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Review Article

Mitochondrial permeability transition pore: an enigmatic gatekeeper

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ABSTRACT
Mitochondrial permeability transition pore (MPTP) is a transient structure formed in the inner mitochondrial membrane (IMM) upon oxidative challenge of the organelle. The transition is a Ca^{2+}-dependent increase of permeability of IMM leading to loss of transmembrane potential ($\Delta\psi_m$), mitochondrial swelling and, finally, rupture of outer mitochondrial membrane leading to release of factors causing apoptosis. Formation of this pore is an indication of the onset of necrotic cell death. In spite of several years of research, the molecular identity of this structure remains a mystery. The current review introduces MPTP, summarizes the research on various proteins that are thought to constitute the pore, enumerates factors that trigger pore formation, and the various experimental approaches that aid in understanding pore behavior.

Keywords: Mitochondrial permeability transition pore, necrosis, PiC, ANT, CyP-D

Abbreviations: MPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; PiC, mitochondrial phosphate carrier; ANT, adenine nucleotide translocase; CyP-D, cyclophilin D; VDAC, voltage-dependent anion channels; IMM, inner mitochondrial membrane; CsA, cyclosporine A; PT, permeability transition; vMIA, viral mitochondrial inhibitor of apoptosis; OMM, outer mitochondrial membrane; PTP, permeability transition pore; CAT, carboxyatractyloside; GST, glutathione S-transferase

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INTRODUCTION

Mitochondria are membranous organelles responsible for energy production through oxidative phosphorylation, heme biosynthesis and porphyrin metabolism, Ca^{2+} homeostasis and apoptotic/necrotic cell death. Mitochondria, as understood by electron microscopy images, is internally organized into distinct membranes and membrane enclosed compartments that are named as outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), intermembrane space, inner boundary membrane, cristae junction, cristae membrane and mitochondrial matrix, respectively. All these structures are evolved for optimal functionality (Benard and Rossignol, 2008). In contrast to OMM, which is selectively permeable, IMM is almost impermeable to small solutes and ions in healthy cells. This impermeability is to ensure that the electrochemical gradient required for ATP synthesis is maintained. Loss of IMM impermeability leads to a net influx of solutes and, in turn, water into mitochondrial matrix due to osmosis. This leads to the swelling of the mitochondrial matrix and rupture of inner and outer mitochondrial membranes. The above sequence of events is called as mitochondrial permeability transition and is thought to proceed through the formation of a megachannel in the IMM called as MPTP. Haworth and Hunter initially described the channel in the late 1970s (Haworth and Hunter, 1979). MTP is a voltage-dependent, CsA sensitive, high conductance inner-membrane channel of unknown molecular identity (Bernardi, P. 1999). Formation of the pore causes mitochondrial failure by uncoupling oxidative phosphorylation and accelerating ATP hydrolysis. Pore formation signals towards cell death (through necrosis), mitochondrial autophagy, and apoptosis (through the rupture of the OMM). Necrotic cell death in reperfused heart and brain cells after ischemic conditions and dysfunction of cardiac muscles during post-infarction modeling has been noticed for a long time which, currently, is ascribed to MPTP formation. The reader is referred to other reviews detailing the pathophysiology of pore formation (Javadov, S. and Karmazyn, M., 2007; Lemasters et al., 1997; Rodriguez-Enrique et al., 2004).

MOLECULAR IDENTITY OF MPTP

In spite of the critical roles played by MPTP, its molecular identity remains unclear. Years of persistent research and indirect evidences has lead to a consensus that adenine nucleotide translocase

(ANT), cyclophilin-D (Cyp-D), voltage-dependent anion channel (VDAC), mitochondrial phosphate carrier (PiC) and other non-specific membrane proteins might form the MPTP structure (Brustovetsky and Klingenberg, 1996). The continued ambiguity about the components constituting MPTP has led He and Lemasters (He and Lemasters, 2002) to propose that there are two conductance modes for permeability transition pore, viz., a regulated one and an unregulated one. They further propose that PT pores are formed by aggregation of misfolded integral membrane proteins damaged by oxidative and other stresses and that overabundance of Cyp-D and ANT in the membrane has led to their direct implication as components of pore formation. Over the years the exact role of proteins speculated to constitute MPT has been worked out and the paragraph below summarizes them systematically.

