concentration can induce PTP opening by sensitization to Ca^{2+}. However, the low pH_i inhibits pore opening, preventing its occurrence during ischemia.

Reperfusion of the heart is accompanied by re-energisation of mitochondria that can therefore take up the accumulated calcium. There is also a burst of ROS generation in the cytoplasm and mitochondria as oxygen re-enters the cell and reacts with accumulated radicals such ubisemiquinone [70-73]. Together these two factors might cause PTP opening if it were not for the low pH_i. However, within a few minutes of reperfusion, pH_i returns to normal values and all restraints on PTP opening are removed. Thus it would be predicted that at this point the PTP would open, which indeed has been observed by several laboratories using different approaches [24, 56, 74, 75]. Direct measurement of PTP opening in the intact

heart by DOG-entrapment technique has detected PTP opening starting from about the second minute of reperfusion which was associated with the normalization of pH [24, 56, 76]. The DOG-postloading method (see section 3.1) detected only about half of the ^{3}H-DOG entrapment determined with the DOG-preloading technique. This strongly supports the concept that a portion of PTP that opens initially upon reperfusion closes again later in reperfusion. Indeed, the extent of this closure appears to determine the post-ischemic recovery of the cardiac function [1, 77]. Such a partial PTP closure might result from the normalization in cytoplasmic and mitochondrial [Ca^{2+}] as oxidative phosphorylation in undamaged mitochondria provides sufficient ATP to restore ionic homeostasis. The resulting reduction in calcium load would be sufficient to induce PTP closure in some mitochondria.

The conclusion that PTPs open only during reperfusion was also confirmed by measurement of the NAD^{+} released from mitochondria after onset of reperfusion [57]. The release of NAD^{+} is considered not only as an indicator of pore opening but also as an indicator of metabolic dysfunction leading to cell death [2, 78]. A loss of NAD^{+} by mitochondria may also have additional effects on the cell. First it has been proposed to attenuate the antioxidant functioning of cells due to depletion of the NADPH that is crucial for the antioxidant defense in both the cytoplasm and mitochondria [79]. Second, it might decrease the rate of the fatty acid oxidation along with further accumulation of the acyl-CoA and acyl-carnitine, lipophylic compounds that may damage membrane lipids and proteins thereby affecting the membrane fluidity and ion transport [80, 81]. Thirdly, loss of NAD^{+} by mitochondria has the potential to cause release of Ca^{2+} from the sarcoplasmic reticulum due to synthesis of cyclic nucleotides such as cADP ribose from NAD^{+} in cytoplasm, and this in turn might further amplify PTP opening [78].

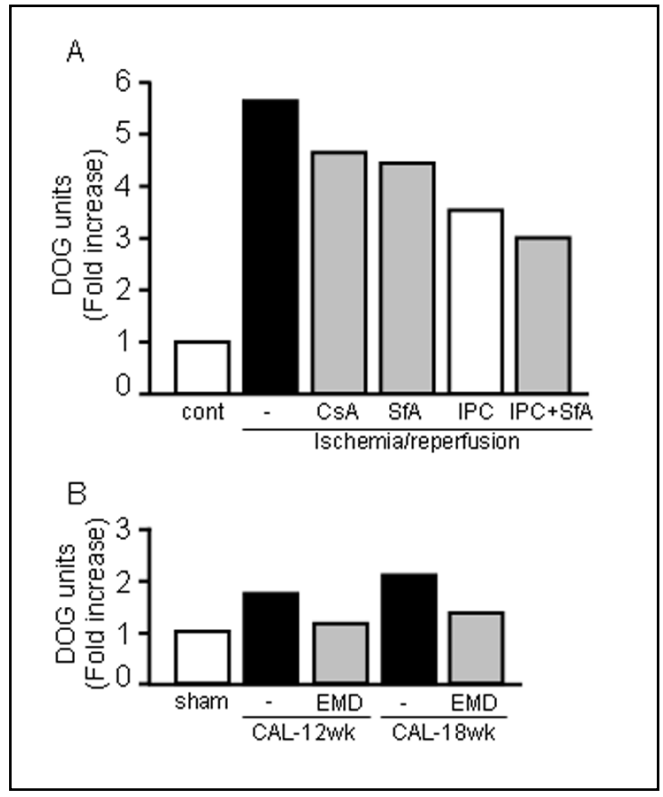

Fig. 2. Mitochondrial PTP opening measured by [^{3}H]-2-deoxyglucose (DOG)-preloading method in isolated and Langendorff-perfused rat hearts. A. Hearts were perfused with or without 0.2 μM cyclosporine A (CsA) or sanglifehrin A (SfA), or subjected to ischemic preconditioning (IPC) by two cycles of 5 min global ischemia interspersed with 5 min reperfusion prior to 30 min global ischemia and 30 min reperfusion. B. Hearts were perfused to measure PTP opening at 12 and 18 weeks after coronary artery ligation (CAL-12wk and CAL-18wk, respectively). Animals were treated with NHE-1 specific inhibitor EMD-87580 (EMD) throughout post-surgical period. Results are represented as fold increase relative to control (cont, A) or sham (B) groups. Data are taken from Refs. [25, 86].

Post-infarction remodeling and heart failure

The concept of post-infarction ventricular remodeling was initially defined as alterations which occur in terms of ventricular geometric architecture as a response to insult which eventually results in, or at least contributes to, defective ventricular function. As such, myocardial remodeling represents an important therapeutic target aimed at mitigating post-infarction deterioration of ventricular function. Although remodeling can occur early after insult, it is generally considered to be a chronic event which progressively increases in severity [*reviewed in* 82-85].

Although it is beyond the scope of this review, it is important to emphasize the complexity of the remodeling process since it is now appreciated that remodeling is associated with various aspects of pathology such as hypertrophy, extracellular matrix deposition, as well as what may be considered as cellular remodeling which encompasses defective cell function including ionic regulation, generation of ROS, energy production and substrate utilization [82-85]. We have proposed that "mitochondrial remodeling" represents an important component of the remodeling program and one which

may determine reversibility of remodeling and functional recovery following infarction [86]. The complexity of remodeling is further exemplified by the contribution of diverse cell signaling pathways whose activation results in the development of the final phenotype.

