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Title Page

Mitochondrial permeability transition pore opening as a promising therapeutic target in cardiac diseases

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Running Title Page

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ABBREVIATIONS

ANT, adenine nucleotide translocase; BKA, bongkrekic acid; CAT, carboxyatractyloside; CsA, cyclosporin A, CyP-D, cyclophilin D; HF, heart failure; IMM, inner mitochondrial membrane; IR, ischemia/reperfusion; MPTP, mitochondrial permeability transition pore; NHE-1, Na^+/H^+ exchanger 1; NCE, Na^+/Ca^{2+} exchanger; PiC, phosphate carrier, PPIase, peptidyl-prolyl *cis-trans* isomerase; ROS, reactive oxygen species, SfA, sanglifehrin A, VDAC; voltage dependent anion channel

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Abstract

In addition to their central role in ATP synthesis, mitochondria play a critical role in cell death. Oxidative stress accompanied by calcium overload, ATP depletion and elevated phosphate levels induces mitochondrial permeability transition (MPT) with formation of non-specific MPT pores (MPTP) in the inner mitochondrial membrane. Pore opening results in mitochondrial dysfunction with uncoupled oxidative phosphorylation and ATP hydrolysis, ultimately leading to cell death. For the last 20 years three proteins have been accepted as key structural components of the MPTP: adenine nucleotide translocase (ANT) in the inner membrane, cyclophilin D (CyP-D) in the matrix and the voltage-dependent anion channel (VDAC) in the outer membrane. However, most recent studies have questioned the molecular identity of the pores. Genetic studies have eliminated the VDAC as an essential component of MPTP and attributed a regulatory (rather than structural) role to ANT. Currently, the phosphate carrier appears to play a crucial role in MPTP formation. MPTP opening has been examined extensively in cardiac pathological conditions including ischemia/reperfusion as well as heart failure. Accordingly, MPTP is accepted as a therapeutic target for both pharmacological and conditional strategies to block pore formation by direct interaction with MPTP components or indirectly, by decreasing MPTP-inducers. Inhibition of MPTP opening by reduction of CyP-D activity by non-immunosupressive analogs of cyclosporin A or sanglifehrin A, as well as attenuation of ROS accumulation through mitochondria-targeted antioxidants are the most promising. This review outlines our current knowledge of the structure and function of the MPTP and describes possible approaches for cardioprotection.

Introduction

Mitochondria play an important role as ATP producers and as regulators of cell death, which make them essential for cell survival. In the heart, mitochondria occupy about 30% of cardiomyocyte volume and provide more than 90% of ATP necessary for cardiac function. One of the key factors regulating mitochondrial function and ATP synthesis is the mitochondrial Ca^{2+} concentration. Cardiomyocyte Ca^{2+} homeostasis is altered under pathological conditions such as ischemia and heart failure (HF) due to decreased ATP levels resulting from inadequate oxygen consumption. Additionally, dysfunction of the electron transport chain, particularly during reperfusion, results in increased generation of ROS. Calcium overload and oxidative stress combine with other factors including high phosphate and low adenine nucleotide concentrations to induce the formation of non-specific mitochondrial permeability transition pores (MPTP) in the inner mitochondrial membrane (Bernardi et al., 1992; Crompton, 1999; Halestrap et al., 2004). Opening of MPTP causes uncoupling of the mitochondria and swelling of the matrix leading to rupture of the outer mitochondrial membrane and ultimately, cell death. In recent years MPTP opening has been considered as a therapeutic target for cardioprotection during cardiac diseases especially those associated with ischemia/reperfusion (IR) primarily to the central role of the MPTP in cell death (Di Lisa and Bernardi, 2006; Javadov and Karmazyn, 2007; Halestrap and Pasdois, 2009).

In this review we will discuss the current understanding of the molecular structure and function of the MPTP (Section 2), mechanisms of MPTP opening and its consequences during IR and HF (Section 3) and MPTP opening as a therapeutic target for cardioprotection (Section 4).

Molecular composition of the MPTP and consequences of pore opening

Structure of MPTP

Despite extensive studies, the exact molecular identity of the MPTP remains uncertain. Until recently three molecules were accepted as key structural components of the MPTP including adenine nucleotide translocase (ANT) in the inner membrane, cyclophilin D (CyP-D) in the matrix and the voltage-dependent anion channel (VDAC, also known as porin) in the outer membrane (Halestrap et al., 1998; Crompton, 1999; Zoratti et al., 2005). Although a number of proteins such as the peripheral benzodiazepine receptor, creatine kinase, hexokinase and Bcl-2 family members have been proposed to play regulatory roles in MPTP formation, the evidence for their involvement is controversial (Halestrap, 2009). Furthermore, the most recent studies from various groups have questioned the role of VDAC as an essential component of the MPTP (Krauskopf et al., 2006; Baines et al., 2007). In addition, the role of ANT has been attributed to a regulatory rather than structural function in pore formation (Kokoszka et al., 2004; Baines and Molkentin, 2009). In 2008, Halestrap's group reported new evidence for the mitochondrial phosphate carrier (PiC) as a possible key component of the MPTP structure (Leung et al., 2008). The possible contribution of the VDAC and PiC to MPTP formation will be discussed in detail below.

