Cyclophilin D controls mitochondrial pore–dependent Ca$^{2+}$ exchange, metabolic flexibility, and propensity for heart failure in mice

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Introduction

Opening of the mitochondrial permeability transition pore (MPTP) is critically involved in regulating cell death by inducing a sustained and irreversible loss of inner mitochondrial membrane potential, coinciding with mitochondrial swelling and rupture (1). The proteins that constitute the MPTP, localized between the inner and outer mitochondrial membranes, remain unknown. Cyclophilin D (CypD), a mitochondrial matrix peptidyl-prolyl cis-trans isomerase, stands as the only genetically verified component of the MPTP that functions in gating the pore (2–5). Cyclosporine A (CsA) and its analogs inhibit CypD, leading to desensitization of MPTP opening functions in gating the pore (2–5). Cyclosporine A (CsA) and its analogs inhibit CypD, leading to desensitization of MPTP opening.

Cyclophilin D (which is encoded by the Ppif gene) is a mitochondrial matrix peptidyl-prolyl isomerase known to modulate opening of the mitochondrial permeability transition pore (MPTP). Apart from regulating necrotic cell death, the physiologic function of the MPTP is largely unknown. Here we have shown that Ppif$^{-/-}$ mice exhibit substantially greater cardiac hypertrophy, fibrosis, and reduction in myocardial function in response to pressure overload stimulation than control mice. In addition, Ppif$^{-/-}$ mice showed greater hypertrophy and lung edema as well as reduced survival in response to sustained exercise stimulation. Cardiomyocyte-specific transgene expression of cyclophilin D in Ppif$^{-/-}$ mice rescued the enhanced hypertrophy, reduction in cardiac function, and rapid onset of heart failure following pressure overload stimulation. Mechanistically, the maladaptive phenotype in the hearts of Ppif$^{-/-}$ mice was associated with an alteration in MPTP-mediated Ca$^{2+}$ efflux resulting in elevated levels of mitochondrial matrix Ca$^{2+}$ and enhanced activation of Ca$^{2+}$-dependent dehydrogenases. Elevated matrix Ca$^{2+}$ led to increased glucose oxidation relative to fatty acids, thereby limiting the metabolic flexibility of the heart that is critically involved in compensation during stress. These findings suggest that the MPTP maintains homeostatic mitochondrial Ca$^{2+}$ levels to match metabolism with alterations in myocardial workload, thereby suggesting a physiologic function for the MPTP.

Authorship note: John W. Elrod and Renee Wong contributed equally to this work.

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Results

Baseline characterization and aging phenotype of Ppif$^{-/-}$ mice. Mice null for the Ppif gene (CypD protein) displayed normal LV dimension...
Baseline characterization and aging of Ppif−/− mice. (A) WT and Ppif−/− mice were evaluated from 2 months to 1 year of age for changes in LV end-systolic dimension (LVEDD). (B) LV percent fractional shortening (FS) from 2 to 12 months of age. (C) Heart weight to body weight (HW/BW) ratio in mice 12 months of age. (D) LV contractility (dP/dt max) in mice 8 months of age following isoproterenol dose escalation. (E) LV relaxation (dP/dt min) in mice 8 months of age following isoproterenol dose escalation. (F) Representative electron micrographs of sarcomeric and mitochondrial structure in LV heart histological sections. (G) FS (%) in isolated adult cardiomyocytes paced at 0.5 Hz. (H) Shortening time (50% time from peak) of isolated adult cardiomyocytes. (I) Relaxation time (50% time from peak) of isolated adult cardiomyocytes. All values are reported as mean ± SEM. Sample size for each group is indicated inside the respective bar. *P < 0.05 versus WT.