In terms of the role of mitochondria in the remodeling process, there have been only a limited number of studies demonstrating the potential contribution of PTP opening to post-infarction remodeling and heart failure. We have recently demonstrated no changes in mitochondrial function of PTP opening 6 weeks after coronary artery ligation (CAL) in rats despite the presence of myocardial hypertrophy [86], although remodeling and hemodynamic abnormalities were evident 4 weeks after initiation of CAL [87]. However, prolongation of post-ligation period to either 12 weeks or 18 weeks resulted in an increase in mitochondrial PTP opening measured by DOG-preloading method [86]. It should be noted that the extent of PTP opening in hearts subjected to CAL *in vivo* was significantly less than detected in hearts after *ex vivo* ischemia/reperfusion that could be explained by development of adaptive response during the post-infarction period (Fig. 2). These mitochondria demonstrated decreased respiratory function and citrate synthase activity as well as enhanced sensitivity to extramitochondrial Ca^{2+} suggesting that the latter may account, at least in part, for the increased PTP opening [86]. Recently, we have also found that these mitochondria were associated with downregulation of various transcription factors including PGC-1α and its downstream pathways [88]. At present, it is difficult to precisely delineate the relevance of altered mitochondrial function to the post-infarction remodeling process. Interestingly, the time required to initiate defective mitochondrial function corresponds to a degree to the transition from reversible to irreversible post-infarction myocardial remodeling. It is therefore attractive to speculate that this transition period is dependent on defective mitochondrial function as manifested by PTP opening and other alterations.

Cardiomyocytes isolated from failing left ventricular myocardium of dogs exhibited enhanced PTP opening which was associated with a low rate of ATP synthesis and a reduction in $\Delta\Psi_m$. CsA inhibited PTP opening, improved respiratory function of mitochondria and protected the heart against heart failure [89, 90].

Mitochondrial dysfunction could also occur in the early stages of the hypertrophic response and not be observed in mitochondrial preparation due to loss of fragile and damaged mitochondria during the isolation procedure. To test this hypothesis and to determine the effect of hypertrophy *per se*, we examined PTP opening in neonatal cardiomyocytes treated with α_1-adrenoceptor agonist phenylephrine for 24h [91]. Phenylephrine-induced hypertrophy was associated with a significant PTP opening and reduction in mitochondrial $\Delta\Psi_m$. The elevation in $[Ca^{2+}]$ concomitant with extensive ROS production appears to be major factors leading to pore opening during hypertrophy. In this respect, PTP opening was associated with an increase of the superoxide production concentration in hypertrophic cells. CsA inhibited pore opening coincident with its anti-hypertrophic effect and normalization of the ROS production and $\Delta\Psi_m$ [91].

It should be noted that although calcineurin-independent effects of CsA to inhibit PTP opening have been demonstrated in various studies, conclusions made only on the basis of inhibition by CsA is not sufficient, especially during hypertrophy, where the Ca^{2+}-calcineurin-NFAT pathway is upregulated. Besides inhibition by CsA, proof of concept of PTP involvement should be provided based on multifaceted approaches including measurement of $\Delta\Psi_m$ loss as well as demonstration of ATP depletion, increased ROS production as well as Ca^{2+} overload.

PTP opening and cell death

As previously discussed, PTP opening is accompanied by mitochondrial depolarization and massive swelling due to an influx of ions and water into the matrix. If the stress experienced by the cell in terms of calcium overload, ROS production and ATP depletion is too severe, the extent of PTP opening will be catastrophic and necrotic cell death becomes inevitable. However, if the insult is more moderate, PTP opening might be only be transient, as some mitochondria reseal during reperfusion. This would allow mitochondria to restore ATP production by oxidative phosphorylation thus possibly allowing the cell to avoid necrosis. However, this does not necessarily mean that the cell will survive because mitochondria also play a crucial role in apoptosis [*reviewed in* 4, 10, 92]. Transient PTP opening has been shown in perfused hearts using the DOG entrapment technique during reperfusion after short periods (10-20 min) of ischemia which was accompanied by total recovery of the heart function and ATP levels [24, 76]. It has also been detected in isolated mitochondria and cultured cells where it was not associated with either matrix swelling or $\Delta\Psi_m$ collapse in a significant proportion of the mitochondria at any one time [59, 93].

