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Mitochondrial Permeability Transition Pore-Dependent Necrosis

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Abstract

Mitochondrial permeability transition pore (mPTP)-dependent necrotic cell death is a form of necrotic cell death that is driven by mitochondrial dysfunction by the opening of the mPTP and is triggered by increases in matrix levels of Ca^{2+} and reactive oxygen species. This form of cell death has been implicated in ischemic injuries of the heart and brain as well as numerous degenerative diseases in the brain and skeletal muscle. This review focuses on the molecular triggers and regulators of mPTP-dependent necrosis in the context of myocardial ischemia reperfusion injury. Research over the past 50 years has led to the identity of regulators and putative pore-forming components of the mPTP. Finally, downstream consequences of activation of the mPTP as well as ongoing questions and areas of research are discussed. These questions pose a particular interest as targeting the mPTP could potentially represent an efficacious therapeutic strategy to reduce infarct size following an ischemic event.

Keywords

MPTP; Necrosis; Ischemia Reperfusion; Mitochondria; Mitochondrial Dysfunction; ANT; BAX; BAK; CypD; ATP Synthase; Calcium; ROS; Permeability Transition

Introduction

The responsibility of cardiac mitochondria is to produce sufficient amounts of ATP to maintain the high energetic demand of the beating heart. Mitochondrial dysfunction is detrimental to this process and has been associated with many cardiomyopathies. Mitochondrial dysfunction is a primary contributor to the cellular damage that occurs during a myocardial infarction (MI). During a MI, the damaged area loses its ability to undergo oxidative phosphorylation due to the lack of O_2 and this energy depletion leads to the

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dysregulation of ions within the myocyte. Following reperfusion of this area, the O_2 starved heart becomes replete with nutrients and O_2, which leads to oxidative damage. These events are the trigger of a regulated necrotic cell death referred to as mitochondrial permeability transition pore (mPTP)-dependent necrosis. The mPTP is a non-selective pore within the inner mitochondrial membrane that is permeable to solutes up to 1.5 kDa in size [1]. Pore opening leads to the dissipation of the mitochondrial membrane potential, organelle swelling, and eventual rupture [2]. In controlled laboratory settings, pharmacological or genetic desensitization of the mPTP is effective at reducing infarct size following ischemia reperfusion (I/R) injury [3–6]. Unfortunately, the molecular identity of the pore-forming component of the mPTP has evaded investigators since its discovery [2]. In this review, we will discuss the triggers of the mPTP—calcium (Ca^{2+}) and reactive oxygen species (ROS)—matrix regulation of the mPTP by cyclophilin D (CypD), the putative pore-forming inner membrane components, the adenine nucleotide translocator (ANT) family and the F_1F_o-ATP synthase, and the outer membrane regulators of the mPTP, the Bcl-2 family. Although the primary focus of this review is the pathological role of the mPTP in the cardiac setting, it bears mentioning that the mPTP also plays an integral physiological role in the heart [7].

Triggers of the mPTP

Numerous studies have focused on the mPTP because it is proposed to be the primary trigger of cell death under many conditions such as I/R injury. The low pH which occurs during ischemia is thought to be inhibitory to mPTP opening, allowing a window of opportunity to deliver drugs to the mitochondria at the beginning of reperfusion to inhibit mPTP-dependent necrosis. Because the identity of the pore-forming components of the pore are still debated, there has been interest in inhibiting mPTP-dependent necrosis by reducing the triggers that activate the mPTP.

Calcium

An increase in mitochondrial Ca^{2+} is the well-established trigger of the mPTP (Fig. 1). In fact, experimentally Ca^{2+} is the common trigger used to stimulate mPTP opening in isolated mitochondrial assays such as Ca^{2+} retention capacity (CRC) and the swelling assay. In CRC, small increments of Ca^{2+} are added to mitochondria in the presence of an extracellular Ca^{2+} indicator. The addition of extra-mitochondrial Ca^{2+} leads to an increase in fluorescence of the Ca^{2+} sensitive dye which then declines back to baseline as the Ca^{2+} is taken into the mitochondria. When the mitochondria exceed CRC, the mPTP opens releasing the accumulated Ca^{2+} which is then detected by the extra-mitochondrial Ca^{2+} indicator. In the mitochondrial swelling assay, a large bolus of Ca^{2+} is added to isolated mitochondria inducing mitochondrial swelling, which is measured as a decrease in mitochondrial absorbance at 540 nm. These common mPTP assays rely on Ca^{2+} to trigger the pore. The primary role for Ca^{2+} in triggering the mPTP has prompted studies to examine the timing of the rise in Ca^{2+} during ischemia and reperfusion as well as studies to identify the transporters responsible for the increase in mitochondrial Ca^{2+} with the goal of inhibiting Ca^{2+} uptake to block PTP.

An increase in cytosolic Ca^{2+} has been shown to occur during ischemia [8,9]. The mitochondrial Ca^{2+} uniporter (MCU) is generally thought to be the primary mechanism for Ca^{2+} uptake into the mitochondria. The driving force for Ca^{2+} uptake into the mitochondria is the negative mitochondrial membrane potential that is maintained by proton extrusion by the electron transport chain. As electron transport is largely inhibited during ischemia it possible that mitochondrial Ca^{2+} uptake via MCU would be inhibited during ischemia and would only occur during reperfusion when oxygen is restored and electron transport resumes. Mitochondrial membrane potential has been measured during ischemia and simulated ischemia and it does not appear to fall until late in ischemia after the heart goes into contracture [10,11]. If Ca^{2+} uptake into mitochondria occurs during ischemia, then inhibition of mitochondrial Ca^{2+} uptake at the start of reperfusion might not inhibit mPTP opening, depending on the amount of Ca^{2+} accumulated during ischemia. There are only a few studies examining mitochondrial Ca^{2+} during simulated ischemia in cells, but these studies suggest some increase in mitochondrial Ca^{2+} during ischemia.

