Depressing Mitochondria-Reticulum Interactions Protects Cardiomyocytes from Lethal Hypoxia-Reoxygenation Injury

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Abstract

Background—Under physiological conditions, Ca\textsuperscript{2+} transfer from the endoplasmic reticulum (ER) to mitochondria might occur at least in part at contact points between the two organelles, and involves the VDAC1/Grp75/IP3R1 complex. Accumulation of Ca\textsuperscript{2+} into the mitochondrial matrix may activate the mitochondrial chaperone cyclophilin D (CypD) and trigger permeability transition pore (PTP) opening, whose role in ischemia-reperfusion injury is well recognized. We questioned here whether the transfer of Ca\textsuperscript{2+} from ER to mitochondria might play a role in cardiomyocyte death after hypoxia-reoxygenation.

Methods and Results—We report that CypD interacts with the VDAC1/Grp75/IP3R1 complex in cardiomyocytes. Genetic or pharmacologic inhibition of CypD in both H9c2 cardiomyoblasts and adult cardiomyocytes decreased the Ca\textsuperscript{2+} transfer from ER to mitochondria through IP3R under normoxic conditions. During hypoxia-reoxygenation (HR), the interaction between CypD and the IP3R1 Ca\textsuperscript{2+} channeling complex increased concomitantly with mitochondrial Ca\textsuperscript{2+} content. Inhibition of either CypD, IP3R1 or Grp75 decreased protein interaction within the complex, attenuated mitochondrial Ca\textsuperscript{2+} overload and protected cells from HR. Genetic or pharmacological inhibition of CypD provided similar effect in adult mice cardiomyocytes. Noteworthy, disruption of ER-mitochondria interaction via the down-regulation of Mfn2 similarly reduced the interaction between CypD and the IP3R1 complex and protected against HR injury.

Conclusions—Our data point first, to a new role of CypD at the ER-mitochondria interface, and second, suggest that decreasing ER-mitochondria interaction at reperfusion can protect cardiomyocytes against lethal reperfusion injury through the reduction of mitochondrial Ca\textsuperscript{2+} overload via the CypD/VDAC1/Grp75/IP3R1 complex.

Key words: mitochondria, calcium, ischemia reperfusion injury, sarcoplasmic reticulum, mitochondria-associated membranes
Introduction

Mitochondria and the endoplasmic reticulum (ER) are separately considered as key players in cell death signaling. Mitochondria and ER are interconnected organelles and form endomembrane network. The contact points through which ER communicates with mitochondria are referred to as mitochondria-associated membranes (MAM). MAM are enriched in phospholipids- and glycosphingolipid-synthesis enzymes, as well as chaperone proteins, which transport lipids and exchange calcium between these two organelles. Several recent studies have identified new proteins enriched at the ER-mitochondria interface, highlighting the emerging understanding of the role of this region within the cell. One of them has identified a macromolecular complex composed of VDAC1, Grp75 and IP3R1 which regulates direct Ca\textsuperscript{2+} transfer from ER to mitochondria. Indeed, ER-mitochondria junctions are aligned with mitochondrial contact points where VDAC1 is abundantly present, thus creating a hotspot for the Ca\textsuperscript{2+} transfer from the ER. While the role of this organelle crosstalk begins to be understood in cell physiology, MAM involvement in cardiac pathologies remains unknown.

Calcium signaling is central for the heart function, through its physiological role in excitation-contraction coupling, and the detrimental impact of Ca\textsuperscript{2+} overload during heart failure and myocardial ischemia-reperfusion. During this latter condition, it is well accepted that the cytosolic accumulation of Ca\textsuperscript{2+} subsequently results in mitochondrial Ca\textsuperscript{2+} overload, which activates the matrix chaperone cyclophilin D (CypD) and triggers the opening of the permeability transition pore (PTP) leading to cell death. Recent reports have confirmed the existence of Ca\textsuperscript{2+} microdomains between ER and mitochondria in the myocardium where the organization of the network between the two organelles is highly ordered. It has also been suggested that mitochondrial Ca\textsuperscript{2+} overload could be mediated through IP3R.
We therefore questioned whether Ca\(^{2+}\) transfer from ER to mitochondria via the VDAC1/Grp75/IP3R1 complex might play a role in mitochondrial Ca\(^{2+}\) overload and subsequent cardiomyocyte death observed during the reperfusion phase following a sustained ischemic insult. We further questioned the relationship between CypD and VDAC1/Grp75/IP3R1 complex in the regulation of the Ca\(^{2+}\) exchange during ischemia-reperfusion. We report a new physiological role of CypD in ER-mitochondria Ca\(^{2+}\) exchange via its interaction with the VDAC1/Grp75/IP3R1 complex, with major impact on cardiomyocyte death upon hypoxia-reoxygenation.

Methods

For further details, see supplemental methods.

Animals

Ppif\(^{-/-}\) mice under C57Bl6/SV129 background were a gift from SJ Korsmeyer’s lab (Dana Farber Cancer Institute, Boston, US)\(^{11}\). Both WT and Ppif\(^{-/-}\) male mice were obtained by homozygous intercross in our laboratory. All experiments were performed on mice at 8 weeks. All procedures conformed to European Parliament Directive 2010/63/EU and the 22 September 2010 Council on the protection of animals and were approved by the local institutional animal research committee (N°BH2012-64).

Duolink® Proximity Ligation in situ Assay (PLA)

Cells were fixed with 4% Paraformaldehyde (10min at room temperature, RT) and permeabilized using 0.1% Triton X100 (15min at RT). Subsequent blocking, antibody hybridizations, proximity ligations and detections were performed according to recommendations from manufacturers (OLINK Bioscience). The cells were incubated with the primary antibodies overnight at 4°C and
then washed three times with TBS-0.05% Tween 20 (TBS-T). Briefly, after incubation with primary antibodies, we applied combinations of corresponding PLA probes for 1h at 37°C. The cells were washed with TBS-T, incubated for 30min with ligase, and finally washed with TBS-T. Then, cells were incubated with polymerase for 100min. Finally, the cells were washed once with SSC 1X, and then with SSC 0,1X. All reactions were performed at 37°C in a humid chamber, with 30μl of reaction mixture/well. Preparations were mounted in DuoLink II mounting medium, containing DAPI (Eurogentec). Fluorescence was analyzed with a Zeiss inversed fluorescent microscope equipped with an ApoTome, using the AxioVision program.

Quantification of signals was done with the BlobFinder software (Centre for Image Analysis, Uppsala University) and expressed as interactions per cell relative to the non-treated. Experiments were performed at least three times, with a minimum of 5 fields taken per condition.

**Ca**^{2+} **measurements in H9c2 cells**

**Ca**^{2+} transfer from ER to mitochondria was assessed by recording mitochondrial **Ca**^{2+} changes in a native cell environment in presence of extracellular calcium (phenol-free DMEM with 1.8mM CaCl_2 and 10%FBS). Cells were plated in a 8-well chamber glass slide (LabTek, Dutsher). To measure mitochondrial **Ca**^{2+}, cells were incubated with Rhod2-AM (2μM, Invitrogen) in phenol-free DMEM with 0.03% pluronic acid (Sigma) for 1h at 37°C. Cells were then washed free of Rhod2 and de-esterified in phenol-free DMEM/10% FBS at 37°C. After being loaded, cells were placed on the stage of a Zeiss LSM 780 bi-photon confocal microscope (Zeiss, Le Pecq France) equipped with a 63X lens (oil immersion, Numerical Aperture, NA=1.4). Rhod2 fluorescence signals were recorded at 30°C by excitation at 561nm and measuring the emitted light at 588nm. Collected images were processed with Zen 2009 Light Edition software (Zeiss).
Isolation of adult murine cardiomyocytes and Ca\textsuperscript{2+} measurements

Ventricular cardiomyocytes were isolated using enzymatic digestion according to previously described procedure\textsuperscript{12}.