Adenine nucleotide translocase

The first evidence implicating ANT in MPTP formation came from studies in which ATP and an inhibitor of the ANT, bongkrekic acid (BKA) inhibited opening of the MPTP by decreasing its sensitivity to [Ca^{2+}]. In contrast, another inhibitor of the ANT carboxyatractyloside (CAT) and adenine nucleotide depletion were both able to trigger pore opening by sensitizing the pores to [Ca^{2+}] (Novgorodov et al., 1991; Halestrap et al., 1997). The divergent regulation of MPTP formation by BKA and CAT is due to opposite conformation of ANT molecule elicited by these inhibitors (Klingenberg, 2008) (see *Section 4*). The sensitivity of pore opening to [Ca^{2+}] increases

with oxidative stress which inhibits adenine nucleotide binding to the ANT through oxidation of two critical thiol groups on ANT close to adenine nucleotide binding site (McStay et al., 2002). It has been demonstrated that CyP-D binds to ANT and, in the presence of Ca^{2+}, induces a conformational change that leads to subsequent pore opening (Griffiths and Halestrap, 1991; Halestrap and Brenner, 2003).

Although there are extensive data supporting ANT as a key molecule of MPTP, studies with knock-out mice showed that ANT was not necessary for pore formation (Kokoszka et al., 2004). Mitochondria isolated from the liver of animals lacking ANT1 and ANT2 exhibited a CsA-sensitive pore opening, although the opening of the pores required much higher [Ca^{2+}] than wild-type mitochondria. The results of these experiments may be explained by an incomplete knock-out of all ANT isoforms, since ANT4 has been discovered in mouse liver (Da Cruz et al., 2005) or by the existence of another molecule apparently playing an important role in MPTP formation. A potential contribution of PiC to pore formation will be discussed in *Section 2.1.3*. Taken together, existing data exclude ANT as a structural component of the pore and attributes to it a regulatory role in MPTP formation.

Cyclophilin D

CyP-D is a nuclear-encoded mitochondrial isoform of cyclophilin with a molecular weight of 18 kDa. It enters mitochondria using a targeting sequence that is cleaved following translocation into the matrix (Connern and Halestrap, 1992; Johnson et al., 1999). At present, extensive data have been obtained in favour of CyP-D as an essential component and key regulator of MPTP using various pharmacological inhibitors and genetic manipulations. The first evidence for the involvement of CyP-D in MPTP formation came from studies showing an inhibitory effect of the immunosuppressant cyclosporin A (CsA), widely used in organ and tissue transplantation, on

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pore opening (Crompton et al., 1988). It has been shown that the effect of CsA is mediated through inhibition of peptidyl-prolyl *cis-trans* isomerase (PPIase) activity of CyP-D (Halestrap and Davidson, 1990; Tanveer et al., 1996). Besides MPTP, CsA might inhibit the calcium-activated protein phosphatase, calcineurin activity. Indeed, CsA in a complex with cytosolic CyP-A binds to the catalytic subunit of calcineurin and inhibits its phosphatase activity, thus preventing dephosphorylation of NFAT and its translocation into the nucleus (Crabtree, 1999). However two lines of evidence demonstrated that the inhibitory effect of CsA on MPTP opening is not related to inhibition of calcineurin. First, another immunosuppressant, sanglifehrin A (SfA) and CsA analogues with non-immunosupressive features (see *Section 4.1*) demonstrated an ability to inhibit pore opening without any effects on calcineurin activity (Clarke et al., 2002; Waldmeier et al., 2002; Hansson et al., 2004). Second, the immunosuppressant FK506 inhibited calcineurin activity but did not affect MPTP and mitochondrial function (Kay et al., 1990). Other evidence for the essential role of CyP-D in pore formation has been reported by several independent groups in studies with CyP-D knockout mice in which mitochondria isolated from these animals exhibited a low sensitivity to Ca^{2+} and as a result, a delayed MPTP opening that was insensitive to CsA (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005).

CyP-D favors MPTP opening by facilitating the Ca^{2+}-triggered conformational change. The inhibitory effect of CsA and its analogs involves interaction with CyP-D that reduces sensitivity of pore opening to Ca^{2+}. Most likely, interaction of CyP-D, Ca^{2+} and the pore is a multifaceted process that also includes enhancement of susceptibility of the MPTP proteins to oxidative stress.

