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The Plymouth Student Scientist - Volume 03 - 2010 The Plymouth Student Scientist - Volume 3, No. 1 - 2010

2010

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Molnar, Z.

Molnar, Z. (2010) 'The mitochondrial permeability transition pore components', The Plymouth Student Scientist, p. 245-254.
http://hdl.handle.net/10026.1/13906

The Plymouth Student Scientist
University of Plymouth

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*The Plymouth Student Scientist, 2010, **3**, (1), 245-254*

The mitochondrial permeability transition pore components

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Abstract

The mitochondrial permeability transition pore (MPTP) is a non-specific channel in the inner mitochondrial membrane (IMM) that opens following ischaemia and reperfusion due to the presence of various stimuli, such as oxidative stress, elevated phosphate concentration, and adenine nucleotide depletion. MPTP opening causes the mitochondria to swell and become dysfunctional. This results in cell death, especially by necrosis, due to the loss of oxidative phosphorylation and the subsequent drop in adenosine triphosphate levels. In search of the identity of the pore numerous studies have been done by several research groups in the last three decades. For many years a widely accepted hypothesis prevailed suggesting the involvement of the adenine nucleotide translocase (ANT) and voltage-dependent anion channel (VDAC) as core proteins of the MPTP. Recent genetic studies, however, contradict this hypothesis and ascribe only a regulatory role to the ANT. Furthermore, there is now sufficient evidence to conclude that VDAC plays no role in mitochondrial permeability transition. In a recent study it was suggested that the mitochondrial phosphate carrier (PiC) may fulfil a role as a pore component. According to a proposed model MPTP is formed as a consequence of a conformational change in the PiC, triggered by calcium binding. Opening of the pore may be enhanced through interactions with the ANT in the “c” conformation and cyclophilin-D, a mitochondrial matrix protein.

Introduction

Research in mitochondrial permeability transition (MPT) stems from the observation that mitoplasts, mitochondria stripped of their outer membrane, swell and become dysfunctional unless placed in a Ca^{2+} ion-free suspension (Hunter *et al.* 1976). Since the mitochondrial inner membrane is impermeable to solutes without specific transport mechanisms, the phenomenon has sparked much interest in mitochondrial research (Bernardi 1999). Reports in the field agree that MPT is mediated by a proteinaceous pore, known as the mitochondrial permeability transition pore (MPTP) (Bernardi 1999; Juhaszova *et al.* 2008). According to Hunter and Haworth (1979)

mitochondria undergoing MPT admit molecules of all sizes with a molecular weight up to 1.5 kDa. MPTP-associated matrix swelling is driven by the osmotic gradient established by the presence of indiffusible matrix proteins (Halestrap *et al*. 2003). A prerequisite to this swelling is that Ca^{2+} is accumulated in the mitochondrial matrix by the Ca^{2+} uniporter, or channel, located in the inner mitochondrial membrane (Zoratti & Szabó 1995). The evidence for the Ca^{2+}-dependent swelling came from a study in which Ruthenium Red (RR), an inhibitor of the mitochondrial Ca^{2+} uniporter was used. Blocking calcium entry into the matrix prevented MPT (Hunter & Haworth 1979). The extent of Ca^{2+} accumulation, or Ca^{2+} load in the matrix compartment required for MPT was found to be organ specific (e.g. mitochondria from liver were more susceptible to permeabilization than heart mitochondria). The rate of MPT and the percentage of mitochondria undergoing MPT were also found to be dependent on the extent of Ca^{2+} load (Zoratti & Szabó 1995). They concluded that the heterogeneity of mitochondria with respect to their susceptibility to permeabilization can be explained by the variation in the presence of different MPT modulators. Indeed, it has been found that Ca^{2+} load alone is often insufficient to induce MPTP opening. Additional factors required may be the presence of reactive oxygen species (ROS), elevated phosphate concentration, mitochondrial depolarization, and adenine nucleotide depletion (Crompton *et al* 1999). These factors correspond to conditions in the heart brought about by ischemia/reperfusion injury that results in tissue damage initiated by inadequate oxygen and nutrient supply (Halestrap 2006). It has been well documented that the necrotic cell death characteristic of ischemia/reperfusion injury is caused by MPTP opening (Crompton 1999). Nevertheless, MPTP-associated swelling of the mitochondria can also induce apoptosis under some conditions (Kim *et al*. 2003). It is clear that the MPTP plays an important role in cell death and understanding the molecular mechanisms of MPTP opening may lead to the development of drugs that target the components of the MPTP. The remainder of this review will summarise the evidence for the MPTP forming components proposed so far.