and performance measured by echocardiography at 2, 4, 8, and 12 months of age (Figure 1, A and B). There was also no difference in heart weights of mice at 12 months of age, nor any defects in myofilament or mitochondrial architecture, as assessed by transmission electron microscopy (Figure 1, C and F). However, while baseline contractility did not differ between WT and Ppif−/− mice at 8 months of age, maximum contractile reserve, revealed by infusion of isoproterenol (a β-adrenergic receptor agonist), was compromised in Ppif−/− mice (dP/dt max, Figure 1D), as was relaxation (dP/dt min, Figure 1E). To determine whether the observed myocardial dysfunction was myocyte specific, we examined sarcomeric shortening and cytosolic Ca2+ transients in isolated adult cardiomyocytes. While the baseline percent fractional shortening was comparable to that in WT myocytes, the shortening time and relaxation time were significantly increased in Ppif−/− myocytes (Figure 1, G–I), consistent with our in vivo findings. In addition, cytosolic Ca2+ transients from adult Ppif−/− cardiomyocytes showed a longer time of decay, substantiating the observed increase in relaxation time (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43171DS1).

Ppif−/− mice are more susceptible to myocardial hypertrophy and heart failure following pressure overload. To explore this maladaptive phenotype further, we subjected Ppif−/− mice to stress stimulation as a means of unmasking underlying problems associated with a loss in functional reserve. Indeed, Ppif−/− mice were unable to compensate for the increase in afterload caused by transaortic constriction (TAC), displaying a 42% reduction in fractional shortening at the 6-week time point as compared with WT mice (Figure 2A). Ppif−/− mice decompensated within 1 week of TAC stimulation and showed significant ventricular dilation by 2 weeks, with equivalent pressure gradients across the aortic constrictions compared with WT mice (Figure 2, B and C). Consistent with these results, hearts from Ppif−/− mice hypertrophied to a greater extent than WT mice after 2 and 6 weeks of TAC stimulation (Figure 2, D and E). Indeed, at the 6-week time point, Ppif−/− mice displayed a significantly greater increase (41%) in heart weight normalized to body weight and LV wall thickness compared with WT TAC controls (Figure 2E and Supplemental Table 1), which correlated with approximatively the same increase in fibrosis following TAC in Ppif−/− mice compared with WT TAC controls (Figure 2G), and indices of congestive heart failure, such as pulmonary edema, were also prominent in Ppif−/− animals (Figure 2H). However, we observed no difference in TUNEL-positive nuclei of cardiomyocytes or interstitial cells at 2 and 6 weeks of TAC stimulation in Ppif−/− compared with WT hearts, suggesting that the rate of cellular demise was unchanged (Supplemental Figure 2, A–D). The more severe cardiac pathology observed in Ppif−/− mice also correlated with stronger activation of the fetal gene program after TAC, including the β-myosin heavy chain gene, atrial natriuretic factor (ANF), and B-type natriuretic peptide (BNP) genes (Figure 2, I–K). Taken together, these results suggest that loss of CypD increases sensitivity to cardiac stress stimulation and disease.

Ppif−/− mice display increased mortality and heart failure in pathologic and physiologic models of hypertrophy. To further characterize this maladaptive phenotype due to hypertrophic stimulation, we crossed Ppif−/− mice with Ca2+/calmodulin-dependent protein kinase II δ transgenic mice (CaMKII-Tg). CaMKIIδ overexpression results in heart failure characterized by a loss of LV function and significant mortality, but more importantly these mice show alterations in intracellular Ca2+ handling and a predisposition to increased...
mitochondrial Ca\(^{2+}\) (17–19). As will be presented in figures below, this predisposition to elevated mitochondrial Ca\(^{2+}\) with CaMKIIδc overexpression is similar to alterations observed in Ppif\(^{-/-}\) hearts, making the cross mechanistically germane. Indeed, hypertrophy associated with the CaMKII-Tg was synergistically increased when mice were crossed into the Ppif\(^{-/-}\) background, and the cross also resulted in earlier lethality (Figure 3, A and B). Importantly, isolation of mitochondria from the hearts of these crossed mice showed that MPTP formation after Ca\(^{2+}\) stimulation was inhibited by genetic loss of Ppif, even with the CaMKII-Tg (Figure 3C).