The VDAC is not a component of MPTP

Initially the VDAC was thought to be an essential component of a "megachannel", MPTP electrophysiological properties of which were similar to those of the VDAC channel (Szabo et al., 1993). Anti-VDAC antibodies prevented Ca^{2+}-induced MPTP opening in liver mitochondria (Shimizu et al., 2001) and chemical inhibition of Ca^{2+}-induced MPTP opening was achieved by the VDAC1-binding ubiquinone analogues (such as UQ_0 and Ro 68-3400) (Cesura et al., 2003) although it was later demonstrated that this drugs do not bind to VDAC and can inhibit pore opening in VDAC1$^{-/-}$ mitochondria (Krauskopf et al., 2006). Strong evidence in favour of the VDAC was also obtained in studies where CyP-D-GST was shown to bind both the VDAC and ANT in solubilised heart mitochondria (Crompton et al., 1998), although it was not confirmed in liver mitochondria (Woodfield et al., 1998). A VDAC-ANT-CyP-D complex acted as a Ca^{2+}-dependent channel which was CsA-sensitive similar to MPTP in heart mitochondria (Crompton et al., 1998). Furthermore, inhibition of MPTP opening was associated with phosphorylation of VDAC by PKA-, PKCε- or GSK-3β (Bera et al., 1995; Baines et al., 2003; Javadov et al., 2009), although others questioned a role of mitochondrial protein phosphorylation (Clarke et al., 2008). Although these results indicate the VDAC as a critical compound of MPTP, recent studies with genetic manipulations have questioned this conclusion. Mitochondria lacking all three isoforms of the VDAC exhibited the same extent of pore opening as normal mitochondria (Baines et al., 2007). Notably, fibroblasts lacking all three isoforms of the VDAC were more sensitive to cell death induced by oxidative stress suggesting a pro-survival role of the channel. The pro-survival effect of the VDAC may be independent of MPTP formation.

Phosphate carrier

Although phosphate has been known as an activator of pore opening for a long time, the potential role of PiC in MPTP formation has only been demonstrated recently in a study showing that CyP-

D binds to PiC in a CsA-sensitive manner and that this interaction is increased by oxidative stress that sensitizes pore opening to Ca^{2+} (Leung et al., 2008). Most notably, these studies have also identified a close correlation between inhibition of MPTP opening and phosphate transport into the mitochondria (Leung et al., 2008; Halestrap and Pasdois, 2009). A possible role of the PiC in pore formation was also confirmed in other studies showing that phosphate is required for inhibition of MPTP opening through blocking CyP-D (Basso et al., 2008). PiC knockout HeLa cells were resistant to staurosporine-induced apoptosis while PiC over-expression induced apoptosis (Alcala et al., 2008). Further studies are required to establish a causative link between PiC and MPTP formation.

Consequences of MPTP opening

The inner mitochondrial membrane permeability is increased in response to stress, leading to formation of the voltage-dependent non-specific pore, MPTP. The pore is permeable to water, ions and any molecule with molecular mass less than 1.5 kDa (Halestrap et al., 1998; Crompton, 1999; Di Lisa and Bernardi, 2006). MPTP opening has several consequences that ultimately lead to mitochondrial dysfunction and cell death (Fig. 1).

Necrotic cell death occurs due to a significant reduction of ATP levels when MPTP opening is very extensive. During pore opening mitochondria become uncoupled and ATPase works in reverse mode which hydrolyzes ATP. With reduced ATP levels, the cells cannot maintain structural and functional integrity including ion homeostasis resulting in irreversible damage and cell death, predominantly through necrosis. Apoptosis may occur when only a part of the mitochondria undergoes MPTP opening and the cells still have sufficient level of ATP to direct cell death through the apoptotic pathway. MPTP opening induces entrance of molecules with low molecular weight into the matrix thus leading to equilibration of the solutes through

both sides of the inner membrane. However, mitochondrial proteins are retained in the matrix and thus increase the colloidal osmotic pressure leading to matrix swelling. Since the surface area of the inner membrane is larger than that of the outer membrane, the outer membrane can rupture while leaving the integrity of the inner membrane relatively intact. Rupture of the outer membrane may be accompanied by the release of pro-apoptotic proteins into the cytoplasm that in turn can induce apoptosis. It should be noted that mitochondria-initiated apoptosis can occur through a mitochondrial pore-independent mechanism due to release of pro-apoptotic proteins from the intermembrane space (Fig. 1). Murine embryonic fibroblast cells and hepatocytes from CypD-deficient mice died normally in response to various apoptotic stimuli, but showed resistance to necrotic cell death induced by ROS and Ca^{2+} overload (Nakagawa et al., 2005). Furthermore, overexpression of Bax resulted in equivalent apoptotic cell death in wild-type and CyP-D knock out fibroblasts, suggesting that Bax and tBid can induce cytochrome c release and initiate mitochondrial-dependent cell death through a mitochondrial pore-independent mechanism (Baines et al., 2005). Conversely, CyP-D overexpression in B50 neuronal cells promoted MPTP opening and necrotic cell death, although it inhibited apoptotic cell death induced by high Ca^{2+} and oxidative stress (Li et al., 2004). Despite the large number of studies, the precise mechanisms underlying cytochrome c release from the intermembrane space still remain to be elucidated. The studies conducted on isolated cells, mitochondria, and artificial membranes demonstrated that the pro-apoptotic protein Bax can form channels in the outer mitochondrial membrane by interacting with Bid (Antonsson et al., 2000; Kuwana et al., 2002; Terrones et al., 2004) or VDAC (Shimizu et al., 1999; Adachi et al., 2004), and inducing release of cytochrome c. Thus, MPTP opening is mostly associated with necrotic cell death rather than apoptosis.

