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Physiologic Functions of Cyclophilin D and the Mitochondrial Permeability Transition Pore

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Abstract

This review focuses on the role of cyclophilin D (CypD) as a prominent mediator of the mitochondrial permeability transition pore (MPTP) and subsequent effects on cardiovascular physiology and pathology. While a great number of reviews have been written on the MPTP and its effects on cell death, we instead focus more on the biology surrounding CypD itself and the non-cell death physiologic functions of the MPTP as regulated by CypD. A greater understanding of the physiologic functions of the MPTP and its regulation by CypD will likely suggest novel therapeutic approaches for cardiovascular disease, both dependent and independent of programmed necrotic cell death mechanisms.

Keywords

Heart; cyclophilin D; ischemic injury; metabolism; calcium; permeability transition; pore; mitochondria; PPIF

PPIF gene – Domains and Structure

Cyclophilin-D (CypD) is a mitochondrial matrix protein encoded by the peptidyl-prolyl cis-trans isomerase F gene (*PPIF*), a member of the greater cyclophilin gene family. Cyclophilins are conserved through all eukaryotic and even bacterial kingdoms and all exhibit peptidyl-prolyl cis-trans isomerase (PPIase) activity, with most members directly binding the immunosuppressive drug cyclosporin A (CsA). There are 17 cyclophilins in the human genome and all are primarily implicated in protein folding and chaperone function (1). *PPIF*, located on human chromosome 10, consists of 6 exons (all coding) and is highly conserved to the yeast homologue (2, 3)). As an aside, the HUGO gene nomenclature committee incorrectly annotated the CypD protein as the *PPIF* gene (peptidyl-prolyl isomerase F), as it should have been named *PPID*. The full-length CypD protein consists of

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207 AA (22 kDa) with the most prominent feature being a 109 AA cyclophilin domain (cl00197, IPR002130) that imparts prolyl-isomerization activity that is conserved among most cyclophilins (4). To illustrate the importance of the isomerase domain, Baines et al. showed that an isomerase-deficient mutant of cyclophilin D (R96G) was unable to rescue mitochondrial swelling or ROS-induced cell death in *Ppif*$^{-/-}$ mouse embryonic fibroblasts (MEFs), yet a wildtype (WT) version fully rescued (5). These results suggest that the isomerase domain of CypD is necessary for modulation of the mitochondrial permeability transition pore (MPTP) (detailed more extensively in upcoming sections).

The remaining coding regions impart properties unique to CypD including the first 29 AA that encode the N-terminal mitochondrial targeting sequence and other extra-PPIase regions that may impart isomerase target specificity or binding regions for modulatory partners (1). The crystal structure of human CypD has been elucidated in complex with CsA (6). Kajitani et al. reported that CypD contained eight β-strands, two α-helices, and one 310 helix, representing a structure similar to that of the other known cyclophilin family members (6). The authors also confirmed that CsA binding sites in CypD were analogous among other cyclophilins and that binding did not require a conformational change.

Phylogenetic Conservation

As previously stated the *PPIF* gene is well conserved across many organisms with predicted homology noted even in plants and fungi (see Table 1, *PPIF* homology across species) (2, 7, 8). In vertebrates the gene is extremely well conserved with the zebrafish homologous gene (*ppifb*, *Danio rerio*) having a 78% protein identity match with *H. sapiens* and functionally being linked to mitochondrial permeability transition (9). The interest in mitochondrial cyclophilin in other species began with the observation that permeability transition may be a highly conserved phenomenon. Indeed, permeability transition has been noted in *Drosophila*, yeast, plants, fish and amphibians; for an excellent review on this topic see reference: (10). In addition to academically deciphering the relevance *in situ*, studying pore function in lower organisms allows high-throughput screening efforts in the search for novel permeability transition inhibitors (drug discovery) and the pursuit of the identity of the elusive ‘pore-forming’ components of the MPTP with genetic screening approaches.

Mitochondrial Targeting and Import

The mitochondrial targeting sequence for human CypD is encoded by the following N-terminal leader sequence (MLALRCGSRWLGLLSVPRSVPLRLPAARA). The sequence computationally predicts to be a mitochondrial transit peptide recognized at the outer mitochondrial membrane (OMM) where processing allows import into the matrix. This post-translational processing results in the mitochondrial mature form (~18 kDa) that accounts for all CypD function. It had been previously hypothesized that multiple mitochondrial isoforms of cyclophilin exist, but expression of [^{35}S]-labeled CypD by Crompton and colleagues experimentally confirmed cleavage of the predicted mitochondrial localization sequence and mitochondrial import of a single isoform (11). With careful western blotting technique we can visualize both the full-length, cytosolic CypD (~22 kDa) and also the truncated, mitochondrial-localized isoform (~18 kDa) in cardiac protein extracts. Although

there is little evidence for transcriptional regulation of CypD, Matas et al. reported alterations in the subcellular distribution of CypD (mitochondrial vs. cytosolic) in rat hearts following volume-overload induced hypertrophy, suggesting another mode of potential regulation (12).