The pore hypothesis

The hypothesis that MPT is mediated through a proteinaceous pore did not receive much attention for a long time. The alternative, prevailing hypothesis was that MPT is due to alterations of the phospholipid bilayer (Zoratti & Szabó 1995). According to this hypothesis MPT is brought about by the accumulation of lysophospholipids, which results in membrane damage. In association with this process the involvement of the enzyme phospholipase A2 (PA2) and lysophospholipid-acyl-CoA-transferase was suggested (Gunter & Pfeiffer 1990). PA2 catalyses the hydrolysis of the sn-2 acyl bond of membrane phospholipids releasing arachidonic acid and lysophospholipids, which are reincorporated by the opposing activity of acyltransferases (Zoratti & Szabó 1995). It was proposed by Gunter and Pfeiffer (1990) that the over-activity of PA2 and the inhibition of acyltransferases might induce MPT. Although experimental observations confirmed that lysophospholipids accumulate in association with MPT, the model failed to account for the “all-or-nothing” nature of the MPT (Zoratti & Szabó 1995). The evidence in support of the pore hypothesis came from the discovery by Crompton *et al*. (1988) that Cyclosporin A (CsA), an immunosupressive drug, could inhibit MPT but it had no effect on PA2 activity. Work in the same laboratory also identified the target protein on which CsA exerts its affect. That protein, now known as cyclophilin D (Cyp-D), was shown to be located in the mitochondrial matrix.

Pore components

Cyclophilin D

Due to its location and the CsA-sensitive affect on MPT the involvement of Cyp-D as a likely candidate in pore formation is evident. Nevertheless, several lines of research have pointed out that Cyp-D has a secondary role in pore formation (Clarke *et al*. 2002; Nakagawa *et al*. 2005; Connern & Halestrap 1996). Cyp-D is a nuclear encoded protein that is targeted to the mitochondrial matrix by its mitochondrial signal sequence. It belongs to a family of peptidyl prolyl cis/trans isomerases (PPIase) that are ubiquitous enzymes involved in promoting protein folding (Woodfield *et al*. 1998). Indeed, the evidence for its role in MPTP formation came from the observation that blocking its PPIase activity with CsA correlated with MPT inhibition (Griffiths & Halestrap 1991). Studies on mitochondria from Cyp-D knockout mice, however, demonstrated that MPTP opening can occur without the involvement of Cyp-D at sufficiently high matrix calcium concentration (Baines *et al*. 2005). In a parallel study by Nagawa *et al*. (2005) it was also revealed that Cyp-D-defficient mice showed resistance to ischaemia/reperfusion injury as well as to ROS-induced necrotic cell death. From this observation emerged the hypothesis that through its PPIase activity Cyp-D may be involved in MPTP opening by facilitating a conformational change in a membrane protein (Juhaszova et al. 2008; Leung & Halestrap 2008). The identity of that membrane protein is, however, less certain. He and Lemasters (2002) debated whether specific membrane proteins are responsible for pore formation. According to their hypothesis the MPTP forms from misfolded integral membrane proteins as a result of oxidative damage or other stresses. The role of Cyp-D in this model is to block conductance at damage sites where misfolded proteins have aggregated forming the MPTP. Above a critical damage level the available Cyp-D is unable to block conductance and MPTP opening occurs. Although this model would elegantly explain why MPTP opening can occur in the absence of Cyp-D the fact that MPTP opening can be modulated by various ligands of the adenine nucleotide translocase (ANT) is inconsistent with such a hypothesis (Zoratti & Szabó 1995).