MPTP opening in ischemia/reperfusion and heart failure

Ischemia/reperfusion

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Myocardial ischemia is defined as an imbalanace between oxygen supply and demand resulting in functional and metabolic abnormalities including mitochondrial dysfunction. Due to the reduction in oxygen mitochondria become unable to synthesize ATP leading to a rapid fall in [ATP] and a rise of [AMP] and [P_i]. Metabolic acidosis induced by accumulated lactate decreases pH_i that in turn activates the Na^+/H^+ exchanger 1 (NHE-1). While the cell attempts to restore pH_i through NHE-1, $[Na^+]_i$ rises due to suppressed Na^+/K^+-ATPase activity. A progressive elevation of $[Na^+]_i$ leads to a further increase in $[Ca^{2+}]_i$ as a result of its reduced extrusion through the Na^+/Ca^{2+} exchanger (NCE) or via reverse mode NCE (Karmazyn et al., 2001). Another major factor causing Ca^{2+} overload during ischemia is inactivation of the sarcolemmal and sarcoplasmic reticular Ca^{2+}-ATPase due to ATP depletion. In addition, despite a reduced oxygen uptake the ischemic cell still has sufficient residual oxygen to generate ROS. It has been shown in isolated perfused rat hearts that ROS levels increase with progression of ischemia (Kevin et al., 2003; Murphy, 2009). Thus, the ischemic heart contains all factors that can induce MPTP opening including ATP depletion, Ca^{2+} overload, as well as accumulation of phosphate and ROS. However, a direct measurement of MPTP opening in intact heart using the [^{3}H]-2-deoxy-glucose (DOG)-entrapment technique (DOG-method) demonstrated that MPTP opening occurs during reperfusion rather than ischemia (Griffiths and Halestrap, 1995). This is explained partially by a low pH_i in the ischemic heart which is a strong inhibitor of pore opening due to its competition with Ca^{2+} at the trigger site of ANT. A burst of ROS generation with further progression of Ca^{2+} overload in the first minutes of reperfusion is associated with MPTP opening. Notably, time-course analysis revealed that MPTP opening rapidly increases immediately after pH_i returns to normal value during the first minutes of reperfusion (Kerr et al., 1999). The use of the DOG-method prior to and after ischemia demonstrates that some MPTPs close at reperfusion (Kerr et al., 1999). This provides evidence that the mitochondria partially recover at reperfusion due to

MPTP closure and the recovery depends on the severity of ischemia. Restoring ATP synthesis and reducing Ca^{2+} overload and ROS accumulation in undamaged mitochondria can return cells to their normal metabolic and functional states. On the other hand, the extent to which the MPTP opens determines the extent of damage upon reperfusion of the ischemic heart. An alternative approach to determine the extent of the MPTP opening is measurement of NAD^{+} released from mitochondria due to pore opening at reperfusion (Di Lisa et al., 2001). A detailed discussion of the main principles and advantages/disadvantages of both methods mentioned above has been provided previously (Halestrap et al., 2004; Javadov and Karmazyn, 2007). The DOG-method is used widely by various groups working in experimental cardiology to study the protective effects of pharmacological and conditional interventions on MPTP opening during IR and HF (Javadov et al., 2005; Ciminelli et al., 2006; Prendes et al., 2008).

Heart failure

Heart failure is the common end-stage of various cardiovascular disorders, including sustained pressure or volume overload, myocardial ischemia or infarction, and inherited or acquired cardiomyopathies. It is accompanied by progressive ventricular remodeling characterized by hypertrophy of cardiomyocytes, impaired myocardial vascularization, abnormal extracellular matrix composition (fibrosis) and elevated cell death (Sutton and Sharpe, 2000; Delcayre and Swynghedauw, 2002). HF is a chronic disease with relentless progression in severity. Central to the loss of contractile function in failing hearts is the inability of mitochondria to adequately supply the myocardium with ATP, resulting in energy deprivation of the cardiac cells (Giordano, 2005). Reduced ATP synthesis has been demonstrated in mitochondria isolated from failing hearts (Sharov et al., 2000). Additionally, in failing hearts, persistent sympathetic tone leads to calcium mishandling that result in prolongation and slowed decay of the Ca^{2+} transient (Houser et

al., 2000). Dysregulation of calcium handling in HF may lead to mitochondrial calcium overload. Furthermore, mitochondria are a major source of ROS in failing myocardium, mostly due to inhibition of complexes I and III which leads to accumulation of superoxide anion (Ide et al., 1999; Choksi et al., 2004).

Thus, progression of HF is associated with diminished energy metabolism, Ca^{2+} mishandling, and ROS generation that together can favor MPTP opening. Indeed, MPTP opening has been demonstrated in HF induced by myocardial infarction in rats (Javadov et al., 2005) and intracoronary microembolizations in dogs (Sharov et al., 2007), as well as in Ca^{2+}-induced cardiomyopathy (Nakayama et al., 2007) and diabetic cardiomyopathy (Oliveira et al., 2003). Notably, our studies have demonstrated that the extent of MPTP opening in failing hearts 12 and 18 weeks after coronary artery ligation (Javadov et al., 2005) is lower than that found following acute IR (Griffiths and Halestrap, 1995; Kerr et al., 1999; Javadov et al., 2003). Measurement of MPTP opening in both models was performed by the DOG-method in intact heart. The difference between two models may be explained by involvement of the compensatory mechanisms in the chronic disease of ventricular remodeling in failing hearts where the extent of ATP depletion, Ca^{2+}-overload and ROS generation is not as great as that found in acute global IR.