Cardiomyocytes were loaded for 20min at 37°C with Fluo4-AM (5μM, Invitrogen). To measure Ca\textsuperscript{2+} transients, cardiomyocytes were field-stimulated at 1Hz with a current pulse delivered via two platinum electrodes. Changes in Fluo4 fluorescence were recorded at 25°C using an LSM510 Meta confocal microscope equipped with a 63x water-immersion objective (numerical aperture: 1.2; Line-scan mode: 1.5 ms/line; Zeiss). Scanning was carried out along the long axis of the cell.

To measure mitochondrial Ca\textsuperscript{2+}, cardiomyocytes were loaded for 40min at 37°C with Rhod2-AM (5μM, Teflabs, Austin, USA). Cardiomyocytes were stimulated under action potential induced-clamp conditions using a whole-cell patch-clamp technique at RT with an Axopatch 200B (Axon Instruments), as described previously\textsuperscript{13}.

Cellular model of hypoxia-reoxygenation and analysis of cell death and mitochondrial Ca\textsuperscript{2+} concentration

H9c2 cardiomyoblasts were subjected to 3h of hypoxia followed by 2h of reoxygenation at 37°C. Cells were randomized to receive either no additional intervention (HR), or 1μM NIM811 (HR+NIM811), or 50μM 2-APB (HR+2-APB), administered at the onset of reoxygenation. To simulate hypoxia, the cell culture medium was replaced with Tyrode containing (in mM): 130 NaCl, 5 KCl, 10 Hepes, 1 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2} at pH 7.4/37°C and cardiomyoblasts were exposed to hypoxia in a controlled hypoxic chamber (Adelbio®, Clermont-Ferrand, France) by 95% nitrogen and 5% CO\textsubscript{2} gas mixture flushing up to partial O\textsubscript{2} pressure of 0.5 to 1%. Reoxygenation was conducted in a normoxic incubator at 37°C, by replacing the ischemia-medium for 2h with
DMEM supplemented with 10%FBS. Time Control (TC) group consists in cells without the hypoxic stimulus, kept in the reoxygenation buffer. Cell death was determined after loading with 1μg/mL propidium iodide (PI) that permeates only the damaged cells, and measured by flow cytometry (FACSCalibur, BD) at the end of the 2h reoxygenation. Similar protocol was used for isolated adult cardiomyocytes, except that the duration of hypoxia was reduced to 45min and cell death was measured by counting PI-positive cells using microscopy after the 120-minute reoxygenation period. For mitochondrial Ca\(^{2+}\) concentration measurements, H9c2 cells were loaded before hypoxia with Rhod2 as described above, and fluorescence measurement was determined by a fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific) at an excitation wavelength of 542nm and an emission wavelength of 590nm. Intensity was normalized by cell density measured with Crystal Violet staining.

**Statistical analysis**

Data are expressed as the mean±SEM. Experiments were performed at least three times, in duplicates or more. Statistical analysis was performed with the GraphPad Prism software using non-parametric tests. Comparisons among more than 2 groups were analysed by the Kruskal-Wallis test. When the Kruskal-Wallis test was significant, the Dunn’s appropriate multiple testing procedure was performed. For all other analysis, data were compared by the Mann-Whitney rank sum test. Statistical significance was defined as a value of p<0.05.

**Results**

**CypD interacts with the VDAC1/Grp75/IP3R1 Ca\(^{2+}\) channelling complex in the heart**

We first questioned if CypD could be present at the heart MAM interface by adapting the protocol of MAM isolation from liver to the heart\(^14\). Absence of cross-contamination and purity
of each fraction was assessed by both electron microscopy (Supplementary figure 1) and Western Blot (Figure 1A). As expected, CypD was concentrated in pure mitochondria and absent of the ER. However, a small fraction of CypD was also detected in the MAM fraction, together with IP3R1, Grp75 and VDAC1. To determine if these proteins could form a macromolecular complex, we performed 2D blue native page separation of heart homogenates. CypD was present in varying amounts in complexes which have a wide range of sizes, but more particularly in a high molecular weight complex where VDAC1, Grp75 and IP3R1, but not ANT, were detected (Figure 1B). A specific interaction of CypD with the IP3R1 channelling complex was also demonstrated by IP3R1 immunoprecipitation in mice heart homogenates (Figure 1C).

To further confirm the involvement of CypD in the IP3R1 channelling complex, we monitored in situ protein-protein interactions at MAM interface by proximity ligation assay in H9c2 cardiomyoblasts. We observed the interaction between IP3R1 and VDAC1, with Grp75 as an intermediate (Figure 1D, first row). To evaluate the potential involvement of CypD in this complex, we analyzed the interactions between IP3R1/CypD, as well as with Grp75. For each couple of proteins, red fluorescent dots were observed, illustrating interactions of IP3R1 and Grp75 with CypD (Figure 1D, middle row). Interestingly, no interaction was observed between CypD and other ER Ca\(^2^+\) channeling proteins, like Serca2 and RyR2, neither with IP3R2 (Figure 1C, last row). This suggests that the CypD interaction with the VDAC1/Grp75/IP3R1 complex is specific and occurs at the ER-mitochondria interface (Supplemental figure 2).

**CypD removal from the ER-mitochondria interface decreases the interactions of partner proteins within the VDAC1/Grp75/IP3R1 complex.**

CypD is known to be a mitochondrial matrix protein able to be recruited at the inner membrane. In order to understand the role of CypD in the MAM, we performed at different time points
(30min, 2h and 16h) a pharmacological inhibition of CypD in H9c2 cells. We used NIM811, a specific inhibitor of CypD (known to detach it from the inner mitochondrial membrane), to determine if it might change this protein localization in the MAM of H9c2 cells. As shown in Figure 2A-B, NIM811 treatment significantly decreased the interactions between CypD and IP3R1 after 2h and 16h with a time-dependent effect. Interestingly, inhibition of CypD by NIM811 also modified the interaction between the other partner proteins of the complex; indeed, Grp75/IP3R1 interactions were reduced as soon as 30min after NIM811 treatment (Figure 2C) whereas interactions between Grp75 and VDAC1 diminished only after 2h of NIM811 treatment (Figure 2D).

In line with this, adult cardiomyocytes of Ppif<sup>−/−</sup> (CypD protein, mice null for the Ppif gene) mice displayed significantly decreased interactions of both VDAC1 and Grp75 with IP3R1 (Figure 2E-F). We further analyzed the composition of heart MAM following the in vivo administration of either NIM811 or CsA in mice. Interestingly, NIM811 significantly decreased the presence of the chaperones CypD and Grp75 at the MAM interface, with a similar trend for CsA (Figure 2G-H).

Alternatively, the inhibition of IP3R by either 2-APB or XestoC also decreased the interactions of IP3R1 with CypD and Grp75 (Supplemental figure 3).