Historical Discovery of the MPTP

The first description of permeability transition can be found in a 1975 manuscript published in *The Journal of Biological Chemistry* by Douglas Hunter and Robert Haworth where they concluded that Ca^{2+} addition to isolated cow heart mitochondria induced a "…nonspecific increase in the permeability of the inner membrane, resulting in entry of sucrose into the matrix space and the observed configurational transition (swelling) of mitochondria." (13). Further they showed that this process required the energized uptake of Ca^{2+} through a ruthenium red-sensitive mechanism (now known to be the mitochondrial calcium uniporter (MCU), whose genetic identity was recently discovered (14–16)) and that permeability transition resulted in the uncoupling of oxidative phosphorylation. The same group followed this discovery with numerous manuscripts detailing the nature of permeability transition and its role in physiology that have largely held to date. The authors concluded in subsequent studies that the channel is gated in a Ca^{2+} specific manner and that opening imparts permeability to solutes up to 1.5 kDa in size (17). Hunter and colleagues also noted that MPTP open probability is reduced by ADP, adenine nucleotides, and other divalent cations such as Mg^{2+} (18–20) (Sr^{2+}, Ba^{2+} and Mn^{2+} have also been shown to inhibit permeability transition). Also see an excellent accounting of these seminal findings, including work predating Hunter and Haworth, in the expert reviews of Dr. Paolo Bernardi (21, 22).

Historical Discovery of CypD as a Component of the MPTP

Mitochondrial permeability transition is best defined as the collapse of the chemiosmotic gradient across the inner mitochondrial membrane (IMM) mediated by opening of a large conductance pore that we call the MPTP. This is not to be confused with OMM rupture, which results in the release of cytochrome *c* and apoptogens from the inner membrane space potentiating apoptotic cell death signaling (see the following in-depth review on OMM permeability (23)). The MPTP has long been postulated to be a channel with finite properties that spans the IMM and OMM simultaneously, although the molecular components that actually constitute this presumed structure remains undefined. Perhaps the most experimentally manipulated feature of the MPTP is that open probability is reduced by the drug cyclosporine A (CsA), which is a potent immunosuppressant originally isolated from a fungus (*Tolypocladium inflatum*) and found to inhibit calcineurin signaling in complex with CypA (24–27).

Preliminary observations leading to the discovery of CsA's modulation of MPTP came from electron microscopic analysis of renal biopsies from three different patients treated with CsA, which showed "giant mitochondria with disoriented cristae and paracrystalline inclusions (28)." While this finding is now viewed in opposition to the noted acute effect of CsA blocking MPTP opening and preventing mitochondrial swelling it did focus attention on the mitochondria as a site of action for CsA and was suggestive of matrix Ca^{2+} overload

being induced by CsA, thus prompting further investigation. Two reports examining CsA's effect on mitochondrial respiration found an inhibitory effect on State 3 respiration and importantly noted that CsA caused a large accumulation of Ca^{2+} in the matrix (29, 30). This set the stage for Crompton and colleagues who first identified that CsA could inhibit the Ca^{2+}-dependent pore of the inner membrane responsible for mitochondrial permeability transition (31). Shortly thereafter, Pfeiffer's group confirmed this finding and proposed that CsA bound a modulatory entity specific to pore formation (32). Halestrap and Davidson followed and were the first to identify a matrix-localized PPIase as the target of CsA and further proposed a unified model of interaction with adenine nucleotide translocase (ANT) to modulate permeability transition, which was considered the IMM component of the MPTP (33). Thereafter Crompton and colleagues identified CypD as the likely PPIase controlling opening of the MPTP using a photolabeling technique (34). Genetic confirmation of CypD as a central component of the pore did not come until 2005 when the Molkentin and Tsujimoto Laboratories simultaneously deleted the *Ppif* gene in the mouse and noted that mitochondria isolated from these animals were resistant to swelling and permeability transition (5, 35). Other genetic loss-of-function models have corroborated these findings solidifying CypD as a bona-fide component of the MPTP (36). However, the in vivo relevance of these data have been questioned (37). Indeed, an alternate hypothesis has been proposed whereby inhibition of CypD by CsA results in the 'unmasking' of an as of yet unknown inhibitory binding site for inorganic phosphate (P_i) (to be examined in more detail in later sections) (38).

Biophysical Characterization of the MPTP

Two independent labs employed mitoplast patch-clamping techniques to characterize the biophysical properties of a giant (1–1.5 nanosiemens), non-specific channel of the inner membrane (39, 40). Zoratti and Szabo confirmed the identity of the large conductance channel as the MPTP by verifying it was inhibited by CsA (41). They went on to suggest in another study that the voltage-dependence of this channel was similar to that of the voltage-dependent anion channel (VDAC) showing that alpidem (a ligand of the benzodiazepine receptor, at the time thought to be comprised of VDAC and ANT and a third component) could elicit currents identical to those observed for the MPTP, thus further supporting the contact-site hypothesis whereby the MPTP spans the inner and outer mitochondrial membranes (42, 43). Szabo and Zoratti also hypothesized that the mitoplast recordings attributed to the multiple conductance channel (MCC, also referred to as the mitochondrial megachannel) and those credited to the MPTP were one in the same (44, 45). Numerous sub-conductance states have been recorded for the MPTP indicating a possible physiological relevance for the 'sub-conductance state' of the channel outside the well-characterized large-conductance opening associated with cellular demise (44, 46, 47). Zoratti and colleagues also employed single channel recordings of the MPTP in $Ppif^{-/-}$ mitochondria and found that for the most part, recordings were indistinguishable from WT controls; with the exception that CsA-inhibition was lost (48). These findings support the known regulatory role for CypD in MPTP function but that it is not an obligatory component for pore formation, as previously reported (37).

Molecular components of the MPTP (CypD Interactors)

While numerous molecular constituents of the MPTP have been suggested to be necessary for pore formation only CypD has held up to genetic scrutiny. Until fairly recently, the model for the pore included the ANT in the inner membrane with the VDAC in the outer membrane together forming a continuous channel across the intermembrane space under the control of CypD (this is sometimes referred to as the 'contact-site hypothesis' and is largely attributed to work by Le-Quoc, Crompton and Halestrap). This model has since been largely discarded given more recent genetic loss-of-function approaches discussed below. However, there remains substantial evidence that numerous components, while not necessary for pore formation, are involved in modulating permeability transition (Figure 1).