The rapid onset of cardiac decompensation in Ppif\(^{-/-}\) mice after TAC stimulation coupled with their inability to even modestly increase cardiac contractility following acute β-adrenergic stimulation suggested a loss in functional reserve. To address this possibility, and minimize the confounding effect of a pathologic insult, we subjected Ppif\(^{-/-}\) mice to swimming exercise to induce physiologic hypertrophy. Remarkably, swimming resulted in significant mortality, with more than 40% of Ppif\(^{-/-}\) mice having died by the conclusion of the training period due to extreme fatigue and drowning (Figure 3D). Histological analysis showed no overt pathology, including no induction of cell death (Supplemental Figure 2, E and F, and data not shown). Swimming also induced significantly greater cardiac hypertrophy and pulmonary edema in Ppif\(^{-/-}\) mice as compared with WT controls, suggesting an inability to compensate for the higher workload (Figure 3, E and F). In total, these results suggest that loss of CypD compromises the functional reserve of the heart and renders it more susceptible to failure, even with physiologic stimulation that is normally cardioprotective.

Cardiac-restricted transgenic rescue of Ppif\(^{-/-}\) pathology following TAC. We hypothesized that the extreme predisposition to decompensation in Ppif\(^{-/-}\) mice with stress stimulation was due to a defect in mitochondrial function within myocytes themselves. However, Ppif\(^{-/-}\) mice lack the gene in all cells; hence, it was formally possible that the cardiac defect observed was not myocyte autonomous. To address this issue, we crossed transgenic mice expressing CypD under control of the α-myosin heavy chain promoter (CypD-Tg) into the Ppif\(^{-/-}\) background. Western blotting demonstrated that Ppif\(^{-/-}\) mice lacked CypD protein in the heart, while CypD-Tg × Ppif\(^{-/-}\) crossed mice had restored myocyte CypD protein, albeit to levels slightly higher than in the WT control (Figure 4A). After 3 weeks of TAC stimulation, Ppif\(^{-/-}\) mice from the same backcross again showed rapid loss of ventricular performance, greater increases in cardiac hypertrophy, and pulmonary edema, all of which were rescued back to WT levels by the CypD-Tg cross (Figure 4, B, C, and D). Importantly, pressure gradients across the aortic constriction did not differ among the groups (Figure 4E). These results suggest that the maladaptive cardiac phenotype observed in Ppif\(^{-/-}\) mice is myocyte dependent.

Ppif\(^{-/-}\) mice display substantial metabolic reprogramming. To further examine the mechanism whereby CypD loss from cardiac mitochondria sensitizes to failure, we performed a genome-wide array analysis using mRNA isolated from LV tissue of 8-week-old WT and Ppif\(^{-/-}\) mice. Affymetrix gene array analysis revealed that 362 probe sets were significantly different (ANOVA, \(P < 0.01\)), with a minimum difference in gene expression of 25% (Figure 5A). Unbiased functional annotation revealed numerous metabolism-associated gene groups that were significantly enriched, suggesting an alteration in mitochondrial function (Table 1). To examine this possible mechanism further, we evaluated substrate utilization in a working heart preparation with \(^{13}\)C NMR spectrometry. The analysis showed that Ppif\(^{-/-}\) hearts had a significant increase in the glucose to palmitate ratio, suggesting a metabolic shift away from fatty acid oxidation toward glycolysis (Figure 5B). We also observed an increase in the succinate to glutamate ratio in Ppif\(^{-/-}\) hearts,
and while the significance of this change is uncertain, it does suggest increased TCA cycle flux (Figure 5C). Associated with these changes in substrate utilization, the activities of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (αKGD), two Ca\(^{2+}\)-regulated mitochondrial enzymes with rate-limiting metabolic properties, were significantly enhanced in Ppif\(^{-/-}\) hearts (Figure 5, D and E). Importantly, protein expression levels for the PDH subunits did not differ between WT and Ppif\(^{-/-}\) hearts, nor were expression levels for αKGD and two other Ca\(^{2+}\)-regulated mitochondrial dehydrogenases changed (Figure 5, F and G), suggesting that the increase in activity was due to increased mitochondrial Ca\(^{2+}\).