**CypD controls the Ca<sup>2+</sup> transfer from ER to mitochondria through IP3R1**

Given that the VDAC1/Grp75/IP3R1 complex has been shown to directly control the Ca<sup>2+</sup> transfer from ER to mitochondria, we hypothesized that CypD would play a role in this Ca<sup>2+</sup> exchange between the two organelles. Assessment of specific mitochondrial Ca<sup>2+</sup> loading with Rhod-2 was performed on H9c2 cells after histamine stimulation, known to induce Ca<sup>2+</sup> release from ER stores (Figure 3A). In untreated H9c2, histamine rapidly increased mitochondrial Ca<sup>2+</sup>
levels, reflecting IP3R-mediated Ca$^{2+}$ transfer from ER to mitochondria. Both the down-regulation of CypD expression by specific siRNA and the pharmacological inhibition of CypD by NIM811 led to a non-significant reduction of the amplitude of the histamine-stimulated increase of mitochondrial Ca$^{2+}$ when compared to siCTL (Figure 3B-D). Inhibition of CypD also accelerated the decay time as depicted by the decreased tau but did not significantly modify the time of rise of mitochondrial Ca$^{2+}$ (Figure 3E-F). So, to rule out an effect on the mitochondrial Na$^+$/Ca$^{2+}$ exchanger, we analyzed the effect of its inhibitor, CGP-37157, on the histamine-induced Ca$^{2+}$ transfer after NIM811 treatment (Supplemental figure 4A). In H9c2 cells treated with NIM811, we observed a similar decrease of Ca$^{2+}$ transfer to mitochondria with prevention of the increase in tau after histamine stimulation when the mitochondrial Na$^+$/Ca$^{2+}$ exchanger is inhibited (Supplementary figure 4B-C). Importantly, neither the inhibition of CypD by NIM811 nor of IP3R1 by 2-APB caused any significant change in the mitochondrial membrane potential, as assessed by TMRM (Supplementary figure 5).

To confirm the involvement of CypD in this Ca$^{2+}$ coupling in the heart, we examined Ca$^{2+}$ transients in isolated adult cardiomyocytes from WT and Ppif$^{-/-}$ mice. In adult Ppif$^{-/-}$ cardiomyocytes, the electrically evoked Ca$^{2+}$ transients displayed an unchanged rate of rise, a significantly reduced amplitude and an increased time constant of decay (Figure 4A-D). Caffeine stimulation revealed a reduction in the SR Ca$^{2+}$ load without any change of the rate of Ca$^{2+}$ release (Figure 4E). Importantly, these alterations of Ca$^{2+}$ homeostasis were mimicked by pharmacological inhibition of CypD activity with NIM811 in isolated WT cardiomyocytes (Figure 4A-E).

To confirm the involvement of CypD in vivo in the Ca$^{2+}$ transfer from SR to mitochondria, we measured mitochondrial Ca$^{2+}$ load by Rhod-2 in isolated cardiomyocytes from...
WT and Ppif−/− mice under patch-clamp conditions (Figure 4F). Ppif−/− cardiomyocytes exhibited a significant reduction of mitochondrial Ca²⁺ loading after both action potential-induced Ca²⁺ transients and caffeine-induced SR Ca²⁺ release (Figure 4G-H). Altogether, these data strongly suggest that CypD is involved in the SR-mitochondria Ca²⁺ coupling in the heart through the VDAC1/Grp75/IP3R1 complex. This SR-mitochondria Ca²⁺ coupling may also contribute to modulate the global Ca²⁺ homeostasis and excitation-contraction coupling in cardiomyocytes.

**Inhibiting the CypD/VDAC1/Grp75/IP3R1 complex protects from hypoxia-reoxygenation injury**

Based on these results, we hypothesized that the IP3R1-dependent Ca²⁺ transfer could also play a role in the mitochondrial Ca²⁺ overload observed during ischemia-reperfusion, and thus may be a new pharmacological target to prevent ischemia-reperfusion injury. Hypoxia-reoxygenation (HR) of H9c2 led to a significant increase of the interactions between CypD, Grp75 and IP3R1 as revealed by in situ PLA (Figure 5A and B, Supplemental Figure 6). These increased protein-protein interactions were associated with a higher mitochondrial Ca²⁺ load (Figure 5C) and a significant increase of cell death as compared to time control (TC) cells (Figure 5D). Interestingly, treatment at reoxygenation with either 2μM NIM811 or 50μM 2-APB reduced CypD/IP3R1 interactions and inhibited HR-induced mitochondrial Ca²⁺ overload and cell death (Figure 5A-D). Similar observations were made following genetic down-regulation of CypD (Supplemental Figure 7) or treatment with Xestospongin C, another inhibitor of IP3R (Supplemental Figure 8). To confirm the involvement of CypD and the VDAC1/Grp75/IP3R1 complex during HR injury, we down-regulated another partner of this complex, Grp75. Under baseline conditions (TC), the down-regulation of Grp75 significantly reduced the Grp75/IP3R1 interactions and tended to reduce the CypD/IP3R1 interactions, and caused a decrease of the
mitochondrial Ca\(^{2+}\) load (Figure 6A-C). After HR, Grp75-silenced H9c2 cells showed a decreased interaction of CypD and Grp75 with IP3R1 and a prevention of the increase in mitochondrial Ca\(^{2+}\) content when compared to siCTL (Figure 6A-C). Cell death was significantly reduced after HR in the siGrp75 group. These results support the idea that modifying the CypD/VDAC1/Grp75/IP3R1 complex at reoxygenation prevents the Ca\(^{2+}\) transfer from ER to mitochondria during HR and attenuate mitochondrial Ca\(^{2+}\) loading and subsequent lethal cell injury.

**Down-regulation of Mfn2 protects H9c2 cells from lethal hypoxia-reoxygenation injury**

Mfn2 has been recently shown to be involved in the tethering of ER to mitochondria and to regulate cardiac bioenergetics at the MAM interface\(^3\)\(^,\)\(^8\). Down-regulation of Mfn2 reduced the interactions between both CypD and VDAC1 with IP3R1, at baseline but also after HR (Figure 7A). In addition, down-regulation of Mfn2 prevented the increase of the mitochondrial Ca\(^{2+}\) content observed after HR (Figure 7B). Eventually, cells depleted in Mfn2 were protected against lethal HR injury (Figure 7C). These data support the proposal that decreasing the ER-mitochondria tethering protects from HR injury by reducing the IP3R1-mediated ER-mitochondria Ca\(^{2+}\) transfer.

**Protection of adult mice cardiomyocytes from lethal reoxygenation injury by targeting the CypD/VDAC1/Grp75/IP3R1 complex**

The organization of the ER/SR and mitochondria is more complex in cardiomyocytes than in H9c2 cells. We then checked whether we could make similar observations in adult WT and *Ppif* \(^{-/}\) primary cardiomyocytes. Cardiomyocytes were subjected to 45 minutes of hypoxia followed by 2 hours of reoxygenation. In WT cardiomyocytes, the analysis of the interaction of both CypD and VDAC1 with IP3R1 by *in situ* PLA showed that HR significantly increased the interaction
between the partners of the IP3R1 Ca\textsuperscript{2+} channelling complex, concomitant with 45% of cell death as assessed by propidium iodide staining (Figure 8A-C), in agreement with our data in H9c2 cells. Treatment at reoxygenation with 1\(\mu\)M NIM811 significantly reduced the interaction between VDAC1 and IP3R1 and tended non-significantly to reduce the CypD/IP3R1 interactions while significantly decreasing HR-induced cell death (Figure 8A-C). Importantly, \(Ppif^{-/-}\) cardiomyocytes displayed a significant reduction of both VDAC1/IP3R1 and Grp75/IP3R1 interactions after HR, concomitant to a reduced cell death (Figure 8D-E).