ANT

The finding that bongkrekic acid, an inhibitor of ANT locking it in the "m" confirmation, was capable of delaying permeability transition, while a different inhibitor of ANT that locks it in the "c" confirmation (atractylaside) could sensitize opening in response to Ca^{2+} suggested that ANT could be a structural component of the MPTP (18, 49, 50). Halestrap and colleagues further developed this hypothesis, venturing that the effects of both CsA and the inhibitory action of ADP on pore opening could be attributed to direct binding of ANT to CypD (33). Using a CypD affinity column both Halestrap and Crompton independently showed molecular interaction with ANT (51, 52). Reconstitution studies by Kroemer's and Crompton's groups found that purified isolates of ANT, CypD, and VDAC (also Bcl2 proteins) could form a Ca^{2+}-responsive, CsA sensitive pore in proteoliposomes (52, 53) and work by Brdiczka's group suggested ANT could itself form a pore-like structure similar to the MPTP (54). The summation of these reports provided strong evidence that ANT was the most likely inner membrane component of the pore. This theory held until mice lacking *Ant1* and *Ant2* (*Ant1/2*$^{-/-}$) were generated and mitochondria isolated from double-null hepatocytes were shown to still undergo permeability transition (CsA sensitive) and remained susceptible to cell death initiated by various agents (55). This singular finding suggested that ANT was not a structural component of the MPTP, although it should be noted that *Ant1/2*$^{-/-}$ mitochondria required a higher concentration of Ca^{2+} to induce opening and that ANT ligands (such as ADP) lost inhibitory capacity on pore open probability. These later observations likely suggest that ANT is part of a larger pore complex in the IMM that can directly influence the properties of the MPTP.

VDAC

The first evidence that VDAC (referred to as porin in the older literature) may be the outer membrane pore forming component of the MPTP came in 1985 when the Le-Quoc's characterized a mitochondrial swelling event that appeared to require the OMM. They found mitoplasts (mitochondria stripped of the outer membrane and IMS constituents by osmotic disruption leaving only the IM and matrix portion of the organelle) did not respond in the classic swelling assay (56). The authors suspected VDAC was involved, possibly by migrating to the inner membrane to permit permeability transition. Crompton and colleagues further suggested VDAC's involvement by utilizing a CypD-GST affinity column, which

showed interaction with VDAC and ANT, as well as that these purified constituents could form a functional pore in proteoliposomes (52). Other reports also supported the VDAC hypothesis through anti-VDAC antibody blockade of permeability transition (57), and inhibition of the MPTP by VDAC phosphorylation (58–60). However, as with ANT, a genetic approach in the mouse eventually dispelled the notion that VDAC was a necessary component of the permeability pore. For example, Baines et al. demonstrated that deletion or knockdown of all three *Vdac* genes (*Vdac1, 2, 3*) did not disrupt MPTP function and furthermore did not alter necrotic or apoptotic cell death (61). In fact, deletion of *Vdac2* enhanced cell death propensity (61, 62).

F_0/F_1 ATP synthase

The mitochondrial ATP synthase (complex V) is a multi-subunit complex of the inner membrane that catalyzes the synthesis of ATP via the proton gradient generated by the electron transport chain. Since the earliest characterization of the MPTP, ATP synthase has been postulated to be a possible component of the pore itself (13), although most of this conjecture can probably be attributed to indirect changes in energetic factors that alter open probability (adenine nucleotides, phosphate, Ca^{2+}, etc.). Girogio et al. has shown that CypD directly binds the lateral stalk (subunit d and b) of ATP synthase and alters its activity (63). Another recent report utilizing siRNA-mediated knockdown found the F_0 c subunit to be required for permeability transition and the authors speculated that it is a principal component of a supramolecular MPTP complex (64). These findings have been confirmed by others showing that CypD interaction decreases ATP synthesis and hydrolysis rates to modulate both energy production and necrotic cell death (65). The exact mechanism whereby CypD may interact with and modulate complex V is complicated by the previously mentioned interactions of CypD with ANT. For example, Chinopoulos and colleagues suggested that the change in adenine nucleotide exchange associated with ATP synthase binding may be partially attributed to a change in ANT flux rates (66).

Phosphate Carrier (PiC)

The phosphate carrier (PiC, *SLC25A3* gene) is the primary transporter of inorganic phosphate (P_i) into the mitochondrial matrix by proton co-transport or in exchange for hydroxyl ions (67). Historically, P_i is a potent modulator of pore opening with countless studies suggesting a definitive relationship between matrix P_i content and MPTP activation (68–73). However, pore regulation by P_i has recently been reappraised, as the entire regulatory action of CypD, and thereby CsA, has been suggested to be P_i dependent (38). However, this hypothesis is in direct contrast to a recent report by Baines et al in which P_i had absolutely no effect in *Ppif* null or CsA-inhibited pore opening (74), a result that is supported by results from Halestrap's group who found that P_i actually enhanced MPTP opening, but independent of CsA (73). Despite the potential effect of P_i, the PiC itself has been suggested to be either an effector of the MPTP, or possibly even a structural component (75). However, definitive proof of PiC's involvement in the MPTP will have to await genetic analysis in *Slc25a3* null mice or null cells.