The Adenine Nucleotide Translocase

The involvement of ANT in MPTP formation was first put forward by Hunter and Haworth (1979). ANT is located in high amounts in the inner mitochondrial membrane. It is an antiporter that transports adenosine diphosphate (ADP) into the mitochondrial matrix where adenosine triphosphate (ATP) is generated through oxidative phosphorylation. The entry of one ADP is coupled to the exit of one ATP via the ANT (Berg *et al*. 2007). It is important to note that for the generation of ATP molecules adequate phosphate (P_i) supply is also necessary. P_i is imported into the matrix by another antiporter, the phosphate carrier (PiC) that couples the influx of P_i into the matrix to the efflux of hydroxyl ions into the inner membrane space (Lodish *et al.* 2008). Indeed, adenine nucleotide and P_i have been known as PT modulators for decades. The long-standing observation is that P_i is an inducer, whereas ATP and ADP (without bound Mg^{2+}) are inhibitors of PT (Zoratti & Szabó 1995). Adenine nucleotide depletion during ischaemia, therefore, may lead to MPTP opening due to the loss of protection by ATP/ADP and a substantial rise of P_i concentration as a result of adenine nucleotide degradation (Halestrap *et al.* 2003). Since MPT-associated swelling may result in the rupture of the outer mitochondrial membrane (OMM) and the subsequent release of pro-apoptotic proteins, such as cytochrome *c* and apoptosis inducing factor (AIF), from the inter-membrane space, research

interest turned to characterise MPT in the context of apoptosis (Crompton 1999). Nevertheless, the studies by Kim *et al.* (2003) revealed that adenine nucleotide depletion is a major factor that drives MPT-associated cellular changes towards necrotic cell death.

Other modulators of MPTP opening used in mitochondrial research involve the respiratory toxins carboxyatractyloside (CAT) and bongkrekic acid (BKA). CAT induces pore opening while BKA has an inhibitory effect on MPT. They act by facilitating opposite changes in the conformational state of the ANT. CAT stabilises the “c” conformation where the nucleotide binding site faces the cytoplasmic side of the membrane. Conversely, BKA enhances the eversion of the binding site to the matrix side (“m” conformation). According to these observations the “c” conformation may be required for pore opening, whereas the “m” conformation is inhibitory (Zoratti & Szabó 1995). From the Ca^{2+} dependency of MPT Halestrap and Brenner (2003) suggested that there may be a Ca^{2+} trigger site exposed on the ANT associated with the “c” conformation. Although there is no conclusive evidence for the identity of this site, they proposed that glutamate and aspartate residues may bind calcium on the matrix side of the ANT. They further suggested that H^+ ions might compete for these binding sites, which might result in the desensitisation of MPTP to Ca^{2+}. Indeed, such a hypothesis appears to be supported by the observation that low pH inhibits MPTP opening (Zoratti & Szabó 1995).

Changes in pH during ischaemia/reperfusion have important implications for cell survival. Data from several lines of research have confirmed that the acidic condition present during ischaemia is cytoprotective against necrotic cell death and cell killing only accelerates after reperfusion, when normal pH recovers (Halestrap 2006). However, according to Crompton (1999) the issue of MPTP-associated cytoprotection by low pH is more complex. He observed that MPTP opening still occurs at pH 6.5, a relatively low pH, which may contribute to a higher rate of H^+ backflow through the open pore leading to the collapse of the membrane potential and the disruption of mitochondrial homeostasis. An alternative explanation may be, as proposed by Halestrap and Brenner (2003), that the higher membrane potential, a widely accepted inhibitor of MPTP opening, may facilitate greater binding of adenine nucleotides to the matrix side of the ANT, which, in turn, will exert its inhibitory effect through inducing the “m” conformation.

More direct evidence that the ANT might function as a pore component came from the demonstration that ANT binds Cyp-D in a CsA-sensitive manner (Crompton *et al*. 1998). It was also suggested that the role of Cyp-D in MPTP formation is to induce a conformational change in the ANT. The supporting evidence for this came from the observation that sanglifehrin A (SfA), a potent MPTP inhibitor, did not prevent Cyp-D from binding to the ANT (Clarke *et al*. 2002). It was also hypothesised that Cyp-D binding facilitates a conformational change in the ANT when triggered by Ca^{2+}. CsA, therefore, inhibits this binding, whereas SfA inhibits the conformational change induced by Cyp-D (Clarke *et al*. 2002). This model is also consistent with the published three dimensional structure of the ANT in its CAT-bound form (Pebay-Peyroula & Brandolin 2004). From this structure it appears that on the cytosolic surface ANT has a large channel that extends deep into the membrane and is blocked by a narrow gate at the matrix side. The conformational change would, therefore, be necessary to wedge this gate open (Leung & Halestrap 2008). Nevertheless, the fact that at very high Ca^{2+} concentration MPTP opening can be

observed without the involvement of Cyp-D renders the evidence for a Cyp-D-induced conformational change in the ANT largely circumstantial (Baines *et al*. 2005).