Ppif\(^{-/-}\) mice display altered mitochondrial Ca\(^{2+}\) exchange. Given the observed metabolic changes and increased αKGD and PDH activity, we suspected an increase in mitochondrial matrix Ca\(^{2+}\). Indeed, we previously showed that mitochondria from Ppif\(^{-/-}\) mice take up more Ca\(^{2+}\) before undergoing MPTP formation (3). Reexamination of this phenomenon with purified mitochondria from Ppif\(^{-/-}\) mice showed nearly 3-fold-greater Ca\(^{2+}\) accumulation prior to MPTP opening compared with that in WT mitochondria (Figure 6A). More importantly, direct measurement of total mitochondrial Ca\(^{2+}\) content of Ppif\(^{-/-}\) hearts showed a 2.6-fold elevation compared with WT mitochondria (Figure 6B). Taken together, these results suggest that in the absence of CypD mitochondrial matrix Ca\(^{2+}\) levels are reset and constitutively elevated, consistent with the observed metabolic alterations.

We hypothesized that the elevated level of matrix Ca\(^{2+}\) observed in mitochondria from hearts of Ppif\(^{-/-}\) mice was due to reduced physiological opening of the MPTP, as its low conductance state has been implicated in affecting Ca\(^{2+}\) homeostasis (20, 21). To address this hypothesis, we used cultured neonatal rat ventricular cardiomyocytes infected with an adenovirus encoding a mitochondrial-targeted ratiometric-pericam (mt-pericam) Ca\(^{2+}\) sensor pro-

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**Figure 3**

Ppif\(^{-/-}\) mice display increased mortality and heart failure in pathologic and physiologic models of hypertrophy. (A) HW/BW ratios in the indicated groups of mice at 4 weeks of age. (B) Kaplan-Meier survival plot in the groups of mice shown. (C) Mitochondrial swelling following Ca\(^{2+}\) addition (indicated by red arrow), measured as change in absorbance (520 nm) over time (representative tracing of 3 independent experiments). (D) Kaplan-Meier survival plot during swimming exercise. (E) Ventricular weight/tibia length (VW/TL) ratios in mice at completion of the swimming protocol. (F) Lung edema (wet weight in mg – dry weight in mg) at completion of the swimming protocol. All values are reported as mean ± SEM. Sample size for each group is indicated inside the respective bar. *P < 0.05 versus WT control (A, B, and D) or rested group (E); †P < 0.05 versus CaMKII and Ppif\(^{-/-}\) (A–C) or versus WT swim (E and F).

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**Figure 4**

Cardiac-restricted transgenic rescue of Ppif\(^{-/-}\) pathology following TAC. (A) Representative Western blot of CypD and oxidative phosphorylation complexes (Com V\(_{\alpha}\), III-core 2, II, I) in heart mitochondrial extracts. (B) LV percent FS following TAC. (C) HW/BW ratio following TAC. (D) Lung edema (wet weight in mg – dry weight in mg) following TAC. (E) Pressure gradient across the aortic constriction 1 week after TAC. All values are reported as mean ± SEM. Sample size for each group is indicated inside the respective bar. *P < 0.05 versus all other groups; †P < 0.05 versus Ppif\(^{-/-}\) TAC.
CypD inhibition is known to allow greater accumulation of mitochondrial Ca\(^{2+}\) in vitro, yet the mechanism proposed for this phenomenon has always been tied to cell death. However, our data taken together with past studies suggest a critical role for CypD and the MPTP in controlling mitochondrial Ca\(^{2+}\) homeostasis for proper metabolic function. For example, past in vitro studies suggested that transient opening of the MPTP might allow the exchange of matrix Ca\(^{2+}\) across the inner membrane without causing cellular demise. Evidence for this hypothesis emerged in 1992 in a study by Altschuld and colleagues, who showed that CsA increased mitochondrial Ca\(^{2+}\) loading and inhibited efflux in isolated rabbit cardiomyocytes (23). This hypothesis was extended by Ichas et al., who presented a biophysical model of Ca\(^{2+}\)-induced mitochondrial Ca\(^{2+}\) release that involved low-conductance MPTP opening for mitochondrial Ca\(^{2+}\) efflux (20). Similarly, Siemen and colleagues used mitoplasts and direct patching to examine MPTP opening in the mitochondrial membrane. They showed a large conductance current, with multiple subconductance states, that was Ca\(^{2+}\) activated and reversibly inhibited with CsA (24). Our results extend this Ca\(^{2+}\) homeostasis model by showing that genetic loss of CypD results in mitochondria with higher resting Ca\(^{2+}\) levels in the heart and that CsA treatment acutely reduces mitochondrial Ca\(^{2+}\) efflux and leak in cultured cardiomyocytes.

