Physiologic Functions of Cyclophilin D and the Mitochondrial Permeability Transition Pore

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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. Although a great number of reviews have been written on the MPTP and its effects on cell death, we focus on the biology surrounding CypD itself and the non-cell death physiologic functions of the MPTP. 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. (Circ J 2013; 77: 1111–1122)

Key Words: Calcium; Cyclophilin D; Ischemic injury; Metabolism; Mitochondria

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 homolog.2,3 As an aside, the HUGO gene nomenclature committee incorrectly annotated the CypD protein as PPIF (peptidyl-prolyl isomerase F), whereas it should have been named PPIF. The full-length CypD protein consists of 207 AA (22 kDa) with the most prominent feature being a 109 AA cyclophilin domain (c00197, 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 the 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 below).

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.6 The crystal structure of human CypD has been elucidated in complex with CsA.7 Kajitani et al reported that CypD contained 8 β-strands, 2 α-helices, and 1 310 helix, representing a structure similar to that of the other known cyclophilin family members.8 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, PPIF is well conserved across many organisms with predicted homology noted even in plants and fungi (Table 1).2,7,8 In invertebrates, the gene is extremely well conserved, with the zebrafish (Danio rerio) homologous gene (ppifb) having a 78% protein identity match with Homo 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 Azzolin et al.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: (ML-ALRCGSRWLGLISSVPRSVPLRLPAARA). 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 [35S]-labeled CypD by Johnson et al 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 Ca2+ 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 Ca2+ through a ruthenium red-sensitive mechanism (now known to be the mitochondrial calcium uniporter (MCU), the genetic identity of which was recently discovered14,15) and that permeability transition resulted in the uncoupling of oxidative phosphorylation. The same group followed this discovery with numerous reports 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 Ca2+-specific manner and that its opening imparts permeability to solutes up to 1.5 kDa in size.16 Hunter et al16–20 also noted that MPTP open probability is reduced by ADP, adenine nucleotides, and other divalent cations such as Mg2+ (Sr2+, Ba2+ and Mn2+ 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 and colleagues.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 (IMS), potentiating apoptotic cell death signaling (see the in-depth review on OMM permeability by Kroemer et al23). 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 remain undefined. Perhaps the most experimentally manipulated feature of the MPTP is that open probability is reduced by CsA, which is a potent immunosuppressant originally isolated from a fungus (*Toiyopodias alatinum*) and found to inhibit calcineurin signaling in complex with CypA.24–27 Preliminary observations leading to the discovery of CsA’s

### Table 1. PPIF Homology Across Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Symbol</th>
<th>Protein Identity %</th>
<th>DNA Identity %</th>
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<tr>
<td>Homo sapiens</td>
<td>PPIF</td>
<td>99.5</td>
<td>99.5</td>
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<tr>
<td>vs. Pan troglodytes</td>
<td>PPIF</td>
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<td>96.9</td>
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<td>PPIF</td>
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<td>PPiF</td>
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<td>72.6</td>
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<td>78.1</td>
<td>69.7</td>
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<td>vs. Drosophila melanogaster</td>
<td>Cyp1</td>
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<td>vs. Anopheles gambiae</td>
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<td>vs. Eremothecium gossypii</td>
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<td>vs. Schizosaccharomyces pombe</td>
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<td>66.2</td>
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<td>vs. Neurospora crassa</td>
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<td>vs. Arabidopsis thaliana</td>
<td>CYP20–2</td>
<td>72.3</td>
<td>61.6</td>
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<td>vs. Oryza sativa</td>
<td>Os05g0103200</td>
<td>66.1</td>
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Cyclophilin D (CypD) interactors and physiological regulators of the mitochondrial permeability transition pore (MPTP). Various molecular components of the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM) have been proposed to form the large, nonselective MPTP. Upon formation solutes ≤1.5 kD in size can 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 whereas adenine nucleotides (ADP and ATP) inhibit pore formation. Inorganic phosphate (P_i), although traditionally viewed as an activator of the MPTP, may have a dual role as an inhibitor under appropriate conditions. (Key is Lower left.) HK II, hexokinase II; ATPase, F_0/F_1 ATPase.