Despite the several lines of evidence implicating the ANT in the formation of the MPTP, experiments performed on ANT1/ANT2 knock-out mice have revealed that the involvement of the ANT in MPT is not an absolute requirement. Kokoszka *et al*. (2004) observed that, albeit at high Ca^{2+} concentrations, MPTP opening still occurred in mouse mitochondria lacking the two isoforms of ANT. Nevertheless, the fact that the mice in which the ANT1/ANT2 isoforms had been knocked out displayed no obvious disruption of urea synthesis and gluconeogenesis, metabolic processes that rely on the export of ATP from the mitochondria, points to the possible involvement of ANT isoforms other than ANT1/ANT2 (Leung & Halestrap 2008). Proteomic studies by Cruz *et al*. (2003) on mouse liver mitochondrial inner membrane, indeed, confirmed the presence of a new ANT isoform, the ANT4. Nevertheless, the fact that the MPTP opening observed by Kokoszka *et al*. (2004) was insensitive to ligands of the ANT suggests that ANT was not involved in pore opening. An explanation suggested by Palmieri (2004) may be that ANT is a member of a large family of mitochondrial carriers with structural similarities and it is possible that other members of this family may be involved in MPTP opening as well.

Another line of evidence questioning the primary role of ANT in MPTP formation has been put forward by Leung and Halestrap (2008). In their study they investigated the interaction between CAT and a thiol reagent, phenylarsine oxide (PAO), with regard to MPTP opening. According to their results both CAT and PAO are able to bind ANT and stimulate MPTP opening. It was also revealed that ANT from rat mitochondria pre-treated with CAT is not able to bind to a PAO-affinity matrix. However, when CAT-treated mitochondria were exposed to PAO, as illustrated in Fig.1, an even greater effect on MPTP opening could be observed (Leung & Halestrap 2008). Therefore, it can be concluded that apart from ANT, which more likely has a regulatory role only, other mitochondrial membrane proteins may be involved in MPTP formation as well.

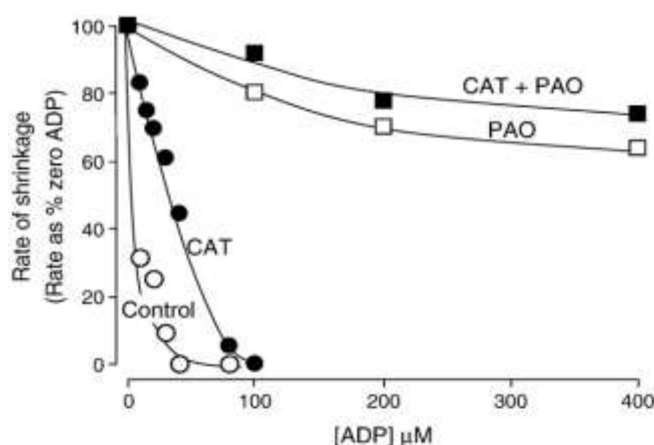

Fig.1 PAO can activate MPTP opening in CAT treated mitochondria. Polyethylene glycol-induced shrinkage was measured as a function of ADP concentration. The rate of shrinkage corresponds to the degree of MPTP opening, that is, the loss of sensitivity to inhibition by ADP. Adapted from Leung and Halestrap (2008).

Other possible components of the MPTP

Until recently, first proposed by Zoratti and Szabó (1994) the voltage dependent anion channel (VDAC) has been known to play a role in pore formation. The VDAC, also known as porin, is located in the outer mitochondrial membrane and forms a channel that is permeable to water soluble metabolites up to the size of 5 kDa (Ha *et al.*1993). The evidence for its involvement in MPTP opening was originally based on the observation that VDAC co-purifies with the ANT and the benzodiazepine receptor (McEnery *et al.* 1992). The association of VDAC with the ANT was thought to occur at contact sites where the outer and the inner mitochondrial membranes are very close to each other (Crompton 2000). Indeed, these sites have been implicated in channelling proteins, metabolites, and ions into the mitochondrial matrix (Ardail *et al.* 1990). Crompton (2000) hypothesised that MPTP forms at these contact sites through the binding of the VDAC to the ANT. This hypothesis was also supported by Cesura *et al.* (2003) who used ubiquinone analogues, potent inhibitors of MPT, in search of the pore forming components. Through radioactive labelling they found that the 32 kDa protein co-purified with the labelled ubiquinone analogue corresponded to the VDAC1 isoform. Recent studies by two research groups, however, have questioned if any of the three known isoforms of VDAC are involved in MPTP opening (Krauskopf *et al.* 2006; Baines *et al.* 2007). They generated mice deficient for the isoforms VDAC1 and VDAC3 (VDAC2 deficiency was non-viable) and assessed their mitochondria with regard to MPT. It was revealed that MPT could be induced in the mitochondria from mice deficient for any of the two or both isoforms of VDAC. Although the results from these genetic studies cast some doubt on the findings of Cesure *et al.* (2003), the fact that ubiquinone analogues inhibit MPT prompted more studies in this line of research.