In the current study, we provide in vivo evidence linking MPTP-mediated Ca\(^{2+}\) efflux to global changes in metabolic substrate utilization, a mechanism that appears crucial to the heart’s ability to maintain contractility in response to increased workload (25). For example, increases in cardiac contractility are tied to greater intra-cellular Ca\(^{2+}\) cycling rates in myocytes, which has the net effect of enhancing mitochondrial Ca\(^{2+}\) load through the Ca\(^{2+}\) uniporter (MCU). Mitochondrial extrusion rates through the mitochondrial Na\(^{+}/Ca\(^{2+}\) exchanger or other transporters/channels lag behind the cytoplasmic Ca\(^{2+}\) transient, resulting in accumulation of Ca\(^{2+}\) at higher beating rates until physiologic opening of the MPTP occurs. The net increase in mitochondrial Ca\(^{2+}\) leads to activation of 3 mitochondrial dehydrogenases important in substrate utilization and oxidative metabolism, thereby increasing energy supply during increased workloads (26). Indeed, simultaneous measurements of mitochondrial Ca\(^{2+}\) and NADH have correlated increased mitochondrial Ca\(^{2+}\) load with increased oxidative phosphorylation and ATP production (27). Moreover, pharmacologic inhibition of the MCU reduced the activity of the aforementioned intra-mitochondrial dehydrogenases by reducing Ca\(^{2+}\) influx (28, 29). Analogous to this model, we found enhanced enzymatic activity for PDH and αKGD at baseline in Ppif\(^{-/-}\) hearts, consistent with the finding of increased basal Ca\(^{2+}\) in the mitochondrial matrix.

Figure 5
Ppif\(^{-/-}\) mice display significant metabolic alterations. (A) Heat map displaying gradient differences in gene expression between WT and Ppif\(^{-/-}\) ventricular tissue at 8 weeks of age (ANOVA, P < 0.01). (B) Ratio of glucose/palmitate (Glu/palm) consumption in working hearts using \(^{13}\)C NMR spectroscopy. (C) Ratio of succinate/cytosolic glutamate (Succ/glut) in working hearts using \(^{13}\)C NMR spectroscopy. (D) PDH enzymatic activity in mitochondria isolated from mouse hearts (y axis indicates change in mOD/min/mg protein where \(r^2 = 1\)). (E) αKGD enzymatic activity in mitochondria isolated from mouse hearts (rate expressed as nmol NADH/min/mg of protein). (F) Western blot of PDH complex expression. (G) Western blot of αKGD and isocitrate dehydrogenase (IDH); mitochondrial cytochrome oxidase subunit II (COXII) was used as a loading control. *P < 0.05 versus WT.
PDH in particular is central in linking glycolysis to the citric acid cycle by converting pyruvate to acetyl-CoA. We hypothesize that the mitochondrial calcium-mediated increase in PDH activity may account for the observed elevation in glucose oxidation. For example, inhibition of PDH kinase, which increases PDH activity, enhances glucose oxidation in rat heart (30). Increased basal Ca\textsuperscript{2+} in the mitochondria mimics higher contractile workloads, which on the surface would not appear to be detrimental, likely explaining why Ppif\textsuperscript{--} mice are relatively unaffected when not stressed. However, our working hypothesis is that such a state of constitutively higher intramitochondrial Ca\textsuperscript{2+} reduces the dynamic range in cardiac metabolism, ultimately decreasing the heart’s metabolic reserve capacity and rendering it more sensitive to decompensation with even subtle stress provocation (such as sustained exercise). At first glance, these results seem contrary to our previous report that Ppif deletion rescued heart disease in β2a-Tg mice, a transgenic model with severe heart failure due to massive Ca\textsuperscript{2+} overload and myocyte necrosis (31). However, the loss of Ppif in β2a-Tg mice was likely protective, blocking the pathologic function of the MPTP in its large-conductance state and secondary myocyte necrosis, hence predominating over the physiologic function of the MPTP in regulating mitochondrial matrix Ca\textsuperscript{2+}.