Griffiths et al., examined mitochondrial Ca^{2+} in isolated adult rat cardiomyocytes (CM) [12]. Indo-1 AM was loaded into the myocytes and manganese was added to quench cytosolic indo-1. Cells were made hypoxic and mitochondrial Ca^{2+} was measured as a function of time after the myocyte went into rigor. In this model of hypoxia, myocytes stop beating and rigor begins after ~30 min and mitochondrial Ca^{2+} does not rise until the onset of rigor. Cellular recovery depends on the duration time in rigor. After 30 min of rigor 36% of the myocytes recovered normal rod shape morphology upon re-oxygenation. Mitochondrial Ca^{2+} prior to ischemia was ~100 nM and by the end of ischemia in cell recovered myocyte mitochondrial Ca^{2+} rose to ~280 nM versus 743 nM in the myocytes that did not recover. In recovering cells during reoxygenation, mitochondrial Ca^{2+} initially declined and then rebounded up to 331 nM and then returned to baseline. Surprisingly, Griffiths et al., found that addition of 20 μM ruthenium red (RR) during hypoxia and reoxygenation led to an increase in mitochondrial Ca^{2+} during hypoxia and a more rapid decrease in mitochondrial Ca^{2+} upon reoxygenation [13]. Additionally, Griffiths et al., tested the effect of cyclosporin A (CsA), a cyclical peptide immunosuppressant drug that inhibits calcineurin and cyclosporine A, on mitochondrial Ca^{2+} during anoxia and reoxygenation. The addition of 0.2 μM CsA increased the mitochondrial Ca^{2+} observed with rigor from 280 nM to 543 nM, whereas 1 μM CsA reduced mitochondrial Ca^{2+} to 153 nM [12]. With both concentrations of CsA, myocytes recovered compared to only 50% recovery without CsA.

Allen et al., loaded perfused rat heart with fura-2-AM, a fluorescent Ca^{2+} indicator, and then isolated mitochondria following hypoxic perfusion and hypoxic perfusion followed by reoxygenation [14]. Free Ca^{2+} was measured in the fura-2-AM isolated mitochondria. Baseline mitochondrial Ca^{2+} was 156 nM, which increased to 360 nM and 574 nM at 50 and 80 minutes of anoxic perfusion respectively. With 20 minutes of reperfusion after 50 minutes of hypoxia mitochondrial Ca^{2+} slightly increased to 501 nM, however, after 80 minutes of hypoxia with reperfusion, mitochondrial Ca^{2+} increased to levels that saturated the Ca^{2+} indicator (>5000 nM). Addition of 2.5 μM, but not 1 μM RR blocked the rise in mitochondrial Ca^{2+} upon reperfusion after 80 minutes of hypoxia.

To measure mitochondria Ca^{2+} in a perfused rat heart Miyamae et al., used the Ca^{2+} indicator indo-1 with manganese to quench the cytosolic fluorescence of the indo-1 [15]. They measured mitochondrial Ca^{2+} at baseline prior to ischemia and found mitochondrial Ca^{2+} to be 230 nM with no difference when RR was added. After 45 min of ischemia and 20 min of reperfusion mitochondrial Ca^{2+} was 360 nM in hearts without RR and 240 in hearts treated with RR. Mitochondrial Ca^{2+} was not measured during ischemia.

Ruiz-Meana et al., measured mitochondrial Ca^{2+} during simulated ischemia and reperfusion in HL-1 cells with and without RR [16]. Mitochondrial Ca^{2+} levels, measured with Rhod-2, doubled during simulated ischemia; however, when 25 μM RR was added, the rise in Ca^{2+} was attenuated by 60%. During simulated ischemia, cytosolic Ca^{2+} showed a steady rise Ca^{2+} (~2 fold increase) by 60 minute. With addition of RR, cytosolic Ca^{2+} rose faster and to a higher level (2.5 fold increase). Addition of RR during simulated ischemia resulted in an increase in LDH release.

Taken together the data seem to suggest that mitochondrial Ca^{2+} increases during ischemia and that RR attenuates this increase. One limitation of these studies is that RR has numerous off target effects and is known to inhibit SR-Ca^{2+} uptake and other Ca^{2+} transporters. Ru360, the active species in RR, was purified and found to have few side effects. Studies utilizing Ru360 were done by perfusing rat hearts with 250 nM of Ru360 for 30 minutes prior to 30 min of global ischemia [17]Upon reperfusion, rate pressure product recovered in the Ru360 treated hearts but not in the untreated hearts. Mitochondria were isolated from hearts upon reperfusion and mitochondria Ca^{2+} was lower in Ru360 treated hearts. Zhang et al., also found that rat hearts perfused with Ru360 (10 μM) had better recovery of post ischemic function [18].

ROS

The importance of ROS and cardiac pathologies was first recognized in the 1970s [19,20]. Upon reperfusion of ischemic tissues, which is overall beneficial when performed in a timely manner, there is additional tissue damage driven by ROS [21–23]. Several groups presented a correlation between the generation of ROS and tissue damage following re-oxygenation [21–25]. This increase in damage post tissue re-oxygenation was coined reperfusion injury and is thought to be driven by toxic levels of ROS as reducing oxidative damage correlates with a reduction in damage [22,26–31].