**Figure 1.**

Biophysical Characterization of the MPTP

Two independent labs used mitoplast (mitochondria stripped of the OMM and IMS constituents by osmotic disruption leav-
ing only the IM and matrix portion of the organelle) patch-clamping techniques to characterize the biophysical properties of a giant (1–1.5 nanosiemens), nonspecific channel of the inner membrane. Szabo and Zoratti confirmed the identity of the large conductance channel as the MPTP by verifying it was inhibited by CsA. 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 IMM and OMM. 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 1 and the same. Numerous subconductance states have been recorded for the MPTP, indicating a possible physiological relevance for the ‘subconductance state’ of the channel outside the well-characterized large-conductance opening associated with cellular demise. Zoratti’s group also used 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. These findings support the known regulatory role for CypD in MPTP function, but not as an obligatory component for pore formation, as previously reported.

Molecular Components of the MPTP (CypD Interactors)

Although numerous molecular constituents of the MPTP have been suggested as 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 IMM with the VDAC in the OMM together forming a continuous channel across the IMS 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 later. However, there remains substantial evidence that numerous components, although not necessary for pore formation, are involved in modulating permeability transition (Figure 1).

ANT

The finding that bongkrekic acid, an inhibitor of ANT by 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 Ca2+, suggested that ANT could be a structural component of the MPTP. Halestrap et al 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. Using a CypD affinity column both Halestrap’s group and Crompton et al independently showed molecular interaction with ANT. Reconstitution studies by Kroemer’s and Crompton’s groups found that purified isolates of ANT, CypD, and VDAC (also Bcl2 proteins) could form a Ca2+-responsive, CsA sensitive pore in proteoliposomes and work by Brdiczka’s group suggested ANT could itself form a pore-like structure similar to the MPTP. 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. 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 Ca2+ to induce opening and that ANT ligands (such as ADP) lost inhibitory capacity on pore open probability. These later observations suggest that ANT is likely 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 characterized a mitochondrial swelling event that appeared to require the OMM. They found mitoplasts did not respond in the classic swelling assay. They suspected VDAC was involved, possibly by migrating to the IMM to permit permeability transition. Crompton et al 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. Other reports also supported the VDAC hypothesis through anti-VDAC antibody blockade of permeability transition and inhibition of the MPTP by VDAC phosphorylation. 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 3 Vdac genes (Vdac1, 2, 3) did not disrupt MPTP function and furthermore did not alter necrotic or apoptotic cell death. In fact, deletion of Vdac2 enhanced cell death propensity. The mitochondrial ATP synthase (complex V) is a multisubunit complex of the IMM 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 as a possible component of the pore itself, although most of this conjecture can probably be attributed to indirect changes in energetic factors that alter open probability (adenine nucleotides, phosphate, Ca2+, etc). Giorgio et al have shown that CypD directly binds the lateral stalk (subunit d and b) of ATP synthase and alters its activity. Another recent study using siRNA-mediated knockdown found the F0 c subunit to be required for permeability transition and the authors speculated that it is a principal component of a supramolecular MPTP complex. 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. 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 et al suggested that the change in adenine nucleotide exchange associated with ATP synthase binding may be partially attributed to a change in ANT flux rates.
Phosphate Carrier (PiC)
The PiC (SLC25A3 gene) is the primary transporter of inorganic phosphate (P) into the mitochondrial matrix by proton cotransport or in exchange for hydroxyl ions. Historically, P is a potent modulator of pore opening with countless studies suggesting a definitive relationship between matrix P content and MPTP activation. However, pore regulation by P has recently been reappraised, as the entire regulatory action of CypD, and thereby CsA, has been suggested to be P-dependent. This hypothesis is in direct contrast to a recent report by McGee et al in which P had absolutely no effect in P-pi null or CsA-inhibited pore opening, a result that is supported by results from Halestrap’s group who found that P actually enhanced MPTP opening, but independent of CypD. Despite the potential effect of P, the PiC itself has been suggested to be either an effector of the MPTP, or possibly even a structural component. 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. Although 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 and ANT 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 earlier), Bax’s role in MPTP formation becomes enigmatic. However, a recent study by Whelan et al suggested that Bax/Bak is a required component of mitochondrial-dependent necrosis. The authors found that mitochondria isolated from Bax/Bak double-null MEFs were resistant to swelling and loss of membrane potential [Ap] in response to Ca²⁺ challenge. They also 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 by Vaseva et al to be a mediator of pore opening by direct interaction and activation of CypD. They present this as a new mechanism whereby p53 can modulate cellular necrosis by directly influencing MPTP formation and 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). Although 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. For example, the authors found no alteration in Ca²⁺-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) as a likely candidate for the pore-forming component of the inner membrane. 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. It is suggested that the β-sheet structure and other biophysical properties impart channel forming potential and a plausible target for CypD. Supporting observations include the apoptotic stimuli Hrk and smARF being required for mitochondrial C1QBP-dependent cell death. In addition, expression of mitoC1QBP in cell culture induced swelling and cristae derangement of mitochondria, suggestive of MPTP activation. In contradiction to these observations, overexpression of C1QBP attenuated ROS-induction of the MPTP and cellular necrosis whereas knockdown was sensitized to MPTP opening. In addition, the same study found C1QBP to be a soluble component of the mitochondrial matrix rather than IMM localization. Thus, although C1QBP appears to be involved with MPTP formation, further studies are required to understand at what level.