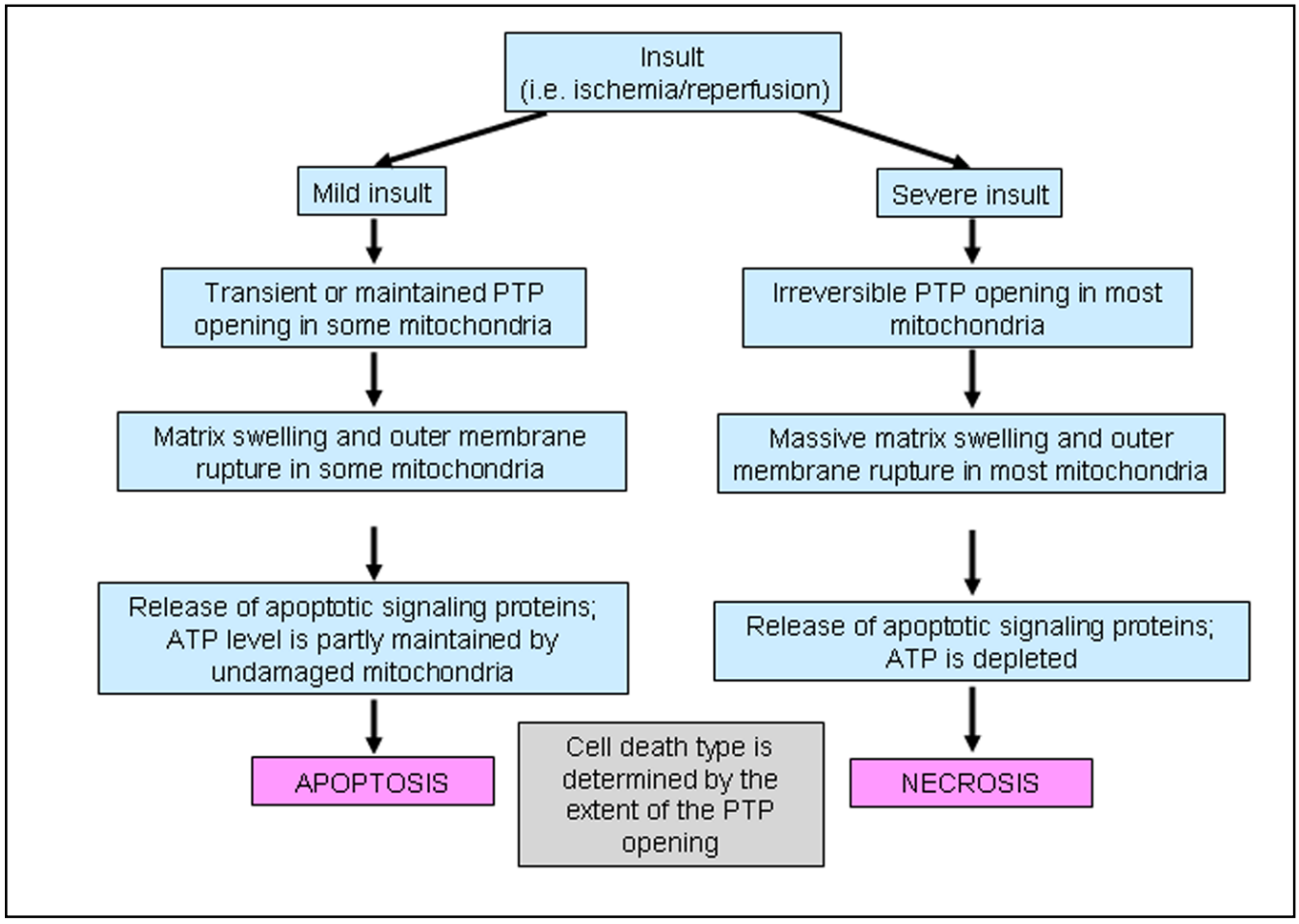

Fig. 3. The proposed mechanism of the PTP-mediated cell death by apoptosis and necrosis. Cell death by either apoptosis or necrosis is dependent on the extent of the mitochondrial PTP opening as a result of the mild or severe insult.

Apoptosis

Upon PTP-induced outer membrane rupture, intermembrane proteins (the intermembrane space contains more than 100 proteins) are released into the cytoplasm including apoptotic factors such as cytochrome c, AIF (apoptosis-inducing factor), Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with low pI), Omi/HtrA2 (mammalian serine protease Omi/high-temperature requirement protein A2) and endonuclease G (EndoG). Once released, these proteins can promote cell death via both caspase-dependent and caspase-independent mechanisms. Cytochrome c binds to the cytosolic protein Apaf-1 in the presence of dATP, which facilitates formation of protein complexes known as "apoptosomes". The apoptosome facilitates activation of pro-caspase 9 which in turn leads to caspase 3 activation and DNA fragmentation [94-97]. In addition, Smac/DIABLO blocks inhibitors of apoptosis (IAPs), thereby allowing activation of caspase 3 and caspase 9 [98, 99]. In recent years AIF and Endo G have been discovered as inducers of the caspase-independent DNA fragmentation and cell death [100, 101]. Although these factors will also be released following a severe insult, cells will die via necrosis rather than apoptosis since apoptosis is an ATP-dependent process and cannot occur if PTP pores remain open and uncouple oxidative phosphorylation (Fig. 3). Thus the relative contribution of apoptosis and necrosis to cell death in ischemia/reperfusion will depend on the severity of the insult which will be heterogeneous across the heart. The centre of the ischemic area is likely to be necrotic but the peripheral region, where collateral flow may produce only partial ischemia, could exhibit apoptosis. Indeed there is strong evidence that the necrotic centre of an infarct is surrounded by a peripheral ring of apoptosis [11].