One potential mechanistic explanation for this correlation is that ROS leads to an increase of membrane permeabilization. It is well established that ROS can peroxidize the lipids in membranes, which can lead to membrane destabilization and the efflux of contents held within. Hicks and Gebicki first established a quantitative relationship between ROS concentration and rate of lipid peroxidation [32]. They demonstrated a proportional relationship between the degree of lipid membrane peroxidation with the amount of solute release from the membranes and the concentration of peroxidizing agents introduced. In addition to direct lipid oxidation, ROS has also been implicated in mPTP opening. Initial investigations revealed that, similar to Ca^{2+}, isolated mitochondria from the heart undergo ROS-induced mPTP opening by treating with t-butylhydroperoxide (Fig. 1) [33]. Further evidence solidified the hypothesis that ROS triggers mPTP opening by demonstrating that

ROS accumulation in cells associates with known characteristics of the mPTP. For example, one hallmark of mPTP opening is the depolarization of mitochondrial membrane potential, which occurs when mitochondria are exposed to ROS. Furthermore, bongkrekic acid (BKA) (a known inhibitor of the mPTP) prevents this ROS-dependent depolarization [31]. Since mitochondria are a major source of ROS generation, ROS-dependent mPTP opening may lead to a feed-forward process of additional ROS release into the cytosol of the cell, this has been termed, “ROS-induced ROS release” [31]. Additionally, ROS is able to trigger mPTP-dependent mitochondrial swelling in the presence of low levels of Ca^{2+}, which is inhibited by CsA [31,34,35].

Although ROS can seemingly induce mPTP opening, there are many critical questions addressing this mechanism. One of these fundamental questions is whether or not ROS-mediated mPTP opening is a completely independent event from Ca^{2+} overload (Fig. 1), or if these two triggers overlap and synergize to lead to mPTP-dependent mitochondrial dysfunction. Another form of necrotic cell death that is dependent on ROS-mediated mitochondrial dysfunction is oxytosis later to be referred to as ferroptosis. This version of cell death leads to a decrease of glutathione peroxidase 4 (GPX4) levels, one of the antioxidant enzymes that reduces oxidized polyunsaturated fatty acids (PUFA), which make up large parts of biological membranes [36]. If GPX4 remains depleted, cells accumulate overwhelming amounts of ROS, which will lead to lipid peroxidation of membranes. This version of cell death has been implicated in I/R injury [37,38]. Following reperfusion of an occlusion, the mechanical and shear stress leads to the lyses of red blood cells. Thus, releasing iron bound hemoglobin, which is taken up by nearby cells. In these cells, the increase of iron leads to the peroxidation of the PUFAs via the Fenton Reaction, in which iron reacts with hydrogen peroxide to form damaging hydroxyl radicals targeting the membrane PUFAs. This causes an ongoing chain of neighboring lipids becoming oxidized eventually leading to membrane collapse. This form of cell death has been reported to occur independently of mPTP activity [39], although inhibition of the mPTP is also protective during I/R injury in numerous animal model systems [3–6]. Potentially, both forms of necrotic cell death may be occurring simultaneously in the heart during I/R injury [40] and ROS levels could be the link between both forms of necrotic cell death. For example, low levels of ROS may facilitate sensitization of Ca^{2+}-mediated mPTP-dependent necrosis, while high levels of ROS may be sufficient to induce ferroptosis independent of the mPTP.

It is clear that there is a relationship between ROS and Ca^{2+} in mediating mPTP opening. ROS has been established to sensitize the mPTP to Ca^{2+}. It is thought that ROS targets mPTP regulator proteins via posttranslational modifications (PTMs) to facilitate this sensitization. These types of PTMs are referred to as oxidative PTMs, since they occur in an oxidative environment. Indeed, low levels of ROS have been implicated to serve as important precursors in various signal transduction pathways mainly related to cellular redox control, and mitochondrial function [41,42]. For example, certain cysteine proteases that are normally residents of the cytoplasm (a generally reduced environment), are susceptible to PTMs via ROS and become active if the cytoplasm becomes overly oxidative. The presence of the thiol group (-SH) in the side chain render cysteine residues particularly susceptible to oxidative modification [43]. Dysregulation of oxidative PTMs in the mitochondria have been linked with numerous disease pathologies including cardiomyopathies and neurological

disorders. The mPTP is composed of numerous proteins and regulators, each of which is potentially susceptible to PTMs, which can affect the sensitivity of the mPTP [44]. S-nitrosylation (SNO/SNOylation) is the addition of a –NO group to thiols of cysteine residues by the donor s-nitrosoglutathione (GSNO) [45]. This modification has been shown to sensitize the mPTP, allowing for the activation of the pore with a lower concentration of Ca^{2+} by modifying the ANT family and voltage dependent anion channels (VDAC) proteins [46].

CypD has been shown to be modified by SNOylation [40,47]. The specific residue of CypD, cysteine 203, was identified by mass-spectrometric analysis to be SNOylated following treatment with GSNO, and is thought to be the potential site of a triggering PTM to sensitize the mPTP. Subsequent research confirmed that in hearts preconditioned to ischemia, increased SNO-CypD was identified perhaps facilitating the protective effect of ischemic preconditioning [40]. Indeed, GSNO pretreatment inhibited mPTP activation when cells were treated with the H_2O_2 [40], providing evidence that SNOylation of CypD desensitizes the mPTP. This study also showed that when cysteine 203 of CypD was mutated to serine, cells were resistant to mPTP activation and mitochondria require significantly more Ca^{2+} to undergo a swelling event. CRC is also increased when liver mitochondria are reconstituted with this mutant version of CypD.

It is clear ROS has the ability to sensitize mitochondria to Ca^{2+}-dependent mPTP opening. Most likely, this is dependent on a ROS-dependent PTM of either a regulator or the actual pore-forming component of the pore. However, high levels of ROS can trigger mitochondrial swelling and dysfunction through lipid peroxidation independently of the mPTP. In the case of I/R injury, additional investigation is needed to determine which form of ROS-dependent mitochondrial dysfunction is dominant. Perhaps it is dependent on ischemic timing or spatial distance from the occlusion.