**Discussion**

We report that the mitochondrial chaperone CypD modulates ER-mitochondria Ca\textsuperscript{2+} crosstalk via the VDAC1/Grp75/IP3R1 complex. This modulation plays an important role in hypoxia-reoxygenation injury. Pharmacological or genetic inhibition of each partner of the complex modified the Ca\textsuperscript{2+} fluxes and blunted lethal reoxygenation injury. Decreasing the tethering of ER to mitochondria by down-regulation of Mfn2 also prevented mitochondrial Ca\textsuperscript{2+} overload and reduced cell death following hypoxia-reoxygenation injury. These findings give new insights into the mechanism of lethal myocardial ischemia-reperfusion injury and open new therapeutic perspectives.

Recently, several MAM-specific proteins were identified, including Mfn2, PACS2, sigma-1 receptor and promyelocytic leukemia\textsuperscript{3, 4, 16, 17}. Most of these proteins are ER proteins, with only a few (e.g. Mfn2) belonging to mitochondria. The identification of CypD in close contact with the VDAC1/Grp75/IP3R1 complex adds a new member to the list of mitochondrial partners in MAM. Several of these MAM-specific proteins are Ca\textsuperscript{2+}-sensitive chaperones\textsuperscript{5}. Interestingly, CypD is a Ca\textsuperscript{2+}-sensitive mitochondrial chaperone and belongs to the peptidyl-
prolyl *cis-trans* isomerase family, confirming the important role of this class of proteins to regulate Ca\(^{2+}\) signalling at ER-mitochondria contact sites. Whether the chaperone activity of CypD is important for its interaction with the VDAC1/Grp75/IP3R1 complex was not specifically addressed in this study. Nevertheless, we found that pharmacological inhibition or siRNA removal of CypD altered the interaction among the other proteins of the VDAC1/Grp75/IP3R1, suggesting that the link between CypD and the complex might not only be functional but also structural. One cannot however rule out that the predominant matrix fraction of CypD might also play a role in Ca\(^{2+}\) transfer from the ER to mitochondria. The mechanism by which CypD physically and functionally interacts with the VDAC1/Grp75/IP3R1 Ca\(^{2+}\) channelling complex needs further investigation. However its inhibition by NIM811, known to detach CypD from the mitochondrial membrane, suggests that its binding to the inner membrane is required to modulate the Ca\(^{2+}\) flux from ER to mitochondria through the VDAC1/Grp75/IP3R1 complex. The observation that, under normoxic conditions, CypD facilitates the transfer of Ca\(^{2+}\) from ER to mitochondria is in line with the well-known role of Ca\(^{2+}\) in cardiac excitation-contraction coupling and bioenergy coupling between ER and mitochondria. Smaili et al. have suggested in intact hepatocytes a role of cyclophilins in the Ca\(^{2+}\) cycling between ER and mitochondria by showing that cyclosporine modifies the IP3-dependent Ca\(^{2+}\) signals. The present data expand this notion since both genetic and pharmacologic inhibition of CypD leads to a decreased Ca\(^{2+}\) transfer from ER to mitochondria through IP3R1, even in the *in vivo* cardiomyocyte model.

IP3R exists as three isoforms. Different studies suggest that several isoforms of IP3R could be enriched in the MAM. In the heart, the type-1 isoform of IP3R is highly expressed, but IP3R2 is recognized as the predominant isoform in the cardiac myocyte.
However, most of the functions of IP3Rs are attributed to the type-1 and our results show that CypD preferentially interacts with IP3R isoform 1 since no interaction with IP3R2 was observed. One can wonder whether the impact of IP3Rs signaling plays an important role in the heart compared to the cardiac RyR2 which is the major Ca\(^ {2+} \) release channel on the sarcoplasmic reticulum in cardiomyocytes. In fact, accumulating evidence suggests that the IP3Rs, and more particularly the type 1 isoform, are involved in cardiac calcium signaling including the excitation-contraction and excitation-transcription coupling in the normal heart but also in some pathologies like hypertrophy.

One may question whether other factors, including mitochondrial membrane potential or SR Ca\(^ {2+} \) load, might contribute to attenuate Ca\(^ {2+} \) transfer from ER to mitochondria upon inhibition of CypD. In contrast to Smaili et al., we found that inhibition of CypD by NIM811 did not modify the mitochondrial membrane potential, indicating that the observed reduction of Ca\(^ {2+} \) transfer from ER to mitochondria was likely not the result of a diminution of the driving force between the two organelles. Following both the chronic (Ppif\(^ {−/−} \)) and acute (NIM811) inhibition of CypD, we showed that cardiomyocytes exhibited a slower cytosolic Ca\(^ {2+} \) transient decay (Figure 4A), indicative of a slower SR Ca\(^ {2+} \) uptake, which in turn may explain the observed reduction in SR Ca\(^ {2+} \) content (Figure 4E). The reduction in SR Ca\(^ {2+} \) content can by itself explain the reduction in SR Ca\(^ {2+} \) transient (Figure 4A-B) since the RyR2-dependent release process does not seem to be affected (Figure 4D). One may question whether the reduced SR Ca\(^ {2+} \) content may further contribute to the limitation in SR to mitochondria Ca\(^ {2+} \) transfer. Such mechanism remains to be investigated in depth in future studies. In summary, we propose that the inhibition of CypD by first limiting the Ca\(^ {2+} \) transfer from SR to mitochondria through the VDAC1/Grp75/IP3R1 complex might create a metabolic uncoupling between SR and
mitochondria, which subsequently results in a deficient uptake of Ca²⁺ by SR, possibly contributing to the disturbances in Ca²⁺ homeostasis. Elrod et al. recently reported that cyclosporine could increase Ca²⁺ uptake by mitochondria²⁷. This apparent discrepancy with our results may be due to different experimental preparations and techniques of Ca²⁺ measurements. Elrod used pericam to measure mitochondrial Ca²⁺ in neonatal rat cardiomyocytes whereas we used Rhod2 either in H9c2 cardiomyoblasts or in adult mouse cardiomyocytes. Mitochondrial Ca²⁺ was measured after single-pulse field stimulation by Elrod, whereas we also used histamine stimulation of IP3R, meaning that we, but not Elrod, specifically addressed the transfer of Ca²⁺ from ER to mitochondria through the VDAC/Grp75/IP3R1 complex. Estimation of the efficiency of mitochondrial Ca²⁺ uptake would have required that we had simultaneously measured cytosolic and mitochondrial calcium; it would have still be difficult to separate the effect of CypD inhibitors on either its energetic or calcium channeling properties.