Another complication when considering the role of

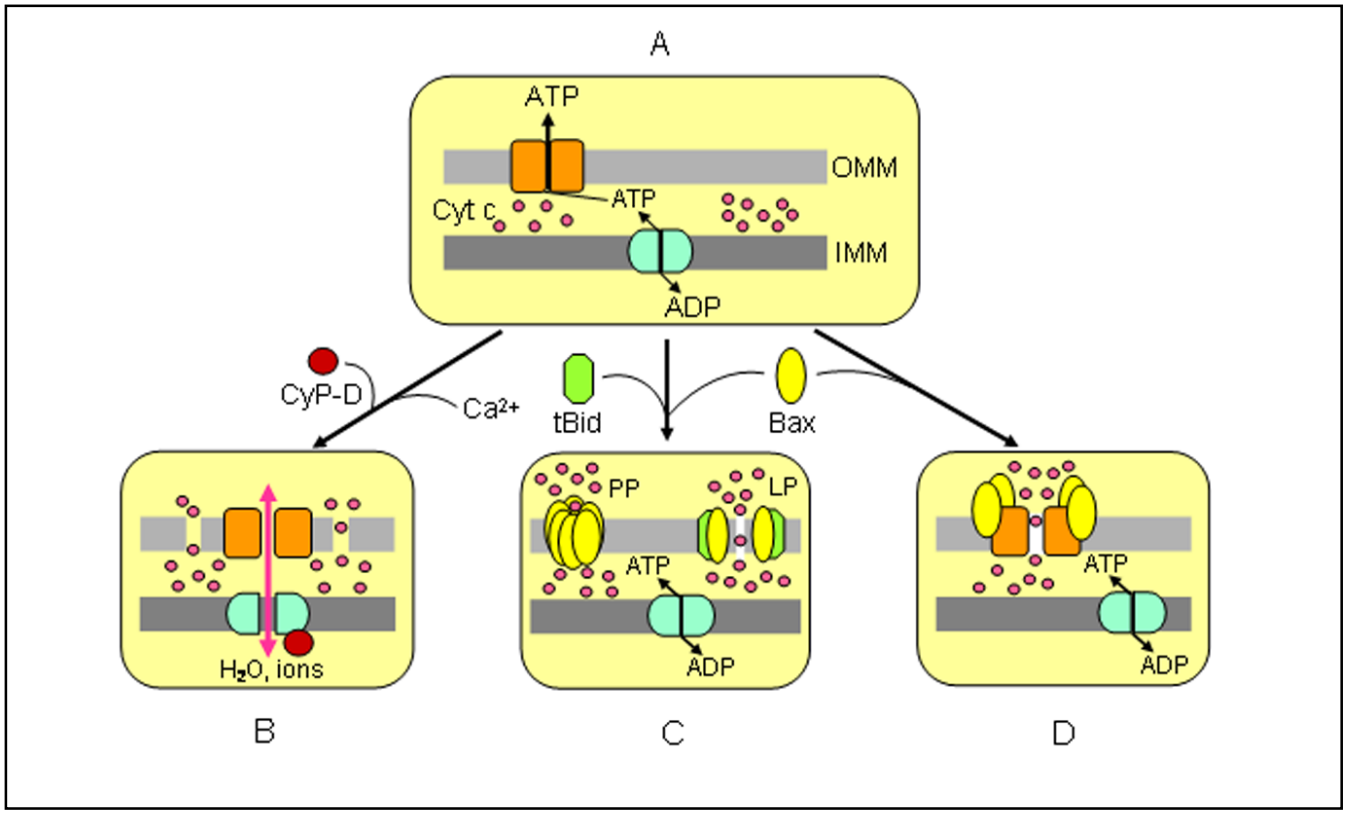

Fig. 4. Hypothetical models of the release of apoptotic proteins such as cytochrome c from the mitochondrial intermembrane space. A. The inner mitochondrial membrane (IMM) is impermeable most metabolites and ions under normal aerobic conditions thus providing optimal conditions for oxidative phosphorylation and ATP synthesis. B. An external stress such as ischemia/reperfusion accompanied by cytoplasmic and mitochondrial Ca^{2+} overload, ROS generation and ATP depletion changes the permeability of mitochondria leading to PTP formation in the IMM. PTP opening is accompanied by mitochondrial depolarization and massive swelling as a consequence of the increase in colloidal osmotic pressure due to influx of ions and water to matrix. C. Supramolecular pore opening in the outer mitochondrial membrane capable of releasing of large molecules can be induced by oligomerized Bax (protein pores-PP) or its monomeric form in the presence of tBid (lipid pores-LP). D. Recombinant Bax and Bak can interact with VDAC allowing cytochrome c to be released.

mitochondria in cardiomyocyte death is based on reports by several groups that different mitochondrial populations in cardiac cells respond differently to calcium overload [102-104], although the cause of these differences is unclear. Nevertheless, at similar levels of Ca^{2+} loading, subsarcolemmal mitochondria released cytochrome c whereas interfibrillar mitochondria did not, even when the capacity of interfibrillar mitochondria for Ca^{2+} accumulation was exceeded [104]. It has been suggested that cytochrome c released from sensitive mitochondria can induce caspase activation in the cytoplasm, which in turn can stimulate PTP opening in other mitochondria thus further amplifying cytochrome c release [105]. In addition, translocation of the pro-apoptotic proteins Bax and Bak to the mitochondria may involve their interaction with the PTP components and stimulation of pore opening [106-108].

It should be noted that under most circumstances where cells die by apoptosis, the release of cytochrome c and other apoptotic proteins from mitochondria may be independent of the PTP opening (Fig. 4). The strongest evidence in favor of this comes from studies on CyP-D knockout mice. These mice appeared quite normal, demonstrating that the apoptoptic processes associated with development were still occurring [31, 33]. Furthermore, cells isolated from the hearts demonstrated no protection against apoptotic insults although they were protected against necrosis [31, 33]. It should be noted that data with CyP-D knockout mice should be interpreted with caution as the function of the knockout protein can

be compensated for by upregulation of other proteins during development [109]. In addition, neuronal cells over-expressing CyP-D were more resistant to nitric oxide- or staurosporine-induced apoptosis but demonstrated hypersensitivity to necrotic cell death induced by Ca^{2+} and oxidative stress [110]. These more recent data complement earlier studies showing that apoptosis can occur without mitochondrial depolarization [111]. Cytochrome c release from mitochondria under such conditions involves translocation to the mitochondria of pro-apopoptic proteins of the Bcl-2 family, such as Bax. However, the mechanism by which Bax induces cytochrome c from the intermembrane space remains controversial. Two possibilities have been suggested. Either Bax itself forms channels in the outer mitochondrial membrane or Bax interacts with an existing outer membrane protein such as VDAC [3, 4]. The first model is based on the observation that oligomerized Bax forms pH- and voltage-dependent ion-conducting channels in artificial lipid membranes that allow cytochrome c release and can be blocked by Bcl-2 [112, 113]. Moreover, addition of Bax (oligomerized Bax alone or monomeric Bax in the presence of tBid) to the outer mitochondrial membrane induced the formation of supramolecular pores capable of releasing large (2 MDa) dextran molecules (Fig. 4C) [114]. There is some evidence that Bid is essential for the Bax-induced pore formation in the outer mitochondrial membrane [115], whilst others have implicated cardiolipin in this phenomenon [114]. The second model is based on ability of Bax to interact with VDAC (Fig. 4D). The addition of recombinant Bax and Bak to VDAC reconstituted into liposomes caused the formation of pores that allowed the passage of cytochrome c, whereas addition of Bcl-X_L closed these pores and prevented cytochrome c release [116]. In addition, VDAC-deficient but not ANT-deficient yeast mitochondria show resistance to cytochrome c release, $\Delta\Psi_m$ loss, and swelling caused by Bax and PTP opening inducers [117]. Furthermore, mitochondrial Bax can form a protein complex with VDAC and this may play a crucial role in acute ethanol-induced hepatocyte apoptosis, which is effectively prevented by anti-VDAC antibodies [118]. However, other studies imply that VDAC is not involved in either the Bax-induced release of cytochrome c or its prevention by Bcl-X_L [119, 120]. Moreover, VDAC2 has been shown to inhibit Bak activation and mitochondria-mediated apoptosis [121, 122]. Recombinant Bax incorporated into mitochondria did not influence Ca^{2+}-induced PTP opening, measured by patch-clamp, and induced mitochondrial swelling only when present at very high (500 nM) concentrations compared to those found physiologically in mitochondria (20 nM) [123]. On the other hand, the anti-apoptotic protein Bcl-2 has been shown to prevent loss of $\Delta\Psi_m$ and mitochondrial dysfunction induced by various agents, including Ca^{2+}, H_2O_2 and ceramide [124, 125]. In the lipid bilayer reconstitution system, Bcl-X_L forms a monovalent cation-selective channel that conducts sodium and is inhibited by Ca^{2+} [126]. These findings suggest that the ratio of death agonists, such as Bax and Bak to death antagonists, such as Bcl-2 and Bcl-X_L plays a crucial role in mitochondria-mediated apoptosis. It must be noted that the majority of studies mentioned above used artificial membranes or isolated mitochondria to elucidate the interactions between apoptotic proteins and mitochondrial membrane components. Therefore caution should be taken in interpretation of these results and application of these mechanisms to mitochondria-mediated apoptosis *in vivo*.