Matrix Regulation of the mPTP by Cyclophilin D

As discussed previously, it was shown in the 1970s that high matrix Ca^{2+} leads to mitochondrial swelling due to activation of a non-specific channel and that this swelling could be reversed by chelating Ca^{2+} [48]. It was later demonstrated that CsA could inhibit the mPTP [49]; the Ca^{2+} activation of mitochondrial swelling required higher Ca^{2+} levels in the presence of CsA. It was later demonstrated that the mitochondrial matrix protein CypD was the target of CsA [50,51]. CypD is a peptidyl-prolyl cis/trans isomerases and genetic deletion of CypD directly confirmed that it was an activator of mPTP opening as mitochondria lacking CypD required much larger amounts of Ca^{2+} to initiate mitochondrial swelling (Fig. 1) [6,52,53]. Although loss or inhibition of CypD increased the amount of Ca^{2+} needed to activate the mPTP, mPTP opening could still occur with higher levels of Ca^{2+}. This was interpreted as showing that although CypD was an activator of the mPTP, with higher levels of Ca^{2+}, mPTP opening was CypD independent.

The demonstration that CypD could enhance mPTP opening initiated studies to identify targets of CypD. It was originally shown that CypD can bind to ANT leading to the suggestion that the ANT might be the pore-forming unit [54], as discussed below. Studies by

Wallace's group showed that CsA inhibitable mPTP opening can still occur in mitochondria with deletion of several ANT isoforms [55]However, much larger amount of Ca^{2+} were needed to active mPTP opening. The mitochondrial phosphate carrier (PiC) was also shown to bind to CypD. However genetic deletion of the PiC did not eliminate mPTP activity [56,57]. CypD has also been shown to bind to the ATPO subunit of the F_1-ATPase supporting a role for the F_1-ATPase as the mPTP or at least a regulator of the mPTP [58]. It should be noted that CypD is a mitochondrial matrix chaperone protein, so it is not surprising that it binds to many proteins.

The mechanism by which CypD activates the mPTP is poorly understood. Inhibitors or genetic ablation of CypD reduce infarct size in animal studies of I/R injury [3–6]. CsA-mediated inhibition of the mPTP and cardioprotection have been demonstrated in cells, isolated hearts, and in vivo models [59]. Furthermore, in a proof of concept, phase II trial, the administration of CsA during acute myocardial infarction decreased infarct size as assessed by MRI [60]; however a subsequent phase III trial showed no benefit to CsA treatment [61]. The potential reasons why CsA has been beneficial in animal studies yet failed to show benefit in the clinical trial have been discussed elsewhere [62,63]. It is worth pointing out that CsA works by binding CypD, which is only a regulator of the pore and robust stimuli can activate mPTP independent of CypD. Alternatively, perhaps a CypD independent mechanism of death (either a second mPTP or an alternative death mechanism) is the primary mechanism of I/R cardiomyocyte death in MI patients.

Amino acids with a proline can exist in either cis or trans conformation and CypD catalyzes this isomerization. The isomerization can occur without CypD but at a slower rate. The role of the CypD prolyl isomerase activity in PTP activation remains unclear. Moreover, other cyclophilin family members are known to have functions that do not involve their isomerase activity [64]. For example, although the drug CsA inhibits the isomerase activity of multiple cyclophilins, mutational analyses have shown that cyclophilin A (CypA) is able to mediate calcineurin inhibition and the immunosuppressive effects of CsA independent its prolyl isomerase activity. There are conflicting data as to whether the isomerase activity is required for CypD activation of PTP. Early inhibitor studies suggested that isomerase activity was not required for mPTP activation [65]. In contrast, Baines et al., expressed a CypD with an R96G mutation, which lacks isomerase activity, in mouse embryonic fibroblasts (MEFs) lacking CypD and showed that the isomerase dead CypD afforded similar protection to CypD-KO when treated with H_2O_2 [53]. Interestingly, overexpression of CypD in HEK293 and rat glioma C6 cells desensitized the cells to apoptotic stimuli, and this protection required CypD isomerase activity [66]. It is noteworthy that the original studies characterizing the CypD-KO mice showed that loss of CypD inhibited necrotic, but not apoptotic cell death [53]. Additional studies will be needed to clarify the role of the isomerase activity in regulating the mPTP.

It is unclear how CypD activates the mPTP (Fig 1). It has been proposed [67] that CypD functions to regulate the mitochondrial synthasome. ATP production by mitochondria requires the transport of ADP into the mitochondria via the ANT family along with the transport of inorganic phosphate via the PiC. ADP and Pi are then converted to ATP by the F_1F_o-ATPase. A complex involving ANT, PiC and the synthase has been shown to form

in the mitochondria and it is proposed that this complex more efficiently converts ADP and Pi to ATP. CypD has been proposed to enhance the dissociation of the synthasome into its components (ANT, PiC and F_1F_o-ATPase). This would be consistent with data showing that CypD results in a reduction in F_1F_o-ATPase activity [58,68], assuming that F1Fo-ATPase activity in the synthasome is higher. Porter and colleagues further propose that neither the F_1F_o-ATPase nor ANT are able to facilitate their mPTP modalities while part of the synthasome complex. Therefore, CypD hypothetically promotes mPTP opening by stimulating the dissociation of the synthasome complex into individual components (ANT and the F_1F_o-ATPase) that can then form the mPTP. It is also possible that as the synthasome disassembles, it exists in a certain configuration that forms the mPTP.

CypD is known to undergo a number of post translational modifications [69–75] (Table 1). Bernardi's group showed that phosphorylation of CypD enhanced its activation of the mPTP [76]. Although, the site of phosphorylation was not identified in this study. In another study phosphoproteomics identified an increase in phosphorylation of CypD-S42 in MCU deleted mice [74]. Furthermore, it was shown that mitochondria from MCU null hearts with increased phosphorylation of CypD exhibited increased susceptibility to mPTP opening and required less Ca^{2+} to trigger the pore. These data suggest that phosphorylation of CypD at S42 can enhance CypD binding to the mPTP exacerbating activation. Other studies showed that phosphorylation of S191 of CypD enhances CypD binding to the oligomycin sensitivity conferring protein (OSCP) of the synthasome and increases mPTP opening [73].