Upon ischemia and reperfusion, the accumulation of Ca²⁺ into mitochondria can trigger PTP opening and cell death²⁸. Ca²⁺ crosstalk between ER and mitochondria has been involved in myocardial reperfusion injury²⁹. We hypothesized that at least part of the death signal represented by mitochondrial Ca²⁺ overload would come from the ER, and that what we had observed under normoxic conditions would be of significant relevance upon the challenge of hypoxia-reoxygenation injury. The interactions among all the partners of the CypD/VDAC1/Grp75/IP3R1 complex were significantly increased after HR and associated with mitochondrial Ca²⁺ overload and cell death. Pharmacological inhibition of CypD by NIM811 administered at the time of reoxygenation prevented the increased interaction between the partners of the IP3R1 Ca²⁺ channeling complex and attenuated the mitochondrial Ca²⁺ overload. This suggests that the enhanced contact between ER to mitochondria and the subsequent
increased transfer of Ca$^{2+}$ through the VDAC1/Grp75/IP3R1 complex during reoxygenation following a prolonged hypoxic insult may contribute to lethal cardiomyocyte injury. Obviously, one may object that the reduction of cell death following inhibition of CypD was due to the sole inhibition of PTP opening within mitochondria. Whereas this point is very difficult to clarify as CypD-independent inhibitors of PTP would also reduce mitochondrial Ca$^{2+}$ overload and cell death, different observations are against this proposal. First, the pharmacological inhibition or genetic ablation of CypD caused disturbances in ER Ca$^{2+}$ homeostasis even under normoxic conditions, i.e. when the PTP is not formed. Second, the mitochondrial CypD is located into the matrix under normoxic conditions, and needs to be translocated to the inner membrane to trigger PTP opening upon hypoxia-reoxygenation. In contrast, it appears that the sub-fraction of CypD located at MAMs is already bound to the VDAC1/Grp75/IP3R1 complex under normoxic conditions. The reason for that is unclear; however, one may hypothesize that the likely high local concentration of Ca$^{2+}$ in the vicinity of the mitochondrial side of the VDAC1/Grp75/IP3R1 channel, favours the binding of local CypD to the inner mitochondrial membrane. Third, we demonstrated that the inhibition of CypD reduced the number of CypD/VDAC1/Grp75/IP3R1 complexes formed during hypoxia-reoxygenation and significantly attenuated Ca$^{2+}$ accumulation into mitochondria. Most importantly, non CypD-related reduction of ER-mitochondria interactions by pharmacological inhibition of IP3R or genetic loss of function of either Grp75 or Mfn2 produced very comparable effects than inhibition of CypD in terms of reduction of Ca$^{2+}$ flux from ER to mitochondria and cell death. There is also no evidence that either protein is linked to the PTP. This strongly suggests that the VDAC1/Grp75/IP3R1 complex plays a major role in hypoxia-reoxygenation injury through the Ca$^{2+}$ transfer from ER to mitochondria. It indirectly indicates that CypD is one target within this complex, apart from the PTP, that might
be used to prevent mitochondrial Ca\(^{2+}\) overload during hypoxia-reoxygenation. With this paradigm, inhibition of CypD would protect against ischemia-reperfusion injury via two synergistic actions (Supplemental Figure 9): 1) the *upstream* limitation of mitochondrial Ca\(^{2+}\) overload by the reduction of Ca\(^{2+}\) transfer from ER to mitochondria, and 2) the *downstream* prevention of PTP formation via the limitation of CypD binding to the inner mitochondrial membrane. This is consistent with a previous report suggesting that Ca\(^{2+}\) cycling from ER to mitochondria may directly favor the PTP\(^{30}\). This is also in agreement with reports showing that some modulators of apoptosis act by modifying Ca\(^{2+}\) transfer from ER to mitochondria and subsequent PTP opening\(^{4,31-33}\).

In summary, our work identifies a new role for CypD as a partner of the VDAC1/Grp75/IP3R1 complex at the contact sites between ER and mitochondria, contributing to the Ca\(^{2+}\) crosstalk between the two organelles. It shows that this Ca\(^{2+}\) transfer complex plays a major role in lethal reoxygenation injury, and thereby identifies new molecular targets for cardioprotection.

**Acknowledgments:** We thank Elisabeth Errazuriz and Christophe Vanbelle for their technical help and advices at the CeCILE Imaging Center (Lyon, France), Claudine Kieda for the gift of the H9c2 cells and Fabien Van Coppenolle and Rizwan Alam for their helpful and critical discussions on the manuscript.

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**Conflict of Interest Disclosures:** None.
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Figure Legends:

Figure 1. CypD is involved in the VDAC/Grp75/IP3R1 complex at the ER-mitochondria interface. A) Detection of CypD by immunoblotting in subcellular fractionation of mice heart. H: total homogenate; ER: endoplasmic reticulum; pM: pure mitochondria; MAM: mitochondria-associated membranes. Serca2 and IP3R1 were used as ER markers, VDAC1, Grp75, ANT, COX IV and cytochrome c as mitochondrial markers. B) Two dimensional blue native separation of native heart extracts from WT mice. The blue native-page was calibrated based on the location of the mitochondrial respiratory chain complexes isolated from mouse heart mitochondria, separated by electrophoresis simultaneously with the total mouse heart lysate. CypD was present in varying amounts in complexes which have a wide range of sizes, but more particularly in a high molecular weight complex where VDAC1, Grp75 and IP3R1 but not ANT were detected (see arrow). C) Immunoprecipitation of IP3R1 from mice heart homogenates specifically pulled down CypD, as well as VDAC1 and Grp75. D) Visualization of in situ interactions between CypD, VDAC1, Grp75 and IP3R1 using proximity ligation assay in H9c2 cells. Nuclei appear in blue and interactions between the two proteins are depicted in red.

Figure 2. CypD removal from the ER-mitochondria interface decreases protein interactions within the VDAC1/Grp75/IP3R1 complex. H9c2 cells were treated with NIM811 (2 μM) during 30 min, 2 h or 16 h and the interactions were analysed by proximity ligation assay, in order to quantify IP3R1/CypD (B), Grp75/IP3R1 (C) and Grp75/VDAC1 (D) interactions compared to the Vehicle (Veh). Panel A shows typical images after 2 h of treatment. Quantification of the Duolink PLA red fluorescent dots was performed using BlobFinder V3.2. Nuclei were stained
with Dapi. *p<0.05 vs. respective Veh, n=3 experiments. E) Typical images of in situ interactions between CypD, VDAC1, Grp75 and IP3R1 using proximity ligation assay in isolated adult cardiomyocytes from WT and Ppif<sup>-/-</sup> mice. Quantification in F) demonstrates a significant decrease in both VDAC1 and Grp75 interactions with IP3R1 in Ppif<sup>-/-</sup> cardiomyocytes. †p<0.05 vs. WT, n=6. G) Analysis of MAM composition after overnight treatment of mice with CsA or NIM (10mg/kg, IP). The corresponding bar graph (H) shows a significant decrease of the amount of both CypD and Grp75 (expressed as a ratio of VDAC content) in the MAM after in vivo CsA or NIM811 treatment. n=4.

**Figure 3.** Inhibition of CypD in H9c2 cells reduces the Ca<sup>2+</sup> transfer from ER to mitochondria through IP3R. A) Representative images of mitochondria staining by Rhod-2 and Mitotracker Green, showing 95% colocalization. B-C) Representative curves of the time course of mitochondrial Ca<sup>2+</sup> after histamine stimulation, after CypD down-regulation by siRNA (B) or CypD inhibition by NIM811 (C). Quantification of the maximal mitochondrial Ca<sup>2+</sup> mean fluorescence (F/F<sub>0</sub>) in (D), of the time constant of decay tau (E) and the time to peak (F) after histamine stimulation. n=8-12.