MPTP is a target to protect the heart against cardiac diseases

MPTP can be targeted by pharmacological agents that interact directly with main pore components and block pore opening, or indirectly through reduction of the level of MPTP inducers including ROS, Ca^{2+} and pH_i (Table 1).

Direct targeting of MPTP

Existing data on the direct effect of the various pharmacological agents to inhibit pore opening may be divided into two groups: agents that bind to CyP-D and agents that interact with ANT.

The first evidence on CyP-D targeting comes from studies where CsA was shown to block MPTP opening in cardiac cells during anoxia/reoxygenation (Nazareth et al., 1991). Similar data were obtained in intact hearts subjected to *ex vivo* global IR (Griffiths and Halestrap, 1995). Subsequently, a protective effect of CsA through direct inhibition of MPTP has been demonstrated in different models of ischemia followed by reperfusion showing that inhibition of pore opening was associated with a greater post-ischemic recovery of heart contractility, decreased LDH release and smaller infarction size (Hausenloy et al., 2003; Argaud et al., 2005a). To eliminate the possible involvement of calcineurin that is activated in hypertrophy and HF, non-immunosupressive analogs of CsA, 6-MeAla-CsA, *N*-Me-4-Ile-CsA (NIM811) and D-3-MeAla-4-EtVal-CsA (Debio-025) have since been developed. All of these agents are potent inhibitors of PPIase activity of CyP-D, although they lack the ability to inhibit calcineurin. Treatment with 6-MeAla-CsA, NIM811 or Debio-025 inhibited MPTP opening and attenuated IR-induced cardiac dysfunction in rats (Griffiths and Halestrap, 1995), as well as reduced infarct size in rabbits (Argaud et al., 2005a) and mice (Gomez et al., 2007). Similarly, SfA also does not inhibit calcineurin activity but blocks MPTP opening via conformational change of the pore (Clarke et al., 2002) and exerts cardioprotection against *in vivo* (Lim et al., 2007) and *ex vivo* (Hausenloy et al., 2003; Javadov et al., 2003) IR. Taken together, these data demonstrate that the protective effects of CsA are mediated through blockade of CyP-D and inhibition of MPTP opening, rather than calcineurin activity.

Similar data on the protective effect of CyP-D inhibition have been obtained in studies using CyP-D knockout mice in which animals lacking *Ppif* gene appear normal and do not demonstrate any baseline phenotype (Nakagawa et al., 2005; Luvisetto et al., 2008). The role of CyP-D under normal physiological conditions remains unknown, although cyclophilins in cytoplasm (e.g. CyP-A) have been shown to participate in protein assembly and signalling as

foldases and chaperones (Min et al., 2005). A feasible role of CyP-D in physiological reversible MPTP formation cannot be excluded. Mice lacking CyP-D demonstrated reduced infarct areas and myocyte injury as measured by LDH release against IR in both heart and brain (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Mitochondria isolated from CyP-D knockout mice were also resistant to MPTP opening induced by oxidative stress, exogenous Ca^{2+} and atractyloside (Baines et al., 2005). Notably, CsA did not exert additional protection in mitochondria isolated from CyP-D knockout mice (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005). Moreover, the protective effect of both preconditioning and postconditioning against IR was not diminished in mice lacking CyP-D (Lim et al., 2007).

Although most of the data on the effect of CsA and other inhibitors of CyP-D were obtained on acute *in vivo* and *in vitro* models of IR, there are several studies performed on other cardiac diseases. CsA reduced mitochondrial dysfunction in cardiomyocytes isolated from dogs with HF (Sharov et al., 2005; Sharov et al., 2007). Additionally, we have demonstrated that CsA blocks MPTP opening and prevents mitochondrial membrane depolarization during cardiomyocyte hypertrophy induced by the α_1-adrenergic agonist phenylephrine (Javadov et al., 2006a). An increased susceptibility of MPTP opening to stress in compensated ventricular hypertrophy was associated with enhanced expression and intramitochondrial translocation of CyP-D (Matas et al., 2009). Cell death and cardiac fibrosis were significantly decreased in hearts with a genetic model of cardiomyopathy induced by overexpression of the β2 subunit of L-type-Ca^{2+} channel in CyP-D knockout mice (Nakayama et al., 2007). Furthermore, both pharmacologic (Zhou et al., 2001) and genetic (Nakayama et al., 2007) inhibition of CyP-D had a protective effect in doxorubicin-induced cardiomyopathy.