CypD has been shown to undergo lysine acetylation. SIRT3, a mitochondrial protein that catalyzes the deacetylation of mitochondrial proteins was reported to deacetylate lysine 166 of CypD [72]. Mice lacking SIRT3 showed increased acetylation of CypD at lysine 166 and at 16 months of age mitochondria from SIRT3 null mice showed reduced mitochondrial CRC. Cysteine 202 (C202) of CypD was also shown to play a role in regulating mPTP opening [69,70]. C202 of CypD is highly conserved among species and can undergo redox-sensitive, post-translational modifications [77]. C202 was found to be S-nitrosylated in cardioprotected hearts. It was proposed that oxidation (e.g. perhaps the formation of a disulfide bond with the mPTP) of C202 might target CypD to the mPTP and that SNO of C202 blocks the oxidation that targets CypD to the mPTP. This hypothesis would be consistent with data showing that oxidation targets CypD to the mitochondrial membrane. A knock-in mouse model was developed using CRISPR/Cas9 in which CypD-C202 was mutated to a serine (C202S) [70]. Infarct size was reduced in CypD C202S Langendorff perfused hearts compared to WT. Cardiac mitochondria from CypD-C202S mice also have higher CRC compared to WT. Cysteines can also undergo S-acylation, a reversible post-translational lipid modification involving a thioester bond, and C202 matches a S-acylation motif. S-acylation of CypD-C202 was assessed using resin-assisted capture. WT hearts are abundantly S-acylated on CypD-C202 under baseline conditions indicating that S-acylation on C202 per se does not lead to mPTP opening [70]. CypD C202S knock-in hearts are protected from I/R injury suggesting further that replacing CypD S-acylation at C202 with a serine is not detrimental and does not induce mPTP opening. The data are consistent with the hypothesis that either acylation, SNO or mutation of C202 can block the ability of CypD to activate the mPTP. All of these modifications would block oxidation of this cysteine. Interestingly, ischemia leads to de-acylation of C202 and Ca^{2+} overload in isolated

mitochondria promotes de-acylation of CypD. Taken together, these data suggest that with ischemia CypD is de-acylated at C202 allowing the free cysteine residue to undergo oxidation during the first minutes of reperfusion, which in turn targets it to the mPTP.

These post translational modifications of CypD are consistent with the hypothesis that CypD integrates signaling to regulate activation of the mPTP. It is intriguing to speculate that perhaps these PTMs are important for integrating metabolic signals to regulate F_1F_o-ATPase activity and as proposed [67] a by-product of this regulation is alter synthasome formation which regulates PTP activity.

The Inner Membrane Pore:

The ANT Family

The ANT family of proteins are members of the larger mitochondrial solute carrier family and are residents of the inner mitochondrial membrane. The ANTs are the most abundant protein found within this membrane. These proteins transport ATP across the inner membrane from the matrix towards the intermembrane space of mitochondria and ADP towards the matrix from the intermembrane space of the mitochondria in a 1:1 ratio. There are four isoforms of ANT (*Ant 1–4*) in humans and three expressed in mice (mice lack *Ant 3*). ANT 1 is most highly expressed in the mitochondria of heart and skeletal muscle. ANT 4 is the predominant isoform in the testis, while ANT 2 and ANT 3 are ubiquitously expressed in other tissues. The first suggestion that ANT may be part of the mPTP came from the observation that Ca^{2+}-dependent mPTP opening is inhibited by ADP [78]. Since ADP binds to the ANT, it was suggested that the specific conformation of this protein can influence the opening of the mPTP [78,79]. Specifically, ADP was found to stabilize the "m" state of ANT, which has the nucleotide-binding site facing the matrix. Additionally, the respiratory toxin, BKA, inhibits the mPTP in the same manner as ADP by stabilizing the ANTs in the m-state [80,81]. Oppositely to the m-state is the "c" state, or cytoplasmic state of the ANTs. Compounds that stabilize the c-state (ATP and carboxyatractyloside (Atr)) are able to sensitize the mPTP to Ca^{2+}. Further, Halestrap and Davidson were first to suggest that the mPTP regulator CypD interacts with ANT [51]. They suggested that the presence of conserved prolines [82] (later identified as proline 62 on the ANTs) in the matrix compartments on this protein may be the site of prolyl-isomerization activity of CypD [51]. Notably, this interaction is ablated with treatments of CsA [51]. Finally, a critical piece of evidence for ANT being the mPTP was demonstrated as ANT reconstituted into lipid bilayers were able to fore pores with similar characteristics as the mPTP (Fig. 1) [82–84].

Although there is substantial evidence to suggest that the ANT family is a pore-forming component or a critical regulator of the mPTP, reports have challenged this hypothesis [85]. Genetic removal of the two major isoforms of the ANT family, *Ant1* and *Ant2,* from mouse liver did not inhibit mPTP opening, however, the mitochondria required significantly more Ca^{2+} to open the pore. The treatment of CsA further desensitized these mitochondria to mPTP opening, suggesting that CypD may function independently of the ANT family in regulating the pore. One caveat to this study was that *Ant4* could potentially compensate for the loss of the other isoforms of *Ants* in the liver. Recently, this caveat was addressed

when researchers deleted all isoforms of *Ants* from the mouse liver. Surprisingly, these mice survived into adulthood with no obvious liver dysfunction [86]. Therefore, an unidentified compensatory mechanism must have been responsible for the transport of ADP/ATP across the inner mitochondrial membrane in these livers. Mitochondria isolated from these livers were extremely resistant to Ca^{2+}-induced mPTP opening, although mPTP opening could still occur with very high levels of Ca^{2+}. However, when CypD was inhibited or deleted on the *Ant* null background, Ca^{2+}-induced mPTP opening is completely inhibited, suggesting, that together the ANT family and CypD are essential regulators of the mPTP or that there may be the presence of two mPTP-like pores. One, consisting of the ANT family and the other requiring CypD activity. Therefore, genetic evidence has confirmed that the ANT family is an important regulatory or partial pore-forming component of the mPTP, however, the family is not essential for Ca^{2+} induced mPTP opening, similarly to CypD.