BH3 Proteins: Bax and Bak

Bax and Bak are proapoptotic, BH3-containing proteins of the greater Bcl-2 family that upon conformational change, oligomerize and insert into the OMM to initiate death (76). While there is substantial historical evidence to suggest that OMM permeability may be a required component of permeability transition, how this is regulated and whether or not it is under the control of CypD or matrix constituents remains unknown. Decade-old studies have suggested direct interaction and localization of Bax with previously proposed components of the MPTP including VDAC (77, 78) and ANT (79, 80) and that these interactions may be required for pore formation. However, when these data are reappraised in the light of the genetic studies showing that ANT and VDAC are dispensable for pore formation (described above (55, 61)), Bax's role in MPTP formation becomes enigmatic. However, a recent study suggested that Bax/Bak is a required component of mitochondrial-dependent necrosis (81). The authors found that mitochondria isolated from *Bax/Bak* double-null MEFs were resistant to swelling and loss of membrane potential ($\Delta\Psi$ in response to Ca^{2+} challenge. The authors proposed that Bax may be altering mitochondrial morphology by influencing mitochondrial fusion as the dynamin-like protein 1 (Drp1) fission inhibitor (Mdivi-1) shifted morphology in *Bax/Bak* double-null cells to the fused state and largely restored MPTP opening. However, how this functions from the OMM to regulate CypD-dependent IMM opening is uncertain. Thus the role of Bax/Bak in regulating or contributing to the MPTP remains largely undefined.

p53

Recently p53 was reported to be a mediator of pore opening by direct interaction and activation of CypD (82). The authors present this as a new mechanism whereby p53 can modulate cellular necrosis by directly influencing MPTP formation. They suggest that the p53-facilitated increase in pore open probability is directly attributed to the mitochondrial localization of p53 and independent of both transcriptional activity and modulation of Bcl2 proteins (work from the same lab suggested that p53 can activate and oligomerize Bcl2 proteins to permeabilize the OMM (83)). While the authors present a provocative +model there are numerous inconsistencies with the known literature casting doubt on the conclusion that p53 regulates classic MPTP formation and programmed necrotic cell death (84). For example, the authors found no alteration in Ca^{2+}-dependent MPTP opening, which is arguably the most fundamental regulator of permeability transition.

Complement component 1, q subcomponent binding protein (C1QBP)

On the basis of structural and computational analysis, Starkov has suggested C1QBP (also known as gC1qR, p32, gC1QR / 33, SF2, HABP1) is a likely candidate for the pore forming component of the inner membrane (85). C1QBP is well conserved and contains an N-terminal mitochondrial localization sequence. Jiang et al. first speculated it to be an MPTP component given predictions from the crystal structure (86). It is suggested that the β-sheet structure and other biophysical properties impart channel forming potential and a plausible target for CypD (85). Supporting observations include the apoptotic stimulants Hrk and smARF being required for mitochondrial C1QBP-dependent cell death (87, 88). In addition,

expression of mitoC1QBP in cell culture induced swelling and cristae derangement of mitochondria, suggestive of MPTP activation (89). In contradiction to these observations, overexpression of C1QBP attenuated ROS-induction of the MPTP and cellular necrosis while knockdown sensitized to MPTP opening (90). In addition, the same study found C1QBP to be a soluble component of the mitochondrial matrix rather than inner membrane localization (90). Thus, while C1QBP appears to be involved with MPTP formation, further studies are required to understand at what level.

Post-translational modification of CypD

Phosphorylation

While many kinase-dependent signaling pathways have been suggested to play a role in the regulation of the MPTP (91) there is only a single report suggesting direct phosphorylation of CypD. Glycogen synthase kinase 3β (GSK3β) has been reported to directly phosphorylate CypD when recombinant proteins were used, although whether CypD is directly phosphorylated by GSK3β in vivo is unknown (92). GSK3β (an often cited modulator of the MPTP) was also shown to translocate to the mitochondrial matrix where it formed a direct interaction with CypD, but not VDAC or ANT (93). However, the role of kinases, in general, as regulators of protein activity/function within the matrix remains a controversial area for several reasons. First, there is often a lack of definitive proof that implicated kinases are actually localized within the matrix, second there is a lack of understanding of what phosphatases are present in the matrix to counteract phosphorylation and provide some sort of dynamic regulation, and third, there is often a lack of associated kinase regulatory proteins or regulatory molecules within the matrix.

Deacetylation

Two independent labs have recently proposed deacetylation as a post-translational modification that regulates CypD. Work from David Sinclair proposed that the NAD+-dependent deacetylase sirtuin-3 (Sirt3) deacetylates CypD on lysine 166, which is adjacent to the binding site for CsA (94). The authors hypothesized that the hypertrophic phenotype associated with aging in *Sirt3* deleted mice is mediated by hyperactivation of the MPTP by acetylated CypD (presumably the homeostatic state is maintained by Sirt3 via direct interaction with CypD). While this hypothesis is intriguing it is complicated by previous observations that *Ppif* null mice are actually predisposed to hypertrophy and heart failure induced by a variety of stressors including swimming (physiological stress) and display an age-associated decrease in contractile reserve (95). This later study suggests that chronic inhibition of the MPTP does not impart a protection against age related hypertrophy but rather the inverse occurs and hypertrophic cardiomyopathy is enhanced. Since the authors failed to perform any experiments to suggest a causal relationship it's difficult to discern if deacetylation is a true physiologic regulator of CypD. A second proposed mechanism for Sirt3 deacetylation of CypD (acetylation at Lys 145) invoked the dissociation of CypD from ANT that in turn leads to hexokinase-II detachment from the mitochondria to stimulate ATP production (96). While both of these reports suggest that deacetylation of CypD may be a control point for MPTP regulation, causal experiments are needed for confirmation.