PTP opening: a target for cardioprotection

If PTP opening represents a critical step in ischemia/reperfusion injury or other cardiac pathologies, then interventions that prevent PTP opening should be beneficial. In general terms two approaches might be used for inhibiting the PTP: direct targeting one of the PTP components (i.e. the ANT and CyP-D) or indirect inhibition of the PTP by modulating calcium overload, ROS accumulation, ATP depletion or intracellular pH.

Cardioprotective effect mediated through regulation PTP components

Since CsA inhibits PTP formation by preventing CyP-D binding to the ANT, it would be predicted to be cardioprotective. Indeed, this has been demonstrated in isolated cardiomyocytes subjected to anoxia and reoxygenation [127-129], Langendorff-perfused hearts subjected to global ischemia/reperfusion [24, 56], as well as in animals subjected to CAL and reperfusion [130, 131]. One problem with the use of CsA is that it can induce additional effects through inhibition of the Ca^{2+}-regulated protein phosphatase, calcineurin. To overcome this, CsA analogues ([MeAla6]CsA, 4-methyl-val-CsA) which are without effect on calcineurin can be used [24, 56, 76] as can SfA [23, 25] (Fig. 2A). Inhibition of the PTP in these studies was associated with the improvement in haemodynamic parameters and a reduction of the lactate dehydrogenase release into effluent. Myocardial ATP/ADP ratios and AMP levels were also higher in the

presence of PTP opening inhibitors. Interestingly, the anti-cancer drug, doxorubicin, induces cardiomyopathy and cardiomyocytes isolated from hearts treated with this drug exhibited a higher susceptibility to calcium-induced PTP opening and cell death, both of which were prevented by CsA [132].

It must be noted that CsA and SfA provide only modest inhibition of the initial PTP opening during reperfusion after prolonged ischemia. This is probably due to a very high concentration of Ca^{2+} and ROS during the first minutes of reperfusion [23, 24] since it is known that CsA does not inhibit the PTP under conditions of high calcium overload and oxidative stress [39]. The failure of CsA to inhibit the pore opening cannot be overcome by using a higher concentration of the inhibitor. Indeed, mitochondria from CyP-D knockout mice still exhibit PTP opening at very high [Ca^{2+}] that is indistinguishable from mitochondria of control rats in the presence of CsA [31]. Furthermore, in both isolated cardiomyocytes and perfused hearts, CsA can only exert its protective effect within a narrow margin of concentrations, the optimal concentration of CsA for protection being about 0.2 μM [25, 56, 57]. Most likely, this reflects either deleterious effects of inhibiting calcineurin related to CsA or additional roles for cyclophilins within cells. Thus cardiomyocytes in which cytosolic cyclophilin-A (CyP-A) had been knocked down demonstrated greater sensitivity to oxidative stress than control myocytes [133], whereas the CyP-D-overexpressed cells exhibited more resistance to oxidative stress than wild-type cells implying a protective role for CyP-D in addition to its involvement in the PTP [134]. It is possible that cyclophilins initially protect the mitochondria from oxidative stress at low ROS concentrations but at higher ROS concentrations, such as during a prolonged or severe insult cyclophilins are transformed into inducers of PTP opening. In this respect, cyclophilins have been shown to induce a reductive regeneration of oxidized peroxiredoxins after each catalytic cycle [135]. Thus CyP-A and CyP-D downregulation might respectively increase cytosolic and mitochondrial ROS levels as a result of peroxiredoxin inactivation. In addition, oxidative stress-induced intramolecular disulfide bonds have been shown to be reduced in chloroplasts by specific binding of a cyclophilin to a thioredoxin [136].

Although cyclophilins act as foldases and chaperones that play a role in protein assembly and signaling [137], the normal role of CyP-D remains a mystery since CyP-D knockout mice appear normal [31, 33]. In such CyP-D-deficient mice, the infarcted area of the heart and brain following ischemia/reperfusion was dramatically reduced and lactate dehydrogenase release was almost completely inhibited [31, 33, 34]. Indeed, as predicted, the protection against pore opening in the CyP-D knockout mice mimicked the effects of CsA in wild-type mice. CyP-D-deficient cells also showed higher resistance to necrotic cell death induced by ROS and Ca^{2+} overload [33]. Furthermore, mitochondria isolated from the heart, liver and brain of CyP-D knockout (*Ppif* null) mice were more resistant to swelling and PTP opening induced by Ca^{2+} and/or atractyloside compared to mitochondria isolated from wild-type mice [31]. An intriguing question for which there is currently no answer is why CyP-D has not been lost during evolution if its loss protects the heart and brain from ischemic damage.