A hallmark of pathologic hypertrophy is metabolic reprogramming back toward a fetal-like gene program, favoring glycolysis over fatty acid oxidation (32). While this metabolic reprogramming was originally postulated to be compensatory, studies in genetically modified mouse models predict that any switching of major metabolic substrate utilization in the heart is detrimental (16, 33). For example, Ppara\textsuperscript{--} mice display an increase in glucose oxidation and a decrease in fatty acid oxidation resulting in myocardial fibrosis and an inability to tolerate increased workloads (34–36). In parallel, Ppard\textsuperscript{--} (PPARY protein) mice display increased glucose uptake, with a decrease in fatty acid β-oxidation and likewise exhibit systolic dysfunction and the rapid onset of heart failure (37). Even more striking, a recent attempt to genetically overcome a shift in fatty acid utilization by overexpression of Glut1 resulted in vast metabolic reprogramming and myocardial pathology (38). The Ppif\textsuperscript{--} phenotype strongly fits this paradigm, in that a shift in substrate utilization precedes any overt cardiac pathology; and upon stress-stimulation the observed deficit in metabolic reserve leads to disease.

In conclusion, we have established that CypD and the MPTP represent an important regulator of mitochondrial Ca\textsuperscript{2+} exchange in myocytes that is pivotal in maintaining metabolic plasticity, thereby facilitating adaptation to disease states or increased workload. Thus, our results suggest that the modulation of mitochondrial Ca\textsuperscript{2+} exchange may be an exciting new avenue for the treatment of heart failure.

**Methods**

Targeted and transgenic mice. The generation of Ppif-null mice and CypD-transgenic mice has previously been reported (3). For our experiments, Ppif\textsuperscript{--} mice were crossed with αMHC-CypD-Tg mice and heterozygous (Ppif\textsuperscript{+/--}) offspring carrying the CypD transgene crossed with Ppif\textsuperscript{--} void of the transgene. The offspring of this cross were again mated to generate the 4 genotypes of mice used in the current study: WT, Ppif\textsuperscript{--}, αMHC-CypD-Tg, and Ppif\textsuperscript{--} × αMHC-CypD-Tg. The generation of CaMKII-Tg mice has previously been reported (17). For our experiments Ppif\textsuperscript{--} mice were crossed

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**Table 1**

Ppif\textsuperscript{--} hearts display significant metabolic reprogramming

<table>
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<th>Functional group</th>
<th>P</th>
<th>Fold enrichment</th>
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<td>12.18</td>
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<tr>
<td>Pigment metabolic process</td>
<td>1.40 × 10\textsuperscript{-3}</td>
<td>10.15</td>
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<tr>
<td>Secondary metabolic process</td>
<td>5.81 × 10\textsuperscript{-3}</td>
<td>6.89</td>
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<td>6.73 × 10\textsuperscript{-3}</td>
<td>23.44</td>
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<td>8.34 × 10\textsuperscript{-3}</td>
<td>21.09</td>
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<td>Substrate-specific transmembrane transporter activity</td>
<td>4.97 × 10\textsuperscript{-2}</td>
<td>1.63</td>
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DAVID functional annotation microarray analysis of gene class changes in Ppif\textsuperscript{--} hearts, which showed many alterations in mitochondrial genes and genes involved in metabolism.
with CaMKII-Tg mice in the same manner as they were with CypD-Tg. All experiments in the current study utilized littermate controls of matched age and sex. The IACUC of Cincinnati Children’s Hospital approved all experiments involving animals.