S-nitrosylation

Nitric oxide can influence cell signaling by S-nitrosylation (SNO, post-translational thiol modification of a protein cysteine residue by covalent attachment of a NO moiety) of target proteins and multiple studies have observed cardioprotection associated with SNO (97, 98). Pertinent to the current review SNO modification of numerous MPTP modulators has been reported (including: F_0F_1-ATPase, creatine kinase, thioredoxin, VDAC and CypD) (98–101). To determine if SNO modification of CypD is critical to its regulatory role in controlling the MPTP the Murphy group mutated cysteine 203 (C203S) and reconstituted *Ppif*$^{-/-}$ MEFs with either WT or mutant protein (102). They found that the mutant cells were resistance to mitochondrial swelling and ROS-induced cell death suggesting that thiol modification at this site may be critical in the regulation of the MPTP. However, the C203S mutant cells behaved similarly to GSNO modified CypD in regards to mitochondrial swelling and Ca^{2+} retention suggesting that the mechanism of SNO modification in MPTP regulation may be to mask Cys-203 from oxidation (a post-translational redox modification often seen as the antithesis to SNO).

Physiological Function of the MPTP

CypD and the MPTP as a $_{mito}Ca^{2+}$ Efflux Channel

Given the inseparable nature between Ca^{2+} and MPTP regulation it was postulated that the pore itself might also affect matrix Ca^{2+}. For example, a mathematical model suggested that under conditions of high intracellular Ca^{2+} cycling during periods of stress, the known $_{mito}Ca^{2+}$ efflux pathways mediated by the H^+/Ca^{2+} exchanger and Na^+/Ca^{2+} exchanger, would not be sufficient to prevent Ca^{2+} overload (103), suggesting that alternate means of removing Ca^{2+} is needed. The first experimental evidence of the MPTP being involved in $_{mito}Ca^{2+}$ efflux came in 1992 when Altschuld et al. demonstrated that CsA could inhibit Ca^{2+} efflux in isolated rat cardiomyocytes (104). This hypothesis was further developed when a biophysical model of Ca^{2+}-induced mitochondrial Ca^{2+} release was presented that involved low-conductance MPTP opening (105–107). We recently bolstered this proposed physiological function for the MPTP by showing that the genetic loss of CypD results in elevated resting levels of Ca^{2+} in the matrix and that acute CsA treatment in cultured cardiomyocytes inhibited mitochondrial Ca^{2+} efflux as shown by an increase in the time rate of decay to baseline following continuous pacing (95). The elevation in matrix Ca^{2+} was found to be associated with enhanced activity of Ca^{2+}-dependent mitochondrial dehydrogenases and a shift in substrate utilization from fatty acid oxidation to glycolysis in the working heart. These results suggest that the MPTP may be a control point linking mitochondrial metabolism (ATP generation) with myocardial workload (functional demand) through Ca^{2+} (Figure 2). In support of this study, Korge et al. recently demonstrated that asynchronous transient MPTP opening (presumably in small mitochondrial subpopulations) allowed depolarization to extrude Ca^{2+}, yet recover $\Delta\Psi$ and ATP generation (108). Additional evidence for CypD/MPTP playing a role in mitochondrial Ca^{2+} came from adult cortical neurons isolated from *Ppif*$^{-/-}$ mice where the authors found elevated $_{mito}Ca^{2+}$ following the activation of plasma membrane Ca^{2+} channels (109). It is interesting to note that the authors only saw this effect with maximal activation suggesting a threshold of Ca^{2+} was necessary before invoking extrusion via the MPTP, presumably because of saturation of

the traditional Ca^{2+} efflux channels. Thus, the MPTP, as regulated by CypD, appears to be a physiologic Ca^{2+} release mechanism that is required for proper metabolic regulation within the mitochondria.

Metabolic Regulator

Direct evidence for CypD modulation of energy homeostasis can be found in the previously mentioned direct-interactions with complex V (ATP synthase) and modulation by hexokinase II (a regulator of the MPTP that binds within the OMM (110–113)). Both of these proteins are intrinsically linked to ATP production and thus are potential sites of CypD regulation of cellular metabolism. In addition, if transient opening of the pore modulates matrix Ca^{2+} levels as discussed above, the matrix-localized dehydrogenases and components of the electron transport chain will be directly affected and ATP production impacted. In further support of CypD as a metabolic regulator, Luvisetto et al. reported that *Ppif*$^{-/-}$ mice develop adult-onset obesity (a finding corroborated by our group, unpublished data) (114). Strikingly the authors found a large increase in body weight at 6 months of age, reaching a maximal difference at 1 year of age as compared to WT controls. This increase in body weight was accompanied by a large increase in white adipose tissue with no apparent change in food intake. This report coupled with our observations of substrate switching in the hearts of *Ppif*$^{-/-}$ mice, along with significant mRNA and proteomic expression changes in metabolism-associated genes, provides a strong case that CypD may play a fundamental role in global energy homeostasis (95, 115). Whether metabolic control is directly linked to the MPTP's role in Ca^{2+} extrusion from the matrix or is mediated by CypD in an MPTP-independent fashion remains to be resolved.

CypD as a Central Regulator of Programmed Necrosis

CypD has been implicated in cell death pathways since it was first identified as the target of CsA (33, 34, 52, 116, 117). The first definitive proof that CypD was critical in necrotic signaling came in 2005 with the previously mentioned reports detailing the genetic deletion of *Ppif* (5, 35, 36). These studies found that *Ppif* null cells were highly resistant to cell death induced by cytosolic Ca^{2+} overload, hydrogen peroxide-mediated ROS stress, and thapsigargin (SERCA inhibitor, ER Ca^{2+} stress) but died similar to control cells when treated with staurosporine, TNFα, and etoposide (topoisomerase inhibitor, causing DNA fragmentation). The death signature elicited by these various agents suggests that CypD is prominent in necrotic cell death but dispensable in apoptotic signaling pathways. These genetic studies provided some of the first conclusive evidence that necrosis, which has historically been viewed as a non-programmed and an accidental death process, may actually be comprised of distinct molecular signaling events. *Ppif* null mice have since been employed in numerous animal models, providing an extensive framework to support a central role for the MPTP in pathophysiology (see Table 2, *Ppif*$^{-/-}$ phenotypes in physiology). The role of the MPTP in programmed necrotic cell death has been extensively covered (118, 119).