The ANT ligands atractyloside and bongrekic acid are known to trap the ANT in two distinct conformations with the ADP/ATP-binding site on either the cytoplasmic side (*c*-state) on matrix side (*m*-state) respectively, and these two states enhance or inhibit PTP opening respectively [5, 46]. However, their usefulness in probing the role of the PTP in cell death is severely restricted by their potent inhibition of ATP/ADP exchange across the mitochondrial inner membrane. Nevertheless, studies on isolated mitochondria and cultured cardiomyocytes have confirmed a protective role of bongkrekic acid via inhibition of pore opening [60, 138, 139] whilst atractyloside had the opposite effect [140, 141]. It has recently been suggested that the NO donor diethylenetriamine may prevent PTP opening in hearts subjected to *in vivo* ischemia/reperfusion by targeting the ANT since administration of atractyloside before coronary artery occlusion completely attenuated NO-induced protection against Ca^{2+}-induced mitochondrial swelling [142]. However, as noted above, the atractyloside may be acting directly to impair recovery by inhibiting the ANT catalytic activity.

Inhibition of the PTP by reducing intracellular Ca^{2+} overload

Since Ca^{2+} is a key inducer of pore opening, attenuation of Ca^{2+} overload may provide protection against PTP opening. In this respect the use of the NHE-1 specific inhibitors especially during ischemia/reperfusion is one of the most promising therapeutic strategies. Activation of the NHE-1 due to intracellular acidosis during ischemia leads to [Na^+] elevation in the cytoplasm that, in turn, causes the Na^+/ Ca^{2+} exchanger to act in reverse mode leading to Ca^{2+} accumulation. Whereas

NHE-1 is activated by intracellular acidosis during ischemia, NHE-1 stimulation during hypertrophy is induced by multiple signaling pathways activated via various protein kinases and G protein-coupled receptors which mediate hypertrophic responses to paracrine, autocrine and hormonal stimuli, leading to accumulation of intracellular Ca^{2+} and Na^{+} [66, 68, 69]. It has been proposed that a mild increase of ROS during short-time ischemia and hypertrophy might induce reversible opening of the PTP and induce Ca^{2+} efflux from the mitochondrial matrix, thus acting as a Ca^{2+}-regulatory mechanism in parallel with mitochondrial Na^{+}/ Ca^{2+} exchanger [*reviewed in* 143, 144]. However, this would require mild uncoupling which would support less oxidative phosphorylation and ATP production, both of which would promote deleterious effects on cardioprotection.

Attenuation of NHE-1 activity by specific inhibitors has been shown to be one of the most effective pharmacological approaches available to protect the heart from ischemia/reperfusion and heart failure [*reviewed in* 68, 69, 145]. Besides attenuating Ca^{2+} accumulation, NHE-1 inhibition will induce a lower intracellular pH during early reperfusion, and low pH is known to inhibit PTP opening. This may explain why maintenance of the acidic intracellular pH through inhibition of NHE in neonatal cardiomyocytes and myocardium offers protection against pH-dependent reperfusion injury ("pH paradox") and facilitates full recovery of cell and heart function [146, 147]. Results of experiments performed in our laboratory showed that inhibition of NHE-1 by the specific inhibitor N-[2-methyl-4,5-bis (methylsulfonyl)-benzoyl]-guanidine (EMD-87580) protects the heart from PTP opening both at 12 weeks and 18 weeks after CAL in rats (Fig. 2B) [86]. Inhibition of PTP was associated with improvement of the mitochondrial respiratory function and reduced myocardial hypertrophy. Mitochondria isolated from EMD-87580-treated ligated hearts were less sensitive to extramitochondrial Ca^{2+} than control hearts subjected to the same procedure indicating desensitisation of the PTP to added Ca^{2+}, perhaps reflecting less Ca^{2+} overload [86]. Furthermore, in neonatal cardiomyocytes subjected to oxidative stress, the NHE-1 inhibitor cariporide also attenuated the mitochondrial and cytoplasmic [Ca^{2+}] overload and prevented mitochondrial depolarization [148]. The protective effect of NHE-1 inhibition on mitochondria is unlikely to be direct because EMD-87580 does not exhibit any beneficial effect when isolated mitochondria are subject to simulated ischemia/ reperfusion [88]. As might be predicted, the presence of the NHE-1 inhibitor also decreased PTP opening in the Langendorff-perfused hearts, and this was associated with improved hemodynamic recovery and decreased release of EndoG and AIF from the mitochondrial intermembrane space (Javadov S et al, unpublished data). In addition to its effects on post-infarction remodeling and heart failure, inhibition of the NHE-1 with EMD-87580 attenuated PTP opening during cell hypertrophy induced by the α_1-agonist phenylephrine in cultured neonatal cardiomyocytes which was associated with reduced accumulation of mitochondria-generated superoxide and mitochondrial depolarization [91].

There are only a few studies showing that inhibition of Ca^{2+} influx into cells may be cardioprotective through inhibition of PTP opening. Recently, a compound identified as an oxygen-bridged dinuclear ruthenium amine complex (Ru_{360}) was used to study its effect on PTP opening in hearts during ischemia/reperfusion [149]. This complex has now been established as the most potent and specific inhibitor of the mitochondrial Ca^{2+} uniporter *in vitro* which has no effect on Ca^{2+} movement in sarcoplasmic reticulum as well as on the sarcolemmal Na^{+}/Ca^{2+} exchanger or L-type calcium channel currents [150]. Ru_{360} treatment dramatically decreased mitochondrial [Ca^{2+}] and inhibited PTP opening which was associated with improved recovery of heart performance [149]. It should be noted that the mitochondrial Ca^{2+} uniporter is a highly selective ion channel with extremely high affinity for Ca^{2+} [151]. Inhibition of PTP opening and attenuation of the mitochondrial [Ca^{2+}] elevation was also observed in perfused guinea-pig hearts treated with S-pyrapyridolol, a novel 5-HT_{1A} receptor antagonist during ischemia/ reperfusion [152]. However, this effect of S-pyrapyridolol on mitochondrial [Ca^{2+}] seems to be indirect.