**Western blot analysis.** Sample preparation and gel electrophoresis were previously described in detail (39). The following antibodies were used in the current study: CypD (Mitosciences), respiratory chain complexes (OXPHOS blots, Mitosciences), PDH subunits (Mitosciences), αKG, COX II (Santa Cruz Biotechnologies Inc.), and isoform dehydrogenase (Abcam).

**Isolation of adult cardiomyocytes, cytosolic Ca\(^{2+}\) current, and myocyte shortening.** Ventricular myocytes were isolated as described previously (31). Myocytes were maintained at room temperature in 5% CO\(_2\) and 95% O\(_2\) and used within 3 hours of isolation. Myocytes were placed in a chamber mounted on an inverted Nikon microscope and perfused with normal physiological solution (Ringer) containing 2 mM Ca\(^{2+}\). Contractions were measured with video edge detection, and intracellular Ca\(^{2+}\) was measured with Fura-2 as described previously (31).

**Mitochondria isolation and analysis of mitochondrial Ca\(^{2+}\) content.** Briefly, the heart was quickly excised and mitochondria isolated in buffers free of EGTA/EDTA, pH 7.4, at 4°C. For measurement of Ca\(^{2+}\) content, mitochondria were isolated and washed repeatedly in EGTA/EDTA-free buffers and resuspended in 0.6 M HCl as previously reported (40). Concentration was then determined spectrophotometrically using the O-Cresolphthalein Complexone assay (TECO Diagnostics).

**Isolated mouse heart preparation and \(^{13}\)C metabolite NMR.** Mice were anesthe-
tized with an intraperitoneal injection of 0.10 ml pentobarbital sodium diluted 1:5 in perfusate. The abdominal cavity was exposed with a transverse incision, and 0.05 ml heparin was administered to the inferior vena cava. The heart was quickly isolated and placed in ice-cold Krebs-Henseleit (KH) buffer (25 mM NaHCO\(_3\), 120 mM NaCl, 11 mM glucose, 4.7 mM KCl, 1.2 mM KHPO\(_4\), 1.2 mM MgSO\(_4\), and 1.75 mM CaCl\(_2\)) to arrest the heart. The heart was perfused via the aorta. A water-filled latex balloon was inserted into the LV to measure hemodynamic parameters using a PowerLab/24 and Chart v5.5 software (AD Instruments). All hearts were perfused with KH buffer gassed with 95% O\(_2\) and 5% CO\(_2\) and maintained at 37°C. \(^{13}\)C metabolite labeling was performed as previously described (41). Following equilibration, hearts were perfused in phosphate-free KH buffer with 8 mM glucose, 2 mM L-\(^{13}\)C-glucose, 0.5 mM uniformly labeled \(^{13}\)C-palmitate, and 3% (v/v) BSA for 20 minutes. The hearts were freeze-clamped, extracted with cold 3.6% perchlo-
ic acid, and centrifuged at 14,000 g. The resulting supernatant was neutralized on ice with 5 M KOH to a pH of approximately 8, centrifuged at 14,000 g, and lyophilized. Samples were dissolved in D\(_2\)O for \(^{13}\)C NMR measurement, and proton-decoupled \(^{13}\)C spectra were acquired on a Varian Inova 500-MHz NMR spectrometer. By analyzing the scalar coupling pattern of the C-4 carbon resonance of glutamate, we were able to measure the relative contribu-
tion of \(^{13}\)C-enriched glucose versus palmitate to the acetyl-CoA pool.