Cyp-D, an 18 KDa protein that is a mitochondrial member of cyclophilin family, is a peptidyl prolyl cis-trans isomerase (PPIase) and plays an important role in protein folding. Cyp-D is encoded by a nuclear gene, *Ppif*, and is subsequently transported to the mitochondria by a mitochondrial signal sequence. The protein is implicated as an important constituent of MPTP because cyclosporin A (CsA), a specific inhibitor of cyclophilin family, inhibits MPTP. Three studies reported the creation of knockout strain of mice lacking Cyp-D to test the indispensability of this protein in MPTP formation (Balnes et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). It was shown that mitochondria from mice lacking Cyp-D were impervious to Ca^{2+}-induced permeability transition and were insensitive to CsA inhibition of MPTP. They were also resistant to Ca^{2+}-overload and oxidative-stress induced cell death characteristic of necrosis. However, beyond a particular Ca^{2+} threshold, MPTP formation ensues in mitochondria from Cyp-D knockout mice as seen in the wild-type. This supports a model whereby Cyp-D is involved in bringing about a Ca^{2+}-triggered conformational change in a membrane protein that constitutes the actual pore. The studies also point out that a Cyp-D-independent conformational change can happen in the presence of high Ca^{2+} overload. These findings make the role of Cyp-D appear regulatory in facilitating channel formation rather than being a component of the pore.

A role for ANT in the formation of MPT is suggested because MPT gets activated by carboxyatractyloside (CAT) and inhibited by Bonkrekic acid, both of which are known modulators of ANT by inducing the “c” and “m” conformation of the protein, respectively. Further, it was shown that oxidative stress alters a cysteine residue in ANT leading to abolishment of binding of adenine nucleotides to the protein, in turn accounting for sensitization of MPTP to calcium by oxidative stress. It was also demonstrated using patch clamp that isolated and reconstituted ANT could be reversibly converted into an unusually large cation selective channel that requires Ca^{2+}. It was shown that the channel was inhibited by low pH and ANT inhibitors (Brustovetsky and Klingenberg, 1996).

However, recent experiments done on liver mitochondria from mice lacking both isoforms of ANT (ANT1 and ANT2) showed the formation of MPTP, albeit at a higher Ca^{2+} than the mitochondria from the wild-type mice (Kokoszka et al., 2004). It was also shown that MPTP formation becomes insensitive to the ligands of ANT in mitochondria from these mice. These experiments suggest a regulatory role for ANT in MPTP formation (Leung and Halestrap, 2008).

VDACs are a family of ion channels localized in the outer membrane of mitochondria. There are three distinct *Vdac* genes, *Vdac* 1, *Vdac* 2 and *Vdac*3, in mammals that exhibit high degree of redundancy as far as their metabolic functions are concerned. While *Vdac* 1 and *Vdac* 3 deletion mutants in mice are viable with minor defects in mitochondrial respiration and abnormalities in mitochondrial ultrastructure, *Vdac* 2 deletions are embryonic lethal and show enhanced activation of intrinsic apoptotic pathway (Baines et al., 2007). Based on experiments where the electrophysiological properties of VDACs incorporated into planar phospholipid membranes were shown to be similar to MPTP (Szabo et al., 1993), they were thought to be an integral component of the pore forming complex. It was also shown that VDACs copurified with ANT on a GST-Cyp-D affinity column, though this results have been contested by other groups (Crompton et al., 1998; Woodfield et al., 1998). It has also been speculated that the ATP/ADP mediated regulation of MPTP is through the enzyme Hexokinase that interacts with VDACs at the outer membrane of mitochondria. Several factors that modulate VDAC channel properties, like addition of Ca^{2+}, NADH and glutamate also affect PTP activity. However, recent literature has clearly ruled out a role for VDACs in constituting the MPTP. It has been shown that deletion of the mammalian *Vdac* genes (*Vdac*1 and *Vdac*3), either individually or in combination, has no effect on the formation of MPTP (Baines et al., 2007). To address for possible compensatory role played by *Vdac*2, the authors knocked down VDAC2 by siRNA. It was shown unambiguously that the induction of MPTP formation in either Ca^{2+}-induced or stress-induced mitochondria was identical between the wild-type and various *Vdac* null mutants ruling