Role is Ischemia-reperfusion injury

In work predating the identification of CypD it was demonstrated that CsA could reduce hepatic ischemic injury, advocating inhibition of the MPTP as a relevant therapeutic approach in cell death-associated diseases (120–124). In addition to the numerous studies citing cytoprotective actions of CsA in ischemia-reperfusion (IR) injury (please see the following for an extensive review (125)), causative evidence of CypD involvement can be found in studies utilizing *Ppif*$^{-/-}$ mice, which were protected in numerous IR models including the heart (5, 35), brain (36, 126), and kidney (127, 128). This potent protection is no doubt linked to direct inhibition of the MPTP as the ischemic milieu consists of the classical MPTP activators Ca^{2+} and ROS.

In addition to the acute effect of inhibiting CypD in IR injury it has also been suggested that pore opening plays a significant role in ischemic-preconditioning (IPC) signaling. IPC is cytoprotective signaling elicited by transient, reciprocal episodes of IR prior to a major ischemic event (129). Interestingly, *Ppif*$^{-/-}$ mice subjected to IPC or pharmacologic preconditioning were found to be refractory to cytoprotection (130). These results suggest that the MPTP may not only play a role in the catastrophic events leading to necrotic cell death but that sequential activation of the pore (perhaps transient opening) can elicit cytoprotective signaling. While this study did not provide a molecular mechanism to account for this phenomenon it's plausible that low-conductance opening of the pore could generate superoxide or Ca^{2+} or modulate a pore-associated component such as GSK3β to activate discrete cytoprotective signaling pathways (some of which has recently been explored (131)). The necessity of CypD for IPC lends further credence to the notion of a physiological function for permeability transition.

Heart failure

We recently showed that *Ppif*$^{-/-}$ mice have progressively worse cardiac disease than control WT mice when subjected to a model of pressure overload-induced heart failure (95). However, our initial hypothesis was that *Ppif*$^{-/-}$ mice would be protected from heart failure by reducing the known accumulative effect of myocyte drop out that contributes to heart failure (132, 133). Mice lacking *Ppif* fared much worse following transaortic constriction displaying a decrease in left ventricular function (LV) and increase in LV dilation, hypertrophy and other markers of disease. Further *Ppif*$^{-/-}$ mice crossed with the Ca^{2+}/calmodulin-dependent protein kinase IIδ-c overexpressing mouse model of cardiomyopathy and mitochondrial Ca^{2+} overload, displayed a decrease in survival and large increase in hypertrophy (134, 135). Even more striking *Ppif*$^{-/-}$ mice subjected to physiological exercise (swimming) also presented with an increase in hypertrophy, lung edema and even mortality (95). Crossing these globally deleted mice with a low-expressing cardiac-specific CypD transgenic line completely rescued the maladaptive phenotype, suggesting a cardiomyocyte driven disease process. Mechanistically, *Ppif*$^{-/-}$ hearts had a significant shift in substrate utilization (from β-oxidation to glycolysis), suggesting metabolic reprogramming as the likely cause of the worsening heart failure.

CypD-Based Treatment Strategies for CV Disease and Conclusions.

Clinical trials have shown that inhibition of CypD and the MPTP with CsA was protective to patients immediately after MI injury when applied during the revascularization phase (136). Clearly this study and a vast array of animal studies indicate that a MPTP inhibitor should be protective to the heart immediately following ischemic injury, although additional clinical trails are needed to solidify a new standard of care treatment with such inhibitors. However, CsA may not be the best choice of inhibitor as it also blocks the activity of calcineurin, which itself is cardioprotective (137–141). Hence, a more selective CypD inhibitor, such as Debio-025 would represent an ideal choice for acute application during revascularization post-MI injury (142–144). While Debio-025 or similar CypD-inhibiting drugs could certainly be cardioprotective in the short term following ischemic injury by preventing myocyte death, long-term use of such drugs might actually contribute to cardiac disease if a pre-existing cardiac condition is present. *Ppif*$^{-/-}$ mice were more prone to metabolic-based heart failure when stimulated, even with exercise. Thus, Cyp inhibitors are probably not applicable for chronic use in heart failure patients, even when myocyte drop out is a significant issue. Moreover, prolonged use of Cyp inhibitors in patients being treated for hepatitis C should possibly be evaluated for CV complications (145–148). Regardless of these issues, acute use of an effective Cyp inhibitor should hold great promise for limiting myocardial damage in patients immediately after infarction injury.