**Dehydrogenase activity assays.** PDH activity was assayed by using an immu-
capture technique and then calculating the mean maximal rate of NADH production (change in mean OD/min where \(r^2 = 1\)) as described in the manufac-
turer’s instructions (Mitosciences). αKG activity was moni-
tored using αKG-dehydrogenase reactions. Briefly, mitochondria were lyzed in 120 mM KCl, 10 mM Tris, 5 mM MOPS, 5 mM KH\(_2\)PO\(_4\), and 1% Triton X-100. The homogenate was immediately added to 5 mM MgCl\(_2\), 5 mM αKG, 0.2 mM coenzyme A, 0.4 mM thiamine pyrophosphate, and 1 mM NAD\(^+\). NADH produced was measured at an absorbance of 340 nm.

**Neonatal myocyte isolation and perfusion imaging and analysis.** Neonatal rat cardiomyocytes were isolated from Sprague-Dawley rat pups as previously described (42). Cardiomyocytes were allowed to mature for 72 hours in media containing 15% fetal bovine serum. Myocytes were then infected with mt-pericam adenovirus for 6 hours (22). Twenty-four hours following infection, myocytes were utilized in Ca\(^{2+}\) experiments. Prior to experi-
ments, myocytes were switched to standard Ringer solution containing 4 mM Ca\(^{2+}\), as previously reported (43). The pericam fluorescence ratio was determined at room temperature using a Delta Scan dual-beam spec-
trofluorophotometer (Photon Technology International), operated at an emission wavelength of 530 nM, with excitation wavelengths of 490 and 415 nM. The stimulating frequency for Ca\(^{2+}\) transient measurements was 0.2 Hz. All data were recorded and analyzed using Chart v5.6 (AdInstru-
ments) and IonWizard (IonOptix) software.

**Isolation of mRNA, Taqman qPCR, and gene array.** mRNA was isolated using the RNeasy Protect Midi Kit according to manufacturer’s instructions (QIAGEN). Reverse transcription was performed in a standard fashion with QuantiTect Reverse Transcription Kit (QIAGEN) supplemented with DNase treatment. Taqman qPCR was carried out according to the manufac-
turer’s instructions using probe sets for αMHC, βMHC, ANP, BNP, and GAPDH. Analysis was carried out using the ΔΔ-CT method with αMHC and GAPDH correction and reported as fold change versus WT sham.

**Gene expression analysis.** Gene expression analysis was performed using Affymetrix Mouse Gene Array 1.0ST. Results were filtered for a raw value greater than 5, followed by ANOVA (P < 0.01) statistical analysis of WT versus Pdpf\(^{-/-}\). A raw filter for values greater than 6 was then applied, followed by a volcano plot for WT versus Pdpf\(^{-/-}\) (fold difference >1.25, P < 0.05). Results were then ana-
lized for functional group classification using DAVID functional annota-
tion microarray analysis (http://david.abcc.ncifcrf.gov/home.jsp) (44, 45).

**Histology, image analysis, and electron microscopy.** For histological analysis, hearts were collected at the indicated times, fixed in 10% buffered for-
malin, and embedded in parafin. Serial 5-μm heart sections from each group were stained with H&E and Masson’s trichrome (to detect fibrosis). Myocyte cross-sectional areas were analyzed in slices stained with wheat germ agglutinin–FITC conjugate at 50 μg/ml to accurately identify sar-
collemmal membrane as previously reported (39). ImageJ (http://rsweb.
nih.gov/ij/) analysis was then performed. All analysis was performed in a blinded fashion. Transmission electron microscopy and TUNEL staining were performed as previously described (11, 39).

**Echocardiography, invasive hemodynamics, swimming protocol, and pressure overload.** Hypertrophy studies using the TAC model, swimming exercise, and invasive hemodynamics have been previously described (39, 46).

**Statistics.** All results are presented as mean ± SEM. Statistical analysis was per-
formed using Prism 4.0 (GraphPad Software) for unpaired 2-tailed t test (for 2 groups) and 1-way ANOVA with Bonferroni correction (for groups of 3 or more). For experiments performed over time, 2-way ANOVA with Bonferroni post-hoc analysis was used. P values less than 0.05 were considered significant.

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