**Figure 4.** Ppif<sup>-/-</sup> cardiomyocytes display an alteration of Ca<sup>2+</sup> homeostasis. A-E) Ca<sup>2+</sup> transients in isolated WT (treated or not with 2μM NIM811) and Ppif<sup>-/-</sup> cardiomyocytes field stimulated at 1.0 Hz and visualized with Fluo-4 AM. A) Representative recordings expressed as ΔF/F<sub>0</sub>; B) quantification of averaged peak Ca<sup>2+</sup> transient amplitude; C) averaged decay time constant (Tau) of Ca<sup>2+</sup> transients; D) rate of rise of Ca<sup>2+</sup> transients; E) averaged SR Ca<sup>2+</sup> content estimated from the caffeine-induced peak in Ca<sup>2+</sup> transients. F) Representative images of patch-clamp
experiment with Rhod-2 loading in adult cardiomyocytes. Mitochondrial Ca\textsuperscript{2+} load was assessed by Rhod-2 after action potential induced- Ca\textsuperscript{2+} transients (G) or caffeine-induced SR Ca\textsuperscript{2+} release (H).

**Figure 5.** Prevention of mitochondrial Ca\textsuperscript{2+} overload through IP3R by NIM811 and 2-APB after hypoxia-reoxygenation. Hypoxia (3hrs) followed by re-oxygenation (2hrs) (HR) was performed on H9c2 cells. A-B) Interactions between CypD/IP3R1 and Grp75/IP3R1 were studied after HR by in situ PLA. Panel A shows typical images of interactions between IP3R1 and CypD. B) Quantification of the interactions per cell was performed. HR induced an increased number of interactions when compared to Time Control cells (TC). Both NIM811 and 2-APB prevented this increase. C) Measurement of mitochondrial calcium by Rhod2 loading after 2 hours of reoxygenation. NIM811 and 2-APB attenuated the increase induced by HR. D) Cell death was measured by FACS analysis of propidium iodide staining. The mortality is presented as a percentage of PI-positive cells. Cell death was slightly but significantly reduced by NIM811 (2μM) and 2-APB (50μM). n=6.

**Figure 6.** Downregulation of Grp75 protects from cell death after hypoxia-reoxygenation. A) Representative in situ PLA images of Grp75/IP3R1 interactions. B) Quantification of CypD/IP3R1 and Grp75/IP3R1 interactions after Grp75 downregulation and HR. At baseline (TC), down-regulation of Grp75 caused a nonsignificant reduction of the interaction of CypD with IP3R1, and expectedly a significant reduction of the interaction of Grp75 with IP3R1. After HR, siGrp75 prevented the increased interactions between CypD and IP3R1 (non-significant trend) and between Grp75 and IP3R1 when compared to siCTL. C) Measurement of
mitochondrial calcium by Rhod2 loading after 2 hours of reoxygenation. D) Assessment of cell death after 3 hours of hypoxia followed by 2 hours of reoxygenation. Downregulation of Grp75 decreased cell death after HR as compared to control (siCTL). n=6.

**Figure 7.** Decreasing ER-mitochondria interactions by down-regulation of Mfn2 protects from cell death after hypoxia-reoxygenation. A) Quantification of CypD/IP3R1 and VDAC1/IP3R1 interactions after Mfn2 down-regulation and HR. At baseline (TC), down-regulation of Mfn2 tended (non-significantly) to decrease the interactions of both CypD and VDAC1 with IP3R1. After HR, siMfn2 significantly prevented the increased interactions between CypD, VDAC1 and IP3R1 when compared to siCTL. Inset shows Mfn2 level after 24 hours of transfection. B) Measurement of mitochondrial calcium by Rhod2 loading after 2h reoxygenation. Down-regulation of Mfn2 significantly decreased mitochondrial Ca^{2+} accumulation after HR when compared to control (siCTL). C) Assessment of cell death after 3 hours of hypoxia followed by 2 hours of reoxygenation. Down-regulation of Mfn2 decreased cell death after HR when compared to control (siCTL). n=6.

**Figure 8.** NIM811 decreases the interaction between the partners of the CypD/VDAC1/Grp75/IP3R1 complex and protects adult mice cardiomyocytes from cell death. A-B) Interactions between CypD/IP3R1 and VDAC1/IP3R1 were studied after HR by in situ PLA. Panel A shows typical images of interactions between CypD (top row) and VDAC1 (bottom row) with IP3R1. B) Quantification of interactions per cell. HR increased the interactions between CypD and IP3R1 and between VDAC and IP3R1 when compared to Time Control cells (TC). NIM811 prevented this increase. C) Assessment of cell death after 45min of
hypoxia followed by 2hrs of reoxygenation. NIM811 significantly decreased cell death after HR. n=6. Quantification of VDAC1/IP3R1 and Grp75/IP3R1 interactions (D) and assessment of cell death (E) after HR in WT and Ppif<sup>−/−</sup> mice cardiomyocytes. Ppif<sup>−/−</sup> cardiomyocytes displayed decreased interactions between VDAC1/Grp75/IP3R1 together with a significantly reduced cell death. n=6.
Figure 3

A

Rhod2  
Mitotracker Green  
Merge

10 μm

B

Histamine

siCTL  
siCypD

Time (s)

C

Histamine

Veh  
NIM

Time (s)

D

Maximal Mitochondrial 
Ca²⁺ Peak (ΔF/F₀)

siCTL  
siCypD  
Veh  
NIM811

p=0.0540  
p=0.0532

E

τ (s)

siCTL  
siCypD  
Veh  
NIM811

p=0.0571  
p=0.002

F

Time to Peak (s)

siCTL  
siCypD  
Veh  
NIM811
Figure 4
Figure 5

A

TC

HR

HR+NIM

HR+2APB

CypD/IP3R1

B

Interactions/cell (fold vs TC)

p<0.001

p<0.001

p<0.001

CypD/IP3R1

Grp75/IP3R1

TC

HR

HR+NIM

HR+2-APB

C

[Ca\textsuperscript{2+}][\textsubscript{m}] (fold of TC)

p<0.001

p=0.049

TC

TC+NIM

TC+2-APB

HR

HR+NIM

HR+2-APB

D

P:\textsuperscript{+} positive cells (fold of HR)

p<0.001

p<0.001

p<0.001

TC

HR

HR+NIM

HR+2-APB
Figure 6

A

siCTL
Grp75/IP3R1
siGrp75

TC

siCTL
Grp75/IP3R1
siGrp75

B

<table>
<thead>
<tr>
<th></th>
<th>CypD/IP3R1</th>
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<td>0.002</td>
<td>&lt;0.001</td>
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<tr>
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D

p=0.043

p<0.001

p<0.001

p<0.001

Δ siCTL
Δ siGrp75

CA_2^{+} (fold of TC)

Pp-positive cells (fold of HR)
Figure 8

A

Negative control

TC

HR

HR+NIM

CypD/IP3R1

VDAC1/IP3R1

B

C

p<0.001

p=0.002

p=0.001

p=0.006

CypD/IP3R1

VDAC1/IP3R1

p=0.004

p=0.005

TC

HR

HR+NIM

% PI-positive cells

D

E

p=0.005

p=0.016

WT

Ppif<sup>-/-</sup>

Interactions/cell (fold vs WT)

% PI-positive cells

VDAC1/IP3R1

Grp75/IP3R1

WT

Ppif<sup>-/-</sup>
Depressing Mitochondria-Reticulum Interactions Protects Cardiomyocytes from Lethal Hypoxia-Reoxygenation Injury
Melanie Paillard, Emily Tubbs, Pierre-Alain Thiebaut, Ludovic Gomez, Jeremy Fauconnier, Claire Crola Da Silva, Geoffrey Teixeira, Nathan Mewton, Elise Belaidi, Annie Durand, Maryline Abrial, Alain Lacampagne, Jennifer Rieusset and Michel Ovize