Most recently, the use of inhibitors for MPTP opening in clinical practice has been initiated. CsA and its non-immunosuppressive analogs, as well as SfA, appear to be potential

candidates for clinical trials on humans with cardiac diseases. Indeed, inhibition of MPTP opening at the onset of reoxygenation with CsA and SfA improved contractile function and cell survival against lethal hypoxia-reoxygenation injury in human atrial tissue harvested from patients undergoing cardiac surgery (Shanmuganathan et al., 2005). Recently, in a pilot trial on 58 patients with acute ST-elevation myocardial infarction, administration of CsA at the time of reperfusion resulted in a significant reduction in infarct size of approximately 40% as measured by creatine kinase release (Piot et al., 2008).

A second attractive MPTP therapeutic target might be ANT, although its role in pore formation appears to be primarily related to regulatory rather than structural. Two inhibitors of ANT, BKA and atractyloside have been identified in MPT-related studies. The first evidence came from studies showing that BKA inhibits Ca^{2+}-induced MPTP opening in mitochondria isolated from heart and liver (Halestrap and Davidson, 1990). However atractyloside had opposite effects and sensitized the MPTP to Ca^{2+} (Haworth and Hunter, 2000; Xu et al., 2001). The opposite effect of the inhibitors was explained on the basis of ability of BKA and atractyloside to trap ANT at two distinct sides of the transporter: matrix and cytoplasmic, respectively and thus, inducing 'm' or 'c' conformations (de Macedo et al., 1993; Halestrap and Brenner, 2003). Agents enhancing the 'c' conformation such as high [Ca^{2+}] favour MPTP opening, whereas 'm' conformations are associated with blockade of the pores. Indeed, the protective effect of nitric oxide against Ca^{2+}-induced mitochondrial swelling (Wang et al., 2005) as well as the effect of calcium preconditioning in cardiomyocytes were prevented by atractyloside (Xu et al., 2001). In contrast, BKA inhibited Gαq-induced cytochrome c release and cardiomyocyte apoptosis (Adams et al., 2000). In other studies BKA prevented hypoxia-induced pore opening, loss of $\Delta\psi_m$, and apoptosis but had no effect on hypoxia-mediated cytochrome c release (Gurevich et al., 2001), suggesting that hypoxia-induced cytochrome c release may not be

mutually dependent or obligatorily-linked to MPTP formation and the reduction in $\Delta\psi_m$. Oxidative stress- and BNIP3-induced MPTP opening and cell death were prevented by BKA in isolated cardiomyocytes (Regula et al., 2002; Akao et al., 2003). It should be noted that despite the fact that BKA blocks MPTP opening, the use of the ANT inhibitors is complicated by their ability to also inhibit catalytic activity of the ADP/ATP carrier.

Indirect targeting of MPTP

Indirect strategies for inhibition of MPTP opening have been aimed at reducing mitochondrial accumulation of inducers (ROS, Ca^{2+}) of pore formation, and/or to enhance intracellular level of pore blockers (ATP, H^+). Oxidative stress apparently is a more powerful inducer of MPTP opening which can occur in the absence of Ca^{2+} overload. MPTP opening during IR has been shown to be mediated through oxidative stress rather than Ca^{2+} overload (Kim et al., 2006). Indeed, the effect of Ca^{2+} depends on the net sum of concentration of other factors favouring or blocking pore opening (Halestrap et al., 1997). The pores can be blocked by low pH_i and high concentrations of divalent cations (Mg^{2+}, Mn^{2+}, Sr^{2+}) even at high [Ca^{2+}] due to inhibition of Ca^{2+} binding to the trigger site of the MPTP complex (Haworth and Hunter, 1979; Bernardi et al., 1992). Therefore, ROS scavengers may be particularly promising from a clinical perspective. The anesthetic propofol, widely used in cardiac surgery, inhibited pore opening in isolated rat hearts subjected to IR and the effect was associated with a great post-ischemic recovery of heart performance (Javadov et al., 2000). In an *in vivo* model of IR in rats, the antioxidant MCI-186 significantly reduced myocardial infarction size and blocked pore opening (Rajesh et al., 2003). Pyruvate inhibited MPTP opening and improved cardiac function in rat hearts subjected to *ex vivo* IR (Kerr et al., 1999). The cardioprotective effects of pyruvate through inhibition of pore opening may be explained by its features to act as a ROS scavenger, as an energy substrate for

ATP synthesis, and as an inducer of acidosis. Numerous agents with antioxidant properties exerted protective effects in HL-1 cells (Vassilopoulos and Papazafiri, 2005) and cardiomyocytes (Takeda et al., 2006) against hypoxia or oxidative stress, and their influence was associated with inhibition of MPTP opening. Cell permeable and mitochondria-targeted ROS scavengers apparently have more capacity to provide the best protection through inhibition of MPTP opening due to prevention of mitochondria-generated oxygen radicals (Adlam et al., 2005).