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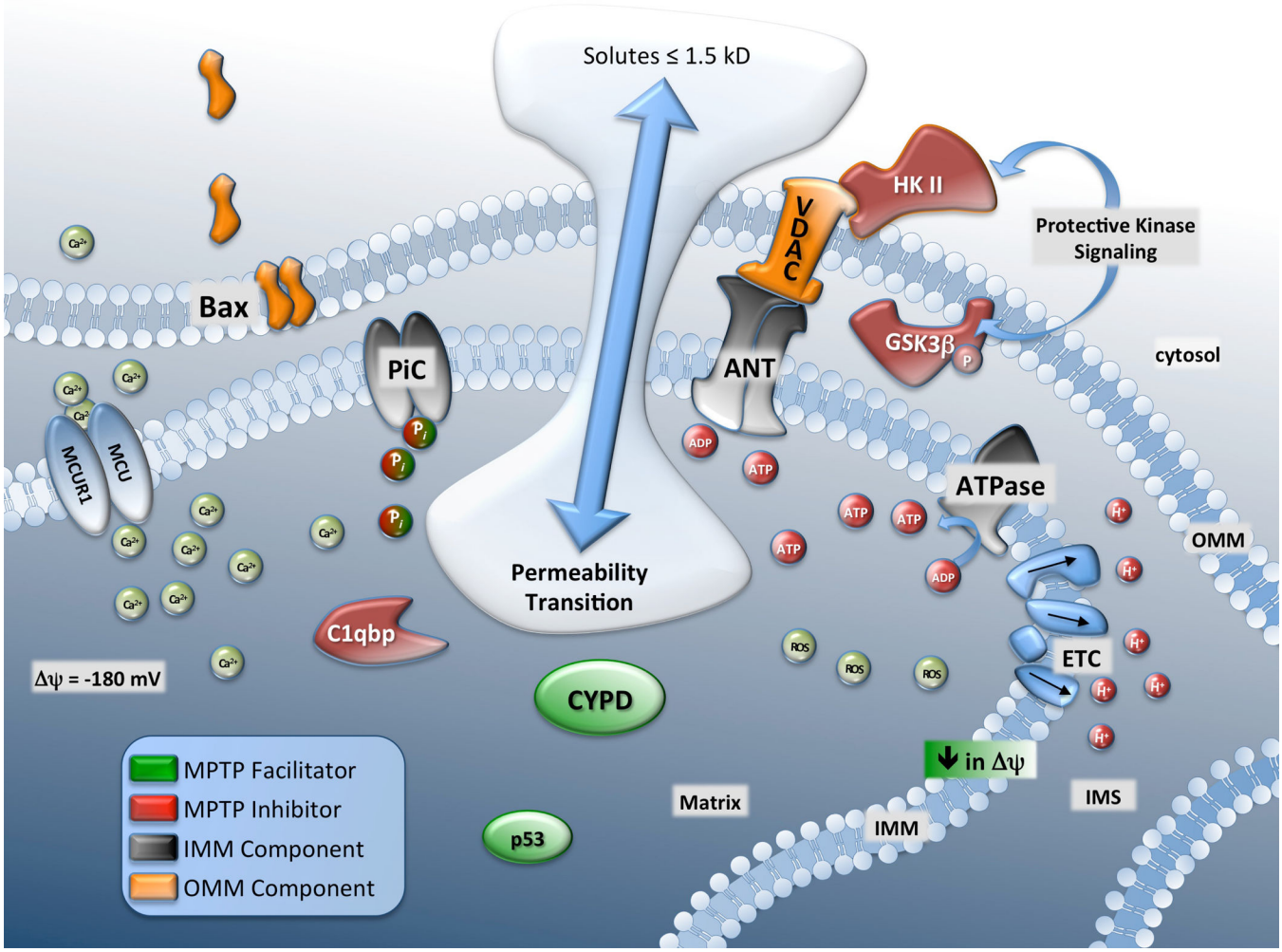

Figure 1. CypD interactors and physiological regulators of the MPTP.

Various molecular components of the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM) have been proposed to form the large, non-specific mitochondrial permeability transition pore (MPTP). Upon formation the passage of solutes ≤ 1.5 kD in size cross the IMM resulting in organelle swelling and eventual rupture, a key event in necrotic cell death. Calcium (Ca^{2+}), the most noted mediator of permeability transition, enters the matrix via the mitochondrial calcium uniporter complex (MCU and MCUR1) driven by the highly negative membrane potential (ΔΨ). Reactive oxygen species (ROS) increase MPTP open probability while adenine nucleotides (ADP and ATP) inhibit pore formation. Inorganic phosphate (P_i) while traditionally viewed as an activator of MPTP may have a dual role as an inhibitor under appropriate conditions. (key is lower left). Additional abbreviations not used in text: HK II, hexokinase-II; ATPase, F1/F0 ATPase.