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DEPRESSING MITOCHONDRIA-RETICULUM INTERACTIONS PROTECTS CARDIOMYOCYTES FROM LETHAL HYPOXIA-REOXGENATION INJURY

Melanie Paillard, Emily Tubbs, Pierre-Alain Thiebaut, Ludovic Gomez, Jeremy Fauconnier, Claire Crola Da Silva, Geoffrey Teixeira, Nathan Mewton, Elise Belaidi, Annie Durand, Maryline Abrial, Alain Lacampagne, Jennifer Rieusset, Michel Ovize

SUPPLEMENTAL MATERIAL
SUPPLEMENTAL METHODS

Isolation of mitochondria-associated membranes (MAM)

Isolation of the MAM fraction was performed by differential ultracentrifugation as previously described. The heart was homogenized with a glass potter in Isolation Buffer (225mM mannitol, 75mM sucrose, 0.5% BSA, 0.5mM EGTA and 30mM Tris-HCl, pH 7.4) and then centrifuged twice at 740g for 5min to remove nuclei and cellular debris. The supernatant was centrifuged at 9000g for 10min to pellet crude mitochondria which were re-suspended in Mitochondria Resuspending Buffer (MRB: 250mM mannitol, 5mM Hepes and 0.5mM EGTA, pH 7.4). Crude mitochondria were further purified through a percoll medium at 95000g for 30min in a SW40 rotor (Beckman). Purified mitochondria were localized at the bottom of the tube as a dense band whereas MAM was a diffused white band above the mitochondria. Pure mitochondria were washed twice by centrifugation at 6300g for 10min and resuspended in MRB. To further purify the MAM fraction, MAM were diluted in MRB and centrifuged at 6300g for 10min to remove any contaminating mitochondria. MAM supernatant was then centrifuged at 100000g for 1h in a 70 Ti rotor (Beckman) and the resulting pellet was resuspended in MRB. Resulting supernatant after crude mitochondria centrifugation was further centrifuged at 20000g for 30min followed by 100000g for 1h to pellet the endoplasmic reticulum (ER). Protein content was assayed by the Lowry method and 30µg of proteins from each fraction was loaded on a 4-15% SDS gel (Biorad).

Blue-Native and SDS-PAGE 2D separation

Hearts from WT mice were gently homogenized three times at 6000 Hz for 10 seconds (Precellys 24, Ozyme), in CP1 buffer (100mM KCL, 50mM Mops, 5mM
MgSO₄, 1mM EGTA, 1mM ATP). To prepare “native” protein lysate, native heart homogenate was solubilized with 1% Lauryl Maltoside (Mitosciences) for 30 min at 4°C. To remove unsolubilized material, the lysates were centrifuged at 10,000 for 10 min. Protein concentration was determined by the Bradford method (Bio-Rad). Native page was performed according to manufacturers’ instructions (Invitrogen). Briefly, 80µg of proteins combined with 0.25% G-250 was loaded onto a 4-12% Criterion Bis-Tris gel (Biorad) for the first dimension. As an internal calibration, 20µg of mice heart mitochondria solubilized with 0.48% Lauryl Maltoside was analyzed. At the end of the first dimension, the gel strips were excised and reduced with 1X NuPage reducing agent in 1X NuPage LDS sample buffer (Invitrogen) for 15 min at RT. Then, cysteines alkylation was done with N, N-Dimethylacrylamide for 15 min at RT. Finally, the reaction was quenched for 15 min at RT with 20% ethanol in 1X LDS and 0.1X Reducing agent. Then, the equilibrated gel strip was applied to the second dimension on a 4-16% Criterion Bis-Tris gel, separated and transferred to the PVDF membrane.

**Cell culture and transfection**

SV40-transformed H9c2 rat cardiomyoblasts were obtained from Centre National de la Recherche Scientifique (CNRS) (C.Kieda, patent 99-16169, France). All cell culture reagents were obtained from Invitrogen. Cells were cultured at 37 °C under 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 mM glucose and supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were plated at a density of 13,000–16,000 cells/cm². Depending on the nature of experiments, H9c2 cells were plated either on 12 wells plates or in a 8-chamber glass slide (Millipore). N-methyl-4-isoleucine-cyclosporin (NIM811) (2µM, Novartis) was
dissolved in 100% Ethanol and used as a non-immunosuppressive and specific pharmaceutical inhibitor of CypD. Cells were also treated with IP3R inhibitors: 2-Aminoethoxydiphenyl borate (2-APB, 50µM) or XestospongionC (XestoC, 1nM) both dissolved in DMSO. Downregulation of CypD, Grp75 and Mfn2 (50nM, Qiagen) was performed using DharmaFECT transfection reagent (Thermo Scientific) according to the technical recommendations. Twenty-four hours post-transfection, the medium was removed and the cells were immediately lysed in appropriate lysis buffer and frozen at -20 °C until processing or used for further experiments.

**Electron microscopy**

Pellets of pure mitochondria, ER and MAM from WT heart were fixed in 2% glutaraldehyde for 2h at 4 °C, postfixed in 1% osmium tetroxide for 1h at 4 °C, dehydrated and embedded in Epon. The fraction was then cut using a RMC/MTX ultramicrotome (Elexience) and ultrathin sections (60-80nm) were mounted on copper grids, contrasted with 8% uranyl acetate and lead citrate, and observed with a Jeol 1200 EX transmission electron microscope (Jeol LTD) equipped with MegaView II high resolution TEM camera. Analysis was performed with Soft Imaging System (Eloïse SARL).

**Protein extracts and Western-blotting**

Total lysates of H9c2 were prepared in lysis buffer (containing 1% NP40, 20mM Tris-HCl, 138mM NaCl, 2,7mM KCl, 1mM MgCl₂, 5% Glycerol, 5mM EDTA, 1mM DTT) supplemented with a cocktail of protease inhibitors (Sigma, Aldrich) and phosphatases inhibitors (PhosStop, Roche Diagnostics). Equivalent amounts of total protein (50-100µg) were separated electrophoretically by SDS-PAGE (6%, 12% or 4-
15% ready gels, BioRad) and transferred to a PVDF membrane (Biorad). The later was blocked in 5% solution of dry milk in TBS-T for 1 h at RT, and probed overnight at 4°C using the following antibodies: IP3R1H80 (1/1000), VDAC1 (1/2500), Grp75 (1/1000), CypD (1/2500), Mfn2 (1/1000, Abcam) and Tubulin (1/500, Santa Cruz). Immunoblots were developed using the ECL plus chemiluminescence assay system (Amersham) with the GelDoc molecular Imager (Biorad). Quantification was performed with the Image Lab software (Biorad).

**Immunoprecipitation of IP3R1**

Heart homogenates (500 µg) were mixed overnight at 4°C with 2µg of anti-IP3R1 antibody or for control with rabbit IgG (Santa Cruz) in buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA, supplemented with protease and phosphatase inhibitors (Sigma and Roche, respectively). On the next day, the mixture was then incubated for 2 h at 4°C with Pure Proteome protein G magnetic beads (Millipore) and bound proteins were then eluted with Laemmli buffer.

**Duolink® Proximity Ligation in situ Assay (PLA)**

Antibodies used for the detection of protein–protein interactions were anti-IP3R1H80 (rabbit, Santa Cruz), anti-VDAC1 (rabbit, Calbiochem and mouse, Abcam), anti-CypD (mouse, Abcam), anti-Grp75 (mouse, Santa Cruz), anti-ANT (mouse, Calbiochem), anti-RyR2 (rabbit, Abcam) and anti-Serca2 (rabbit, Cell Signaling) at a concentration of 1/200. All antibodies were first confirmed by immunofluorescence (data not shown).