The cardioprotective effects of the Ca^{2+} channel blockers (Ca^{2+} antagonists) in cardiac diseases have been elucidated extensively in both clinical practice and experimental studies, although the contribution of MPTP opening to their beneficial effects has not been determined. Ru360, a specific inhibitor of the Ca^{2+} uniport in mitochondria which does not affect Ca^{2+} movement across the sarcoplasmic reticulum or the sarcolemma, reduced mitochondrial [Ca^{2+}] and blocked MPTP opening. The effect of Ru360 on mitochondria was associated with a significant recovery of cardiac function after ischemia (de Jesus Garcia-Rivas et al., 2005). NHE-1 inhibition may represent one of the prospective pharmacological approaches for inhibition of MPTP opening during IR and HF. Indeed, inhibition of NHE-1 may block pore opening during IR through two mechanisms including prevention of Ca^{2+} overload and delay of pH_i recovery. As mentioned in Section 3.1, IR enhances NHE-1 activity leading to Ca^{2+} overload, and accordingly, inhibition of NHE-1 activity reduces $[Ca^{2+}]_i$ (Karmazyn et al., 2001). In addition, inhibition of NHE-1 induces acidosis which in turn slows pH_i recovery in the first minutes of reperfusion. A delayed pH_i recovery has been shown to correlate with inhibition of MPTP opening in IR (Kerr et al., 1999). We and others (Javadov et al., 2008; Prendes et al., 2008) demonstrated that cardioprotection induced by NHE-1 specific inhibitors against IR was associated with inhibition of MPTP opening. Hearts treated with NHE-1 inhibitors demonstrated reduced myocyte injury as shown by decreased low LDH release and greater recovery of cardiac function at reperfusion

(Javadov et al., 2008). A protective effect of the NHE-1 inhibitor cariporide to attenuate oxidative stress-induced mitochondrial Ca^{2+} overload and $\Delta\psi_m$ loss has been shown in neonatal cardiomyocytes (Teshima et al., 2003). Unlike acute ischemia, activation of NHE-1 during hypertrophy and HF is a multi-faceted process due to actions of autocrine, paracrine and hormonal factors leading to cellular Ca^{2+} and Na^{+} overload (Karmazyn et al., 2008). Ca^{2+} accumulation and ROS generation observed in hypertrophied and failing hearts appear to be major inducers of MPTP opening. Long-term (12 and 18 weeks) treatment of rats with the NHE-1 inhibitor EMD87580 reduced post-infarction remodelling which was associated with inhibition of MPTP opening, as well as improved mitochondrial respiration (Javadov et al., 2005) and biogenesis (Javadov et al., 2006b). Moreover, the anti-hypertrophic effects of NHE-1 inhibitors were associated with the inhibition of pore opening in isolated cardiomyocytes in response to phenylephrine (Javadov et al., 2006a), angiotensin II and endothelin-1 (Garciarena et al., 2008). It should be pointed out that mitochondrial ROS accumulation in response to pro-hypertrophic agents was abrogated in the presence of NHE-1 inhibitors (Javadov et al., 2006a; De Giusti et al., 2008; Garciarena et al., 2008).

In addition to pharmacological approaches, conditional interventions such as preconditioning (Hausenloy et al., 2002; Javadov et al., 2003; Argaud et al., 2004), postconditioning (Argaud et al., 2005b; Lim et al., 2007) and hypothermia (Bopassa et al., 2006; Khaliulin et al., 2007) have been shown to mediate their cardioprotective effects through inhibition of MPTP opening during IR. A key mechanism underlying the protective effect of these interventions may include reduction of oxidative stress.

Conclusion

In recent years MPTP opening has emerged as a promising target for various pharmacological and conditional interventions in cardiac therapeutics. Development of novel specific inhibitors of CyP-D activity or mitochondria-targeted ROS scavengers represents potentially effective approaches to reduce mitochondria-mediated (especially MPTP-mediated) cardiac dysfunction in IR and HF. Indeed, pilot clinical trials in patients with myocardial infarction have already demonstrated the ability of CsA to reduce cardiac injury following myocardial infarction. However, challenges exist in the development of pharmacological agents which specifically target MPTP opening. The development of such specific agents will require the elucidation of the precise molecular structure of the pore as well as the mechanisms underlying pore formation and its regulation.

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Footnotes

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Legends for Figures

Figure 1. Proposed mechanism of MPTP opening and its consequences. ANT, adenine nucleotide translocase; BKA, bongkrekic acid; CsA, cyclosporin A, CyP-D, cyclophilin D; OMM, outer mitochondrial membrane; P_i, phosphate; PiC, phosphate carrier, SfA, sanglifehrin A

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Table 1. Direct and indirect inhibitors of MPTP opening and possible mechanisms of their action (see text for details).