Inhibition of PTP opening by free radical scavengers

In addition to ATP synthesis, the electron transport chain of the mitochondria is a significant source of ROS, mainly superoxide, which is generated at two sites: the reduced flavin mononucleotide of NADH dehydrogenase in complex I and the ubisemiquinone radical intermediate ($QH^{\cdot}$), formed during the Q cycle at the Q_o site of complex III [71]. In addition, alternative $P66^{Sch}$-mediated redox reactions have been recently demonstrated to generate mitochondrial ROS as signaling molecules for apoptosis [153]. ROS production also occurs in the cytoplasm through enzymes such as NADPH oxidase, nitric oxide synthase and xanthine oxidase. ROS accumulation plays a crucial role in cardiac diseases especially in myocardial infarction and heart failure, not least because of

their ability to activate the PTP. Indeed recent data has shown that in isolated cardiac myocytes the critical trigger for PTP opening after simulated ischemia/and reperfusion is the increase in ROS rather than mitochondrial calcium overload [154]. Interestingly, in adult cardiomyocytes PTP opening is accompanied by a greater increase in ROS production by mitochondria, a process termed mitochondrial "ROS-induced ROS release", although the mechanism underlying this phenomenon is unclear [60]. Therefore it is not surprising that free radical scavengers can be effective therapeutic tools in cardioprotection. The use of antioxidants during ischemia/reperfusion may inhibit mitochondrial PTP directly by preventing ANT oxidation or/and indirectly through normalization of the function of ion pumps and channels thus re-instating intracellular ionic homeostasis [38, 155]. In recent years, the effect of various free radical scavengers on PTP opening has been investigated in different models of ischemia/reperfusion. Pyruvate may protect the heart against ischemia/reperfusion due to its free radical scavenging activity although it is also a good fuel for oxidative phosphorylation which, unlike glucose, does not require ATP-dependent activation before oxidation [156, 157]. Indeed, in the Langendorff-perfused rat heart it was demonstrated directly that addition of pyruvate to the perfusion medium before the onset of ischemia significantly inhibited PTP opening which was associated with a greater recovery of cardiac function [77]. Pyruvate treatment also caused an accumulation of intracellular lactate during ischemia and slowed the normalization of pH_i during reperfusion, which may provide yet another mechanism by which it inhibits the PTP and so provides protection [77].

Propofol, which is widely used in cardiac surgery as an anesthetic drug, also acts as a free radical scavenger [158] and propofol-treated hearts have been shown to exhibit protection against oxidative stress and ischemia/reperfusion injury [159]. The drug may also exert additional effects through inhibition of plasma membrane calcium channels [160] and at higher concentrations may inhibit *in vitro* PTP opening directly [161]. The DOG-entrapment technique has been used to demonstrate directly that in hearts subjected to *ex vivo* ischemia/reperfusion in both Langendorff-perfused and working mode, propofol inhibits PTP opening and this is accompanied by a greater post-ischemic recovery of cardiac function [162]. In addition, mitochondria isolated from propofol-treated hearts exhibited less pore opening than control mitochondria in the presence of similar $[Ca^{2+}]$, while propofol added directly to isolated mitochondria failed to exert a protective effect against PTP opening. These data are consistent with the effects of propofol being mediated indirectly by a reduction in oxidative stress and $[Ca^{2+}]$ overload [162].

A similar protective effect was observed in HL-1 cells subjected to hypoxia in the presence of antioxidants such as trolox, ascorbic acid and melatonin. These agents abrogated PTP opening, cytochrome c release and Bax translocation, and restored ATP level and abolished DNA laddering [163]. Serofendic acid, an established neuroprotective agent against NO- and H_2O_2- induced cytotoxicity has been shown to prevent PTP opening and depolarization of mitochondrial membrane and thereby, to suppress cell death in neonatal cardiomyocytes subjected to H_2O_2-induced oxidative stress [164]. This study demonstrated that preservation of mitochondrial integrity can be achieved by partial inhibition of mitochondrial Ca^{2+} overload and ROS accumulation. More recently, antioxidants have been specifically targeted to the mitochondria by virtue of a positive charge that causes their membrane potential-dependent accumulation. These agents are especially potent at protecting the heart from ischemia/reperfusion injury [165].

Several studies have confirmed the protective effects of antioxidants on ischemia/reperfusion injury in *in vivo* models. For example, the combination of propofol and pyruvate has been shown to provide substantial protection from reperfusion injury in a pig model of open heart surgery [166]. Similarly, the antioxidant MCI-186, a potent scavenger of hydroxy radicals, inhibited PTP opening in parallel to a reduction of myocardial infarction area, cytochrome c release, DNA fragmentation and apoptosis in hearts subjected to CAL followed by reperfusion [167].

The use of ROS scavengers has also been shown to be protective during non-ischemic cardiac injuries in various animal models that may involve PTP opening. For example, mitochondria isolated from rat hearts with doxorubicin-induced cardiomyopathy demonstrated an extensive PTP opening and carvedilol, a β-adrenergic receptor antagonist with strong antioxidant properties that is used clinically for the treatment of congestive heart failure and myocardial infarction, inhibited PTP opening and preserved ATP synthesis and myocardial ultrastructure [168]. The authors attributed the cardioprotection to inhibition of pore opening by carvedilol acting as an antioxidant, as opposed to its β-adrenergic antagonist properties. This conclusion was supported by the demonstration that carvedilol decreased the number of oxidized protein thiol groups in heart mitochondria [169].