Post-Translational Modification of CypD
Phosphorylation
Although many kinase-dependent signaling pathways have been suggested to play a role in the regulation of the MPTP, 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. CypD is 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. 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 kinases 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 laboratories have recently proposed deacetylation as a post-translational modification that regulates CypD. Work from David Sinclair’s group proposed that the NAD⁺-dependent deacetylase sirtuin-3 (Sirt3) deacetylates CypD on lysine 166, which is adjacent to the binding site for CsA. Those 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). Although this hypothesis is intriguing, it is
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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 \(\text{mitoCa}^{2+}\) Efflux Channel

Given the inseparable nature of Ca\(^{2+}\) and MPTP regulation, it has been 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 \(\text{mitoCa}^{2+}\) efflux pathways mediated by the H\(^{+}\)/Ca\(^{2+}\) exchanger and Na\(^{+}\)/Ca\(^{2+}\) exchanger would not be sufficient to prevent Ca\(^{2+}\) overload, suggesting that an alternate means of removing Ca\(^{2+}\) is needed. The first experimental evidence of the MPTP being involved in \(\text{mitoCa}^{2+}\) efflux came in 1992 when Altschuld et al demonstrated that CsA could inhibit Ca\(^{2+}\) efflux in isolated rat cardiomyocytes.

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. 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

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. The latter study suggests that chronic inhibition of the MPTP does not impart protection against age-related hypertrophy, but rather that the inverse occurs and hypertrophic cardiomyopathy is enhanced. Because the authors failed to perform any experiments to suggest a causal relationship, it is 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. Although 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 (SNO)

Nitric oxide (NO) can influence cell signaling by 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. Pertinent to the current review, SNO modification of numerous MPTP modulators has been reported (including F\(_{0}\)F\(_{1}\)-ATPase, creatine kinase, thioredoxin, VDAC and CypD). 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 \(Ppif^{-/}\text{-}\) MEFs with either WT or mutant protein. 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).

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Figure 2. MPTP-mediated mitochondrial calcium (\(\text{mitoCa}^{2+}\)) efflux. The flow chart details a possible physiological function for the MPTP in maintaining \(\text{mitoCa}^{2+}\) 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) Black arrows = homeostatic conditions, (Right) red arrows = pathological conditions. MPTP, mitochondrial permeability transition pore.
Cyclophilin D and Mitochondria Pore Formation