Effect of inhibitors	*Mechanism*	*Reference*
Direct action via MPTP components		
CsA and its analogues (6-MeAla-CsA, 4-MeVal-CsA, *N*-Me-4-Ile-CsA (NIM811), D-3-MeAla-4-EtVal-CsA (Debio-025)	Inhibit CyP-D binding to PiC (ANT?)	Nazareth et al., 1991; Griffiths and Halestrap, 1993; Hausenloy et al., 2003; Argaud et al., 2005a, 2005b; Gomez et al., 2007.
SfA	Inhibits PPIase activity of CyP-D	Clarke et al., 2002; Hausenloy et al., 2003; Javadov et al., 2003; Lim et al., 2007;
BKA and ADP	Induces "m" conformation of ANT	Halestrap and Brenner, 2003; Halestrap and Davidson, 1990
H^+ and divalent cations (Mg^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+})	Antagonise Ca^{2+} binding to ANT	Haworth and Hunter, 1979; Bernardi et al, 1992
Indirect action MPTP opening		
ROS scavengers (e.g. propofol, pyruvate, MCI-186)	Prevent ROS accumulation and therefore, oxidation of the critical thiol groups on the ANT	Javadov et al, 2000; Kerr et al, 1999; Rajesh et al, 2003
Low pH_i inducers (NHE-1 inhibitors cariporide and analogues, pyruvate)	Reduce pH_i that inhibits Ca^{2+} binding to ANT	Kerr et al, 1999; Teshima et al, 2003; Javadov et al, 2005, 2008,
Ubiquinone analogues (e.g. UQ_0, Ro 68-3400)	Unknown. May act via complex I or bind to ANT and PiC	Cesura et al., 2003; Krauskopf et al, 2006; Leung et al, 2008
Preconditioning/ postconditioning	Prevent oxidative stress, normalize mitochondrial metabolism	Hausenloy et al, 2002; Javadov et al, 2003; Argaud et al., 2004, 2005b; Lim et al., 2007

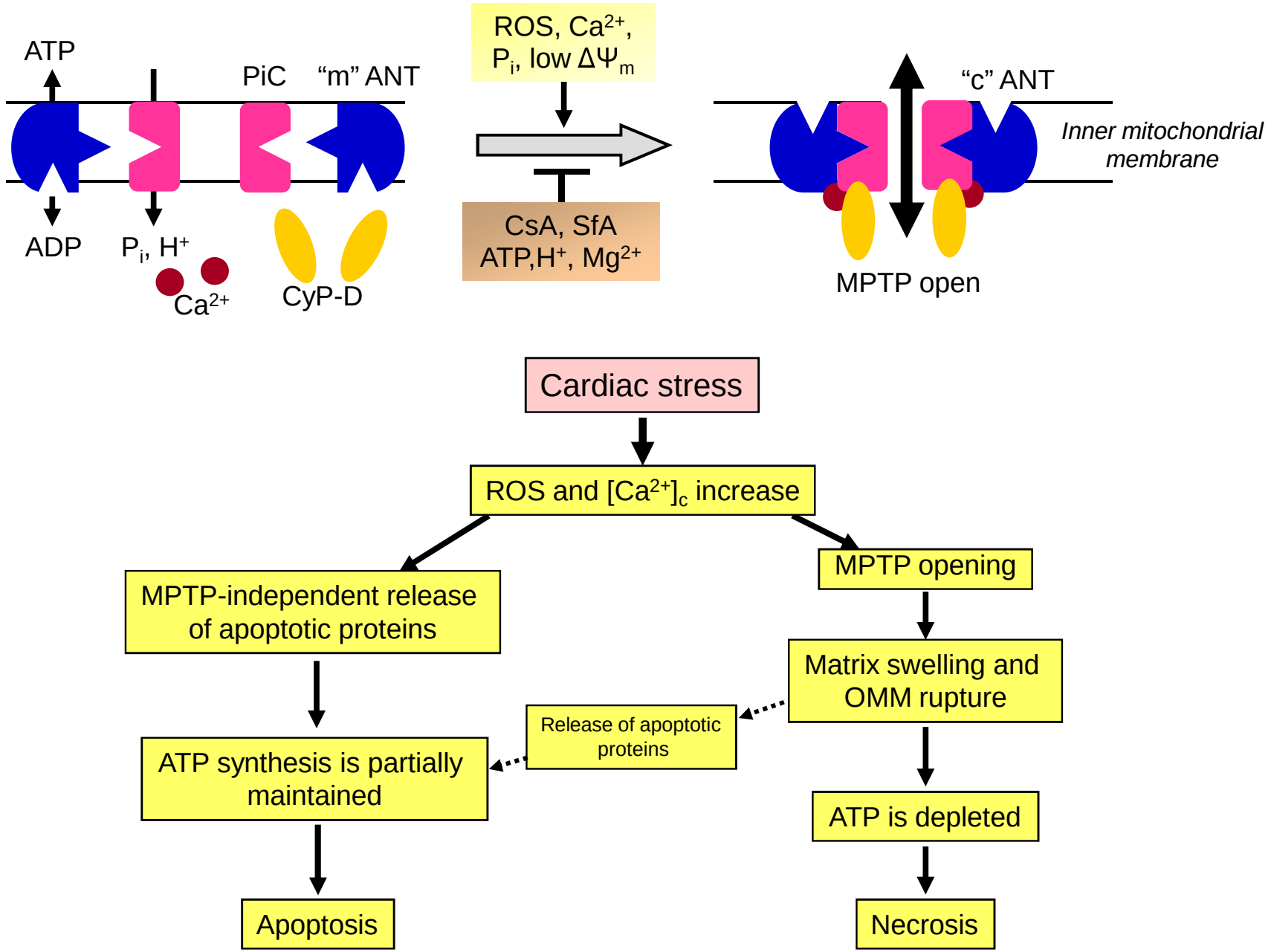

Figure 1