Ischemic preconditioning-mediated inhibition of PTP opening

One of the most effective protocols for cardioprotection is ischemic preconditioning (IPC) when hearts are subjected to two or three brief (3-5 min) ischemic periods with intervening recovery periods before a prolonged period of ischemia is initiated. IPC induces biphasic protection against ischemia/reperfusion injury: a first and immediate window of strong and brief (2-4h) protection, and second window approximately 24h later which is less robust but of longer (48-96h) duration. Although the precise mechanisms underlying the protective effect of IPC are still being debated, numerous receptor-dependent (adenosine, opioids, norepinephrine, prostaglandins, angiotensin II, bradykinin and endothelin-1) and receptor-independent (NO, free radicals, calcium) triggers, and intracellular mediators (PKC, K_{ATP} channels) have been proposed to play a crucial role in preconditioning [*reviewed in* 170-172]. Since PTP opening is a critical factor in ischemia/reperfusion-induced cell death, it might be expected that IPC leads to inhibition of PTP opening and this is now known to be the case. The protective effects of ischemic- [140], calcium- [129] and diazoxide-induced [173] preconditioning were first linked to the inhibition of PTP opening in isolated mitochondria and cultured myocytes models. These results were later confirmed by direct measurement of PTP opening in isolated hearts subjected to IPC with subsequent global ischemia/reperfusion [25]. DOG-preloading and DOG-postloading techniques (see section 3.1) demonstrated that IPC reduces PTP opening during reperfusion and increases subsequent pore closure. Interestingly, under these conditions IPC has a greater cardioprotective effect compared to targeting the PTP directly with CsA or SfA (Fig. 2A). In rabbit cardiomyocytes, IPC- or CsA-induced inhibition of PTP opening was shown to be accompanied by reduced apoptosis [64].

It should be noted that the mitochondria isolated from preconditioned hearts exhibited a higher or unchanged sensitivity to *in vitro* PTP opening by external Ca^{2+} thereby, showing that IPC induces inhibition of PTP *in situ* most likely by indirect mechanisms of attenuating Ca^{2+} overload and ROS generation [25, 174]. However, others have proposed that inhibition of PTP opening may be the result of direct interaction between PKCε and MAPKs, stimulated by IPC, and VDAC [141, 175]. Furthermore, it has been suggested that PKC-induced phosphorylation of the glycogen synthase kinase-3β (GSK-3β) may have an inhibitory effect on pore opening due to its interaction with VDAC and ANT [176]. More recently, it has been shown that the inhibition of PTP opening by both bradykinin [177] and the adenosine A_3 receptor agonist N_6-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA), [178] may involve PI3-kinase/Akt-mediated inactivation of GSK-3β. However, the molecular mechanism of the translocation of these signal protein kinases from cytoplasm to mitochondria and their ability to induce phosphorylation of the PTP compounds (VDAC and ANT) in the outer and inner mitochondrial membranes still remain controversial. Notably, an apparent interaction GSK-3β with VDAC and ANT may not be surprising due to the abundance of these proteins in the mitochondria [179]. There also would appear to be an inherent problem of translocation of PKC across the outer and inner mitochondrial membranes since these processes usually require specific targeting sequences [180].

A particularly controversial area in regards to how IPC may exert its effects is whether and how activation of mitochondrial K_{ATP} (mitoK_{ATP}) channels may lead to inhibition of PTP opening during ischemia/reperfusion [181]. Increased mitochondrial K^+ uptake might have several consequences [*reviewed in* 182-184]. First, it might depolarize the inner mitochondrial membrane, which in turn could reduce the driving force for mitochondrial Ca^{2+} uptake and thereby attenuate matrix Ca^{2+} overload and/or ROS production. However, depolarization would also cause a decrease in oxidative phosphorylation that would be predicted to be detrimental to the heart, increasing damage rather than being cardioprotective. Second, mitochondrial matrix swelling might occur with subsequent improvement of the contact between inner and outer membranes thereby providing lower ATP and ADP conductance, and preventing ATP hydrolysis. However, direct measurement of matrix volume in either isolated mitochondria or the perfused heart does not lend much support for this view [185, 186]. A major problem surrounding the role of mitoK_{ATP} channels in cardioprotection is the lack of specific pharmacological agents which inhibit or activate these channels [181]. Further elucidation of their role and any relationship between their activation and inhibition of PTP opening will require the use of mitoK_{ATP} openers and blockers with greater specificity. However, with no molecular identity and questions about the very existence of these channels, this may not be a simple task [181, 186, 187].

Another hypothesis relating IPC to inhibition of PTP opening at reperfusion is that transient PTP opening during preconditioning may cause mitochondrial depolarization and thus protect as has been proposed for mitoK_{ATP}

channel opening. This proposal was based on data showing that inhibition of the PTP by either CsA and SfA during preconditioning prevented the beneficial effect of IPC [188]. However, these data are hard to reconcile with the observation that CsA and SfA bind tightly to mitochondria and remain attached even after extensive washing. Thus it would be anticipated that the drugs would remain present during reperfusion and protect the heart from reperfusion injury in their own right rather than inhibiting protection induced by IPC [189].

Irrespective of the mechanisms underlying IPC, a growing body of evidence supports the concept that the inhibition of the PTP by IPC plays a key role in bestowing protection of the heart against ischemia/reperfusion injury. However, the existing data reveal no consensus as to how protein kinase pathways and mitoK_{ATP} channels may interact and inhibit PTP opening to mediate protection.

Conclusions

Mitochondrial PTP opening has been demonstrated under pathological conditions including cardiac ischemia/reperfusion, post-infarction remodeling and heart failure, when mitochondria are overloaded with Ca^{2+} in parallel with ROS generation and ATP depletion. Existing data support the central hypothesis that PTP plays a crucial role in determining life or death. Furthermore, the mode of cell death, apoptosis or necrosis, is also determined by the extent of PTP opening, which in turn depends on the duration and severity of pathological conditions. In addition to its negative effect on energy production and transport of ATP, PTP opening promotes the release of apoptotic proteins from the intermembrane space and thus, induces apoptosis. However, due to its dependence on ATP, apoptosis is unlikely to occur during severe insults when PTP opening is extensive: under such conditions, cell death will likely proceed via necrosis rather than apoptosis. In either case the PTP represents a logical target for pharmacological interventions to enhance cardiac survival under pathological conditions including ischemia/reperfusion and heart failure. In view of the growing body of evidence of PTP opening as an endpoint of cell damage, elucidating the precise mechanisms of pore opening and its consequences will be necessary for further development of specific inhibitors to be used as therapeutic tools in the clinical setting.

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