**Isolation of adult murine cardiomyocytes and Ca²⁺ measurements**
Briefly, WT and 
Ppif\textsuperscript{-/-} mice were anesthetized with sodium pentobarbital (70mg/kg) (Sanofi Santé Animale, France) and the heart was quickly harvested and retrogradely perfused at 37°C for 6-8min with a perfusion buffer (in mM: 113 NaCl, 4.7 KCl, 0.6 KH\textsubscript{2}PO\textsubscript{4}, 0.6 Na2HPO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 12 NaHCO\textsubscript{3}, 10 KHCO\textsubscript{3}, 10 Heps, 30 Taurine, pH 7.4) containing 10mM 2,3-Butanedione, 5.5mM Glucose, 12.5µM CaCl\textsubscript{2}, 0.1mg/mL of liberase (Roche) and 0.14mg/ml trypsin (Sigma). Isolated myocytes were then transferred on laminin (10µg/ml) precoated dishes with M199 medium (Invitrogen) and allowed to attach for 2h in humidified 5%CO\textsubscript{2}–95% air at 37°C and then washed once to remove unattached cells before being used.

Analysis of Ca\textsuperscript{2+} transients was performed on different parameters: amplitude of Ca\textsuperscript{2+} transients (change in fluorescence ΔF was divided by the fluorescence F\textsubscript{0} detected before the electrical stimulation pulses), rising phase of Ca\textsuperscript{2+} transients (normalizing the peak amplitude over the time to peak) as an index of ryanodine receptor (RyR) function and the decay of electrically stimulated Ca\textsuperscript{2+} transients as an index of sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) activity.

For patch-clamp measurements, pipettes (2-3MΩ) were filled with recording solutions, in mM: KCl 130, HEPES 25, ATP(Mg) 3, GTP(Na) 0.4, EGTA 0.5, pH adjusted to 7.2 with KOH. The cardiomyocytes were perfused with Tyrode’s solution. Action potentials (APs) were elicited by 0.2ms current injections of supra-threshold intensity. Cells were stimulated routinely at 0.1Hz until APs stabilized. Data acquisition and analyses were performed using PClamp version 10.1 (Axon Instruments).

Measurement of membrane potential in H9c2
H9c2 cells were loaded with 10nM TMRM (Molecular Probes) for 40min at 37°C, and then followed by 20min washout before recordings with a Zeiss LSM 780 bi-photon confocal microscope (Zeiss, Le Pecq France) equipped with a 63X lens (oil immersion, Numerical Aperture, NA=1.4). Analysis was performed on at least 5 different cells per field.

SUPPLEMENTAL BIBLIOGRAPHY

Depressing mitochondria-reticulum interactions protects cardiomyocytes from lethal hypoxia-reoxygenation injury
Melanie Paillard, Emily Tubbs, Pierre-Alain Thiebaut, Ludovic Gomez, Jeremy Fauconnier, Claire Crola Da Silva, Geoffrey Teixeira, Nathan Mewton, Elise Belaidi, Annie Durand, Maryline Abrial, Alain Lacampagne, Jennifer Rieusset, Michel Ovize
Supplemental Figure 1: Electron microscopy of ER, pure mitochondria (pM) and MAM fraction. Subcellular fractions were Percoll-purified from WT heart as described, fixed, processed and analyzed by TEM. Bars, 0.5 μm.
Supplemental Figure 2: Simultaneous staining of mitochondrial network and CypD/IP3R1 interactions.
CypD/IP3R1 interactions (red dots) are aligned along mitochondria (stained in green), as shown by the merge. This suggests that this interaction likely occurs at the ER-mitochondria interface in the heart.
Supplemental Figure 3: Inhibition of IP3R in H9c2 cells alters the interaction of CypD with the VDAC1/Grp75/IP3R1 complex. Effect of 2-APB treatment (50 µM, 2h) and Xestospongin C (XestoC, 2µM, 2h) on interactions between CypD, Grp75 and IP3R1, using in situ PLA. n=4-5.
Supplemental Figure 4: Inhibition of the mitochondrial Na⁺/Ca²⁺ exchanger by CGP-37157 does not modify the decreased histamine-induced Ca²⁺ transfer to mitochondria in NIM-treated H9c2 cells. 
A) Representative curves of the time course of mitochondrial Ca²⁺ after histamine stimulation, in condition of inhibition by NIM811 (2µM) with or without CGP-37157 (10µM). Quantification of (B) the maximal mitochondrial Ca²⁺ mean fluorescence (F/F₀) and (C) the time constant of decay tau, after histamine stimulation. n=8.
Supplemental Figure 5: Inhibition of CypD by NIM811 or IP3R1 by 2-APB does not alter mitochondrial membrane potential.
Representative curves of the time course of mitochondrial membrane potential assessed by TMRM fluorescence after inhibition by 2 µM NIM811 (A) or 50 µM 2-APB (B), followed by 1 µM FCCP. C) Quantification of the TMRM mean fluorescence (F/F₀) after NIM and 2-APB stimulation. n=3 independent experiments with 4-5 cells/each.
Supplemental Figure 6: Increased ER-mitochondria interaction in the perinuclear region after HR. Representative images of CypD/IP3R1 interactions after HR, by in situ PLA.
Supplemental Figure 7: Downregulation of CypD prevents mitochondrial Ca\(^{2+}\) overload and protects against hypoxia-reoxygenation injury

Hypoxia (3h) followed by reoxygenation (2h) (HR) was performed on H9c2 cells. A) Interactions between CypD/IP3R1 and Grp75/IP3R1 were studied after HR by in situ PLA. At baseline (TC), depletion in CypD decreased interactions of IP3R1 with both CypD and Grp75. siCypD prevented the increased interactions after HR. Inset shows CypD protein level after 24h of transfection. B) Measurement of mitochondrial calcium by Rhod2 loading after 2h reoxygenation. siCypD prevented the increase after HR. C) Cell death was measured by FACS analysis of propidium iodide staining. The mortality is presented as a percentage of PI-positive cells. Cell death was reduced by downregulation of CypD (siCypD). n=6.
Supplemental Figure 8: Effect of Xestospongin C on mitochondrial Ca\(^{2+}\) and cell death after HR.
A) Measurement of mitochondrial calcium by Rhod2 loading after 2h reoxygenation. B) Assessment of cell death after 3h hypoxia followed by 2h reoxygenation. n=3.
Supplementary Figure 9: Proposed theoretical model for the regulation by CypD of the IP3R1 Ca\(^{2+}\) channeling complex during ischemia-reperfusion and cardioprotection. Under baseline conditions, physiological Ca\(^{2+}\) transfer from the reticulum to mitochondria occurs through the VDAC1/Grp75/IP3R1 complex which might be regulated by CypD. Ischemia-reperfusion stress leads to an increased formation of IP3R1 complexes, favoring mitochondrial Ca\(^{2+}\) overload and possibly cell death through PTP opening. CypD is present at the IP3R1 complex and also recruited upon reperfusion at the PTP. Beyond the direct inhibition of PTP opening, cardioprotection could be induced by preventing mitochondrial Ca\(^{2+}\) overload (and subsequent opening of the PTP) either through the inhibition of the partners of the IP3R1 Ca\(^{2+}\) channeling complex (including CypD) or by decreasing reticulum-mitochondria tethering.