out a role for VDAC in MPTP formation.

PiC, a member of the family of hexa-transmembrane proteins that facilitate the transport of metabolites across the IMM, is a protein recently speculated to constitute MPTP. A CsA-sensitive binding of PiC to Cyp-D and a CsA-insensitive binding of ANT to PiC was seen in pull-down and co-immunoprecipitation studies with GST-Cyp-D and ANT, respectively. The above associations, given the regulatory role of ANT and Cyp-D, implicate PiC as an essential component of MPTP. It should also be noted that inorganic phosphate, an activator of MPTP, binds PiC with high affinity (Leung et al., 2008). Other circumstantial evidence implicating PiC as an essential component of MPTP are that a genome-wide cDNA screen has identified that overexpressed PiC can efficiently trigger the intrinsic apoptotic pathway (Alcala et al., 2008). It is also shown that cytomegalovirus-encoded protein vMIA, an inhibitor of apoptosis, may interact with PiC as shown by the reduced uptake of [^{33}P] phosphate into isolated mitochondria (Poncet et al., 2006)

The other proteins that are tentatively speculated as components of MPTP are peripheral benzodiazeine receptors (PDBR), hexokinase, creatine kinase, Bcl-2 and Bax as summarized in the review by Halestrap (Halestrap, A.P. 2009)

Electrophysiological studies of PTP show that its characteristics are compatible with a dimeric structure formed by two cooperative channels (Zoratti et al., 2009; Zoratti et al., 2005) while there are studies that suggest that PTP might be formed by inorganic polymers made of poly-3-hydroxybutyrate and calcium polyphosphate (Pavlov et al., 2005). The last word on what constitutes the pore is yet to be pronounced.

TRIGGERS OF PORE FORMATION

The principal trigger for pore formation is intramitochondrial Ca^{2+} concentration. Ca^{2+} influx causes the pore to open rapidly while chelating agents such as EDTA or EGTA can reverse this effect. Other divalent cations like Mg^{2+}, Mn^{2+}, Ba^{2+} and Sr^{2+} are competitive inhibitors of Ca^{2+}-induced pore formation (Bernardi et al., 1992). It has also been shown that H^+ ions strongly inhibit MPTP formation by competing for the same site as calcium and this might explain the potent inhibitory effect of low pH on MPTP opening. The pore opens only at pH >7. This might explain the fact that during ischemia, the acid accumulation as a result of anaerobic metabolism serves as a strong inhibitor of MPTP and, hence, cell death. During reperfusion, the sudden increase in pH triggers MPTP formation leading to cell death. The concentration of calcium ions required for PTP formation might vary depending on several factors that modulate both the rate and extent of pore opening. High oxidative stress (induced by agents like t-butylhydroperoxide, diamide or phenylarsine oxide and processes like reperfusion after ischemia), inorganic phosphate and carboxyatractyloside (CAT) act as activators of pore formation and reduce the amount of Ca^{2+} required to trigger PT. On the contrary, presence of adenine nucleotides ATP/ADP is inhibitory to pore formation and PT happens at higher Ca^{2+} when adenine nucleotides are present. This inhibition is specific to the di- and tri-phosphorylated form of adenine nucleotides because AMP, guanine nucleotides and the magnesium-complexed forms of ATP/ADP are not known to inhibit the permeability transition. ATP and ADP are proposed to act either through ANT or by acting through Hexokinase that is tightly associated with VDACs at contact points on the outer mitochondrial membrane. As mentioned above, low pH (acidosis) also necessitates higher calcium ion concentration for PT. Cyclic immunosuppressant peptide CsA and the macrolide sanglifehrin A (SfA) also inhibit MPTP formation (Zenke et al., 2001; Halestrap, A.P., 2009).