Perhaps the unknown compensatory mechanism that is responsible for the physiological function of the ANT family that engages in their absence is also responsible for the remaining mPTP activity. Additionally, both of these genetic loss-of-function approaches relied on the same Cre/LoxP approach for the deletion of *Ant2* in the liver, since the complete loss of *Ant2* is embryonically lethal. These systems are inherently problematic when deleting essential genes in tissues that are highly regenerative, such as the liver. There remains a possibility of incomplete deletion of *Ant2*, which may give these hepatocytes a competitive proliferative advantage to maintain liver functionality and may explain the remaining mPTP activity. Additionally, these data put into question the relationship between CypD and the ANT family and further investigation is needed to determine if their interaction has a functional consequence on mPTP sensitivity.

F_1F_o-ATPase

The F_1F_o-ATPase has been proposed by two groups to be the pore-forming unit of the mPTP (Fig. 1). Briefly, recent data have suggested that the F_1F_o-ATPase can, under certain circumstances, form the mPTP. Bernardi's group has proposed that dimers of the F_1F_o-ATPase can form the pore-forming unit of the mPTP [87]. Jonas' group has suggested that de-lipidation of the c-ring of the F_1F_o-ATPase can form the mPTP [88]. Although there are strong data in support for both hypotheses, there are also data in opposition [89,90]. Data from Walker's group has shown a CsA-inhibitable mPTP-like swelling is still present in cells lacking the c-ring and other components of the F_1F_o-ATPase that are required for dimerization. Furthermore, patch clamping results from the Jonas lab indicating similar conductance activity as the mPTP to the c-subunit ring pore showed that this pore is oligomycin (F_1F_o-ATPase inhibitor) sensitive [91], however; Ca^{2+} induced mPTP opening by the mitochondrial swelling assay was not inhibited by oligomycin treatment [92].

One explanation for the discrepancy is that there are two or more CsA-inhibitable pores [86]. As discussed above data from over 30 years ago proposed a role for ANT as a key component of the mPTP. CypD was shown to bind to ANT and adenine nucleotides were known inhibitors of the mPTP. BKA and Atr, two inhibitors of ANT that lock ANT in different conformations, were shown to regulate mPTP opening. BKA locks ANT in the matrix facing conformation and inhibits mPTP opening whereas Ca^{2+} binds to the cytosolic

facing conformation and activates PTP. The hypothesis that ANT was the pore-forming component of the mPTP fell out of favor in 2005 when Wallace's group reported that a CsA-inhibitable PTP, albeit requiring higher Ca^{2+} for activation, was present when *Ant1* and *Ant2* were deleted [93]. Recent studies have revisited a role for ANT as a pore component of the mPTP. Karch et al., showed that mitochondria with deletion of *Ant1*, *Ant*2 and *Ant4* were desensitized to Ca^{2+}-induced PTP opening, and PTP opening was completely inhibited by the further addition of CsA [86]. They proposed that there are two distinct pore-forming components of the mPTP: consisting of the ANT family and a second pore (Fig. 1). Both components may be activated by CypD, although the non-ANT component has a strict requirement for CypD.

Another feature of the mPTP that may also suggest two independent channels or regulatory components is its bistable conductance states, high and low [94]. Low conductance mPTP is thought to occur in more physiological settings due to the ability for the membrane potential to remain intact during this transient opening and closing. The high conductance state is considered to be pathological due to the depletion of membrane potential. Perhaps the ANT family or the F_1F_o-ATPase favors a high vs low mPTP channel. Neginskaya et al., reported that mitochondria deficient for the c-subunit of the F_1F_o-ATPase lack the high conductance mPTP channel, while a low conductance CsA sensitive channel still persists [95]. More insight is required on determining how the ANT family or the F_1F_o-ATPase influences the high vs low conductance mPTP channels and how this may affect physio- and pathological conditions.

Outer Membrane Regulators of the mPTP

The mPTP is defined as an inner mitochondrial membrane pore that is triggered mainly by increases in matrix Ca^{2+} levels. Although the pore is regulated from the matrix and takes place in the inner membrane, evidence suggests that the outer mitochondrial membrane plays a significant role in its sensitivity. In the early models of the mPTP, it was hypothesized that the mPTP spanned the inner mitochondrial space and occurred at inner and outer membrane contact sites. Some early evidence of this originated when it was demonstrated that ANT was isolated and identified at contact sites between the inner and outer membranes [96,97]. Furthermore, at these sites it has been shown that there is a pore that has similar conductance characteristics to that of the mPTP [97]. Together, these results provided evidence for an outer membrane component of the mPTP and research began into potential identities of this pore.

One such protein that became a suitable candidate is the voltage dependent anion channel (VDAC). VDAC is the most abundant protein on the mitochondrial outer membrane. This protein family form the main channel that regulate ion and metabolite flux between the outer mitochondrial membrane and the cytosol. Early evidence that the VDAC family may be part of the mPTP came from patch clamping experiments that showed that opening of the mPTP was voltage dependent [98]. The conductance characteristic of the mPTP was then compared to that of bilayers with reconstituted VDAC and similar conductance patterns were observed [99]. Later evidence showed affinity capture experiments that identified VDAC and ANT as binding partners of a GST-CypD fusion protein [100]. Additionally, reconstitution of CypD,

ANT, and VDAC produced the characteristic trace of mPTP opening in the presence of Ca^{2+}, which could be inhibited by pretreatment with CsA [100].