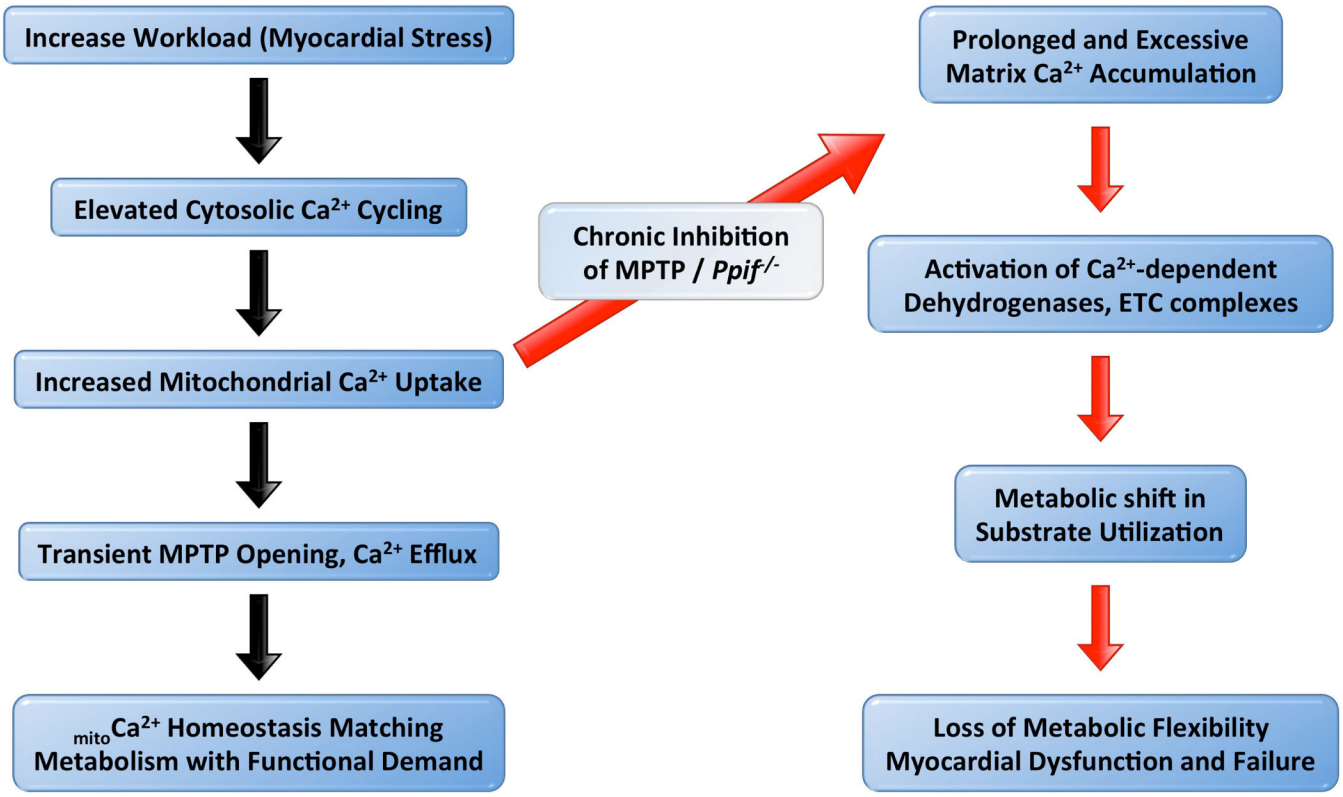

Figure 2. MPTP-mediated mitochondrial calcium ($_{mito}Ca^{2+}$) Efflux.

The flow chart details a possible physiological function for the MPTP in maintaining $_{mito}Ca^{2+}$ homeostasis in the heart during times of stress. Low-conductance or transient opening of the pore may represent an important route of Ca^{2+} extrusion from the matrix. (*left side, black arrows* = homeostatic conditions, *right side, red arrows* = pathological conditions).

Table 1.

PPIF gene homology across species.

HomoloGene Pairwise Alignment Scores		**Identity *%***	
Species	**Symbol**	**Protein**	**DNA**
H.sapiens	PPIF		
vs. P.troglodytes	PPIF	99.5	99.5
vs. M.mulatta	PPIF	97.1	96.9
vs. B.taurus	PPIF	91.4	88.3
vs. M.musculus	Ppif	90.3	89
vs. R.norvegicus	Ppif	89.8	88.7
vs. G.gallus	PPIF	83.1	72.6
vs. D.rerio	ppifb	78.1	69.7
vs. D.melanogaster	Cyp1	72.4	68.1
vs. A.gambiae	AgaP_AGAP000462	71.9	68.9
vs. S.cerevisiae	CPR1	70.4	64.6
vs. K.lactis	KLLA0D16676g	69.8	65.2
vs. E.gossypii	AGOS_AGL177C	70.4	67.5
vs. S.pombe	ppi1	75.6	66.2
vs. M.oryzae	MGG_10447	63.9	64.3
vs. N.crassa	NCU00726	61.1	61.4
vs. A.thaliana	CYP20–2	72.3	61.6
vs. O.sativa	Os05g0103200	66.1	62.6

Table 2.

Ppif$^{-/-}$ phenotypes in physiology.

Pathology and Physiology of Ppif gene-deleted Mice		
Mouse model	**Phenotype as compared to WT controls**	**Reference**
Myocardial IR	decreased infarct size	(5, 35)
Permanent LCA ligation (myocardial infarction)	increased survival, decreased scar size	(149)
transaortic constriction (pressure-overload heart failure)	increased pathology, decreased LV function	(95)
CamKIIδc-transgenic model (Ca^{2+}-overload induced cardiomyopathy)	increased pathology, decreased survival	(95)
β2a-transgenic model (hyperactivation of L-type Ca^{2+} channel)	decreased pathology, increased survival	(150)
Carotid artery injury model	accelerated thrombosis	(151)
Renal IR	cytoprotection	(127, 128)
Acetaminophen hepatotoxicity	cytoprotection	(152)
Pdx1 haploinsufficient mice (heritable diabetes model)	preservation of pancreatic cells	(153)
Cerebral IR	decreased infarct size	(36, 126)
Cerebral Neonatal Hypoxia	increased injury	(126)
EAE (multiple sclerosis)	axon preservation (cytoprotection)	(154)
Amyloid-β mutant model (Alzheimer's)	cytoprotection, improved learning and memory	(155, 156)
SOD1 G93A mutant model (ALS)	delayed disease onset, improved survival	(157)
Glutamate-excitotoxicity (*ex vivo)*	cytoprotection	(158)
MPTP neurotoxin administration (Parkinson's)	acute model cytoprotective, chronic model no change	(159)
Sgcd$^{-/-}$ (muscular dystrophy)	reduced skeletal muscle pathology, cytoprotection	(143)
Lama2$^{-/-}$ (muscular dystrophy)	reduced pathology, increased survival	(143)
Col6a1$^{-/-}$ (muscular dystrophy)	reduced pathology, cytoprotection	(160)
Aging	adult-onset obesity	(114)
Aging	behavioral defects	(114)
Aging	decreased LV contractile reserve	(95)
Behavioral testing	impairments in memory	(161)
Endurance exercise (swimming)	increased hypertrophy and mortality	(95)