Table 2. Pathology and Physiology of Ppif Gene-Deleted Mice

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<th>Mouse model</th>
<th>Phenotype vs. Wildtype controls</th>
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<tr>
<td>Myocardial I/R</td>
<td>Decreased infarct size</td>
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<td>Permanent LCA ligation (myocardial infarction)</td>
<td>Increased survival, decreased scar size</td>
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<td>Transaortic constriction (pressure-overload heart failure)</td>
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<tr>
<td>CamKIIα-transgenic model (Ca2+ overload induced cardiomyopathy)</td>
<td>Increased pathology, decreased survival</td>
<td>95</td>
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<tr>
<td>β2a-transgenic model (hyperactivation of L-type Ca2+ channel)</td>
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<td>Carotid artery injury model</td>
<td>Accelerated thrombosis</td>
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<td>Preservation of pancreatic β cells</td>
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<td>Cytoprotection, improved learning and memory</td>
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I/R, ischemia-reperfusion; LCA, left coronary artery; LV, left ventricular.

generation) with myocardial workload (functional demand) through Ca2+ (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 Ca2+, yet recover Δψ and ATP generation. Additional evidence for CypD/MPTP playing a role in mitochondrial Ca2+ came from adult cortical neurons isolated from Ppif−/− mice where the authors found elevated mitoCa2+ following the activation of plasma membrane Ca2+ channels. It is interesting to note that the authors only saw this effect with maximal activation, suggesting a threshold of Ca2+ was necessary before invoking extrusion via the MPTP, presumably because of saturation of the traditional Ca2+ efflux channels. Thus, the MPTP, as regulated by CypD, appears to be a physiologic Ca2+ release mechanism that is required for proper metabolic regulation within 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). 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 Ca2+ levels, as discussed earlier, 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). Strikingly, those authors found a large increase in body weight at 6 months of age, reaching a maximal difference at 1 year of age as compared with 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, together 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. Whether metabolic control is directly linked to the MPTP’s role in Ca2+ 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. Those studies found that Ppif null cells were highly resistant to cell death induced by cytosolic Ca2+ overload, hydrogen peroxide-mediated ROS stress, and thapsigargin (SERCA inhibitor, endoplasmic reticulum Ca2+ stress), but died similarly to control cells when treated with staurosporine, tumor necrosis alpha, 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 accidental death process, may actually be comprised of distinct molecular signaling events. Ppif null mice have since been used in numerous animal models, providing an extensive framework to support a central role for the MPTP in pathophysiology (Table 2). The role of the MPTP in programmed necrotic cell death has been extensively covered.118,119

Role in Ischemia-Reperfusion (I/R) 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 the cytoprotective action of CsA in I/R injury (please see Hausenloy et al125 for an extensive review), causative evidence of CypD involvement can be found in studies utilizing Ppif−/− mice, which were protected in numerous I/R models, including the heart,5,35 brain,16,126 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 Ca2+ and ROS.

In addition to the acute effect of inhibiting CypD in I/R 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 I/R 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. Although the studies did not provide a molecular mechanism to account for this phenomenon, it is plausible that low-conductance opening of the pore could generate superoxide or Ca2+ or modulate a pore-associated component such as GSK3β to activate discrete cytoprotective signaling pathways (some of which has recently been explored131). 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 dropout 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 Ca2+-calmodulin-dependent protein kinase IIδ-e overexpressing mouse model of cardiomyopathy and mitochondrial Ca2+ 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 fatty acid oxidation to glycolysis), suggesting metabolic reprogramming as the likely cause of the worsening heart failure.

CypD-Based Treatment Strategies for Cardiovascular Disease and Conclusions

Clinical trials have shown that inhibition of CypD and the MPTP with CsA was protective to patients immediately after myocardial infarction (MI) when applied during the revascularization phase.136 Clearly that 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 trials are needed to solidify a new standard of care treatment with such inhibitors. However, CsA may not be the best choice of inhibitor because it also blocks the activity of calcineurin, which itself is cardioprotective.137,138 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 Although 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 preexisting 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 dropout is a significant issue. Moreover, prolonged use of Cyp inhibitors in patients being treated for hepatitis C should possibly be evaluated for cardiovascular complications.145 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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Cyclophilin D and Mitochondria Pore Formation


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