Though there is significant evidence to suggest VDAC is the outer membrane regulator of the mPTP, genetic evidence challenged this hypothesis. Baines *et. al,* utilized a dual deletion approach of two of the three isoforms of the VDAC family expressed in the heart. (*Vdac1 and Vdac3*) to demonstrate that mitochondria isolated from cardiomyocytes were still susceptible to Ca^{2+} induced mPTP-dependent swelling [101]. Unlike the deletion of CypD or the ANT family, the deletion of these isoforms did not alter the sensitivity of the mPTP to Ca^{2+} [101]. Utilizing *Vdac1* and *Vdac3* double deleted MEFs and an siRNA approach to abolish the expression of *Vdac2*, they found that VDAC family null MEFs were not protected against necrotic or apoptotic stimuli, suggesting that they are dispensable for mPTP activity [101].

In addition to the VDAC family, the Bcl-2 family is another set of proteins that can greatly affect the permeability of the outer mitochondrial membrane and has been implicated in regulating the mPTP (Fig. 1). The Bcl-2 family consists of over 25 family members characterized by containing at least one Bcl-2 homology (BH) domain and consist of both pro-apoptotic and anti-apoptotic members [102]. During homeostasis, the pro-apoptotic BH3-only family members are in balance with the anti-apoptotic BH1-4 containing members [102–104]. The downstream ramification of these family members is the inactivation or activation of BAX and BAK (BH1-3, effector Bcl-2 family members) [102]. Upon activation, BAX and BAK form homo-/hetero oligomers, which leads to increased permeability of the outer mitochondrial membrane allowing molecules up to 100 kDa to freely cross the outer membrane vs the normal 5 kDa molecules [105–107].

The first evidence that BAX may be a component of the mPTP was determined when depletion of BAX from cells inhibited the ability to undergo permeability transition [108]. This same study showed that BAX and ANT interact in a yeast two-hybrid system and cells with reconstituted BAX could not induce cell death in cells lacking ANT [108]. It was also shown that recombinant BAX could lead to a dissipation of the inner membrane potential, which is associated with mPTP opening [108,109]. Strong supportive evidence for the role of BAX and BAK in the mPTP came from showing that these proteins are necessary for cells to engage regulated necrosis. These studies demonstrated that cells lacking both *Bax* and *Bak1* are resistant apoptotic cell death as well as multiple forms of regulated necrotic cell death, including mPTP-dependent necrosis [110–113]. Further, mitochondria isolated from these cells were unable to undergo Ca^{2+} -induced swelling and displayed significantly increased CRC [112,114]. During apoptosis BAX/BAK form large pores within the outer mitochondrial membrane, however, this role of BAX and BAK is not required for mPTP-dependent cell death. Indeed, double deleted cells reconstituted with oligomeric-dead mutant versions of BAX restore the ability to undergo some forms of regulated necrotic cell death, but not apoptosis [110–112]. Notably, the inner membrane pore is still intact and measurable by patch clamping mitoplasts isolated from *Bax* and *Bak1* deleted cells. Thus suggesting, that BAX and BAK are eliciting their mPTP sensitizing effects by increasing outer mitochondrial membrane permeability.

In a recent paper, it was shown that cells are more susceptible to Ca^{2+}-induced necrosis when treated with BH3-mimetic compounds targeting and inhibiting various members of the anti-apoptotic Bcl-2 family (Bcl-2, Mcl-1, and Bcl-xL) [114]. Further, isolated mitochondria treated with the same BH3-mimetics displayed decreases in CRC due to the sensitization of the mPTP [114]. Indeed, mitochondria lacking both BAX and BAK remained unaffected by the treatment of the various BH3-mimetics [114]. Together, these data support the hypothesis that upstream Bcl-2 family members are able to affect mPTP sensitivity by influencing the activation status of BAX and BAK, which alters outer mitochondrial membrane permeability. Mechanistic understanding of how outer mitochondrial membrane permeability directly affects matrix Ca^{2+} capacity and mPTP sensitivity is required. Potentially, active BAX and BAK may lead to the release of a critical regulator of the mPTP found within the inner membrane space, similarly to cytochrome-c release during apoptosis.

Conclusion

MPTP-dependent necrosis is dependent on mitochondrial dysfunction via prolonged mPTP opening. If the heart does not produce sufficient levels of ATP, it cannot maintain contractibility or ion homeostasis, which can lead to osmotically driven cellular rupture and necrosis. Although mPTP-dependent necrotic cell death is a primary mode of cell death during I/R injury, the process of permeability transition can also lead to other forms of cell death as well (Fig. 2). For example, if a limited population of mitochondria undergo mPTP opening and lose membrane integrity, this can lead to cytochrome-c (Cyt-C) release [115,116] or mtDNA release, which can initiate apoptosis or pyroptosis respectively [117]. Additionally, these dysfunctional mitochondria need to be processed through mitophagy and the energetic deficit caused by their dysfunction can lead to an increase in macroautophagy, which may lead to autophagy or lysosomal-dependent forms of cell death [118,119]. Furthermore, compensation for the loss of oxidative phosphorylation through glycolysis leads to a decrease in NAD^+ pools within the cell, which are required for PARP-dependent DNA repair, which may sensitize a cell to undergo parthanatos, another form of necrotic cell death [120].