Leung and Halestrap (2008) suggested that the ubiquinone analogues may exert their inhibitory effect on MPTP opening by binding to the ANT and the PiC. They demonstrated that, when run on SDS-PAGE, both the ANT and the PiC were in the 30-34 kDa region, therefore, the two intermembrane proteins may be potential candidates for the protein identified by Cesura *et al.* (2003). Indeed, Leung *et al.* (2008) stated that according to their results ubiquinone analogues bind to the ANT and the PiC. Since PAO facilitates MPTP opening it was logical to assume that these proteins also bind to a PAO affinity matrix. As already mentioned above, the ANT binds to PAO unless the mitochondria are pre-treated with CAT. As expected the binding of PiC to PAO was also demonstrated and it was found that CAT treatment did not influence this binding (Leung & Halestrap 2008). Thus, PiC may fulfil the role of the inner membrane pore component of the MPTP. Although the presence of two other inner membrane proteins could also be detected in the region of 30-35 kDa, the idea that they are involved in MPT as a pore component was not supported. One of the proteins, adenylate kinase 2 (AK2) is a soluble inter-membrane space protein that cannot function as a membrane component. In addition it was demonstrated that inhibition of its activity by P1,P5-di(adenoside-5') pentaphosphate did not affect MPTP opening. The other protein was NIPSNAP-2, a protein of unknown function (Leung & Halestrap 2008). As opposed to these two proteins, several lines of evidence have been reported in support of the involvement of the PiC in MPT. According to Leung and Halestrap (2008), apart from pore opening ubuquinone analogues can also inhibit phosphate transport into the mitochondrial matrix. Research by Krämer (1998) demonstrated that if crosslinked between two cysteine residues, the PiC from *S. cerevisiae* mitochondria expressed in *E. coli* can function

as a non-specific anion channel, a characteristic of MPTP. Furthermore, the data from studies by Leung *et al*. (2008) indicate that the PiC binds Cyp-D in a CsA-sensitive manner. The same group also revealed that the PiC can be co-immunoprecipitated with the ANT from CAT-treated mitochondria suggesting that the PiC, ANT, and Cyp-D may form a complex during the MPT. In view of their recent findings Leung and Halestrap (2008) proposed a model in which the PiC is the MPTP forming component. PiC can be induced by an elevated Ca^{2+} concentration to undergo a conformational change that is needed for pore formation. Although this conformational change is also facilitated by Cyp-D and the ANT in the "c" conformation, they are not required for MPTP opening at a much higher Ca^{2+} load.

Conclusion

In spite of several decades of research the molecular nature of the MPTP components remains debated. Studies in the field have been carried out mainly in the context of ischaemia/reperfusion injury because the molecular triggers that induce MPT are also present in the condition. Through biochemical and genetic approaches the involvement of several mitochondrial proteins of known function has been suggested. According to a recent finding the PiC has been identified as the major pore forming component. Its involvement as a potential candidate has been supported by data from several lines of research; nevertheless, from gene knockout experiments it still awaits confirmation (Leung & Halestrap 2008). Similar knockout studies have ruled out the ANT as an MPTP component, however, it may have a regulatory role that requires further characterisation. Biochemical and genetic studies have confirmed that CyP-D and VDAC are not involved in pore formation; nonetheless, CyP-D may have an effect on MPTP opening through its PPIase activity. Although MPTP opening may result in the rupture of the OMM and the subsequent release of pro-apoptotic proteins such as cytochrome *c* and AIF, there is accumulating evidence that MPTP opening is mainly responsible for causing necrotic cell death. Nevertheless, further studies aimed at characterising both apoptotic and necrotic cell death may contribute to this field of mitochondrial research.

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