Ubiquinone and its analogues (UQ_0 and Ro 68-3400) are also shown to regulate MPT, as is the antipsychotic drug trifluoperazine. Both the above-mentioned regulators are shown to inhibit PTP formation. MPTP opening is inhibited by negative mitochondrial membrane potential and can be induced by an uncoupling agent after loading the mitochondria with Ca^{2+} (Halestrap, A.P., 2009).

Readers are referred to some excellent reviews for further information on aspects related to MPTP regulation (Halestrap, AP. 2009; Halestrap, AP. 2010; Crompton, M. 1999; Zorov et al., 2009)

EXPERIMENTAL APPROACHES

Following are the various experimental approaches that can be utilized to understand the impairment of IMM impermeability, the principal cause of which is the formation of MPT. Some techniques are appropriate for studying isolated mitochondria while others are useful in studying mitochondria in the tissues.

The rapid and irreversible loss of mitochondrial membrane potential is a hallmark of MPT formation and this feature can be utilized with several distinct potential-sensitive probes that accumulate in mitochondria driven by the membrane potential. A few examples of such probes are rhodamine derivatives (TMRE and TMRM), rosamine derivatives (MitoTracker-red and -orange) and cabocianines. Yet another technique is based on the differential accessibility of calcein AM and its quencher, cobalt, to the mitochondrial matrix. The above-mentioned techniques are summarized in a

paper by Galluzi et al (Galluzi et al., 2008).

An alteration in permeability of the IMM also leads to mitochondrial volume changes that can be measured by light scattering experiments. The scattering of light by osmotically-responsive mitochondria reduces as their volume decreases. The wavelength range used for such experiments is usually between 500-600 nm to avoid interference by mitochondrial chromophores (Fiskum et al., 2000; Massari, S. 1996).

Hot-Dog technique is more appropriate to the study of MPTP behavior in intact tissues and organs. In the technique, the organ is perfused with radiolabelled 2-deoxyglucose (DOG) that gets entrapped in the cytosol as DOG-6 phosphate. Homogenization of the tissue in the presence of EGTA (to prevent further pore opening) and quantifying the radiolabel in the mitochondrial fraction is used as a criterion to gauge the extent of PT pore opening before homogenization (Griffiths and Halestrap, 1995).

MPTP opening is preceded by an influx of calcium ions into mitochondria. Mitochondrial uptake of calcium can be determined by fluorimetric techniques involving the use of calcium indicators Calcium Green 5N (Molecular probes). Quantification of extramitochondrial calcium after incubation of the indicator with mitochondria reflects mitochondrial calcium uptake (Schinzel et al., 2005).

Patch-clamp experiments are also employed by several groups to study the behavior of voltage-dependent MPTP (Szabo and Zoratti, 1991).

FUTURE DIRECTIONS

Given the central role MPTP plays in conditions as diverse as cell death, ageing and cancer, it is very important to understand the various components that constitute the pore to devise appropriate intervention strategies. Though the pore complex is well understood in terms of its function, the mystery surrounding its molecular nature is disturbing. The efforts have been so frustrating as to lead some groups to claim that there might not be a specific channel at all, though such claims do not present convincing evidence as to the specific regulation (inhibition and activation) displayed by several agents. The number of research articles claiming to have unraveled the molecular nature of the pore, and equal number of scientific reports refuting those claims, indicates to the complexity of the question in spite of rapid advances in genetic, biophysical and molecular biology tools. The most pressing question in the field is to unravel the molecular nature of the pore given the central role mitochondrion plays in ageing, cardiac diseases, cell death and cancer.

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