The lack of molecular characterization of the mPTP has hampered the progress and impact of this cell death mechanism. Potentially, pinning down the identification has challenged researchers due to the possibility of being composed of more than one pore-forming component. Many ongoing questions concerning the regulation of the mPTP have been highlighted throughout this review (Fig. 1). Identifying the molecular composition of this pore will be the catalyst for answering most of these remaining questions. Regardless, it is clear that the mPTP is a significant contributing factor to the diseases of the heart and identifying novel strategies to target mPTP regulation will be therapeutically beneficial.

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Highlights

- Ischemia reperfusion injury is a consequence of mPTP-dependent necrosis
- Prolonged mPTP opening leads to mitochondrial dysfunction
- mPTP opening is triggered by calcium and reactive oxygen species
- The mPTP may consist of multiple pore-forming entities
- Outer mitochondrial membrane permeability through the Bcl-2 family alters mPTP sensitivity to calcium

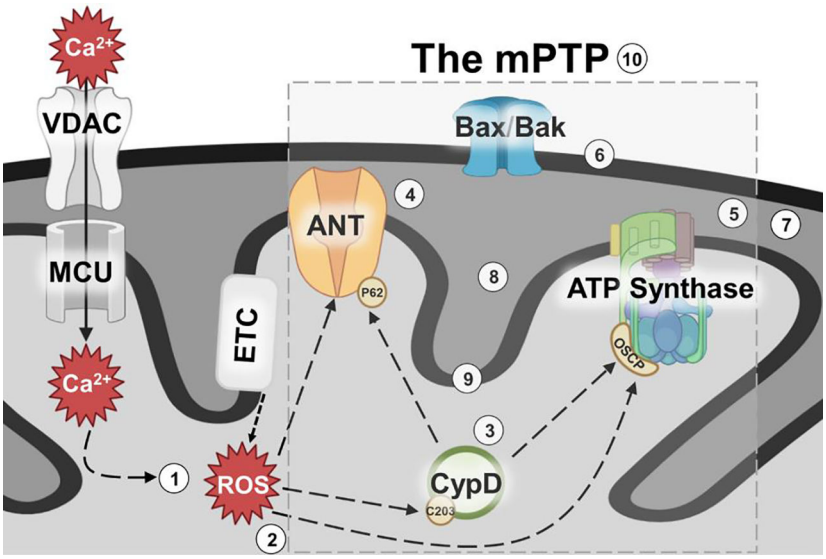

Figure 1: Outstanding questions surrounding the regulation, identity, and pathological relevance of the mPTP.

Schematic representation of the current model of the mPTP, its regulators and its triggers. Though it is known that Ca^{2+} and ROS trigger the mPTP, and various protein components of the mPTP have been proposed, there are essential questions that remain to be addressed: ① How does Ca^{2+} trigger mPTP? ② How does ROS sensitize mPTP to Ca^{2+}? ③ What is the target of CypD activity? ④ Is ANT a regulator or pore forming component of mPTP? ⑤ Is ATP synthase a regulator or pore forming component of mPTP? ⑥ How does outer mitochondrial membrane permeability influence matrix calcium retention capacity? ⑦ Are contact sites essential for mPTP activity? ⑧ Is the mPTP composed of two pore forming entities (ANT and ATP synthase pores)? ⑨ Are there additional pore forming components of the mPTP? ⑩ Can complete inhibition of mPTP protect humans against myocardial infarct injury?

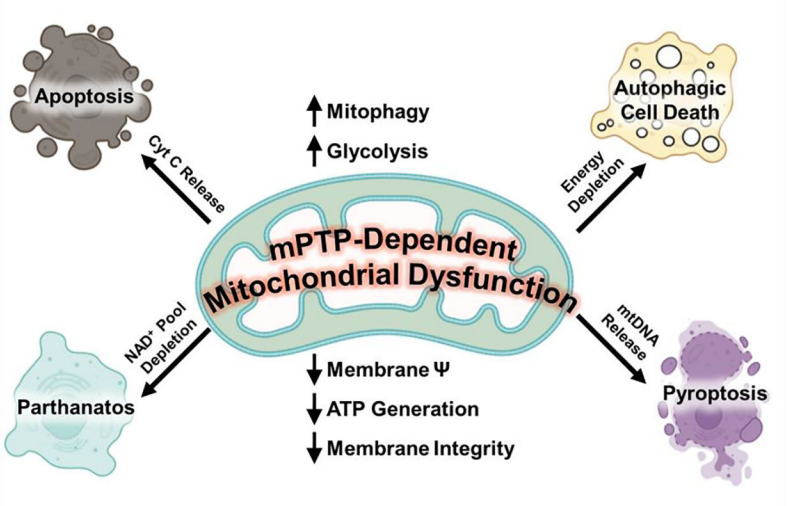

Figure 2: mPTP dependent mitochondrial dysfunction can potentially feed into other cell death pathways.

Following mPTP opening, mitochondrial membrane potential and eventually integrity decreases. This leads to a decrease in mitochondrial ATP production, which can lead to an increase in glycolysis as a compensatory mechanism to maintain the energetic demand of the cell. These events culminate in the reduction of the NAD+ pools which can sensitize a cell to parthanatos. Energetic depletion may also result in the over activation of autophagy, which may result in autophagic or lysosomal dependent cell death. In addition, mitochondrial membrane rupture can trigger other cell death modalities. For example, rupture can lead to Cytochrome C (Cyt C) release, which may initiate apoptosis or mtDNA release, which can initiate pyroptosis.

Table 1:

Modifications of Cyclophilin D and its effect on mPTP opening

SITE	MODIFICATION	EFFECT	REFERENCE
C202	SNO	inhibit	[69]
C202	palmitoylation	inhibit	[70]
C104	sulphenylation	inhibit	[71]
K166	acetylation	enhance	[72]
S191	phosphorylation	enhance	[73]
S42	phosphorylation	enhance	[74]
S31	phosphorylation	inhibit	[75]