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

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Background—Under physiological conditions, Ca\(^{2+}\) transfer from the endoplasmic reticulum (ER) to mitochondria might occur at least in part at contact points between the 2 organelles and involves the VDAC1/Grp75/IP3R1 complex. Accumulation of Ca\(^{2+}\) into the mitochondrial matrix may activate the mitochondrial chaperone cyclophilin D (CypD) and trigger permeability transition pore opening, whose role in ischemia/reperfusion injury is well recognized. We questioned here whether the transfer of Ca\(^{2+}\) 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 pharmacological inhibition of CypD in both H9c2 cardiomyoblasts and adult cardiomyocytes decreased the Ca\(^{2+}\) transfer from ER to mitochondria through IP3R under normoxic conditions. During hypoxia-reoxygenation, the interaction between CypD and the IP3R1 Ca\(^{2+}\) channeling complex increased concomitantly with mitochondrial Ca\(^{2+}\) content. Inhibition of either CypD, IP3R1, or Grp75 decreased protein interaction within the complex, attenuated mitochondrial Ca\(^{2+}\) overload, and protected cells from hypoxia-reoxygenation. Genetic or pharmacological inhibition of CypD provided a similar effect in adult mice cardiomyocytes. Disruption of ER-mitochondria interaction via the downregulation of Mfn2 similarly reduced the interaction between CypD and the IP3R1 complex and protected against hypoxia-reoxygenation injury.

Conclusions—Our data (1) point to a new role of CypD at the ER-mitochondria interface and (2) suggest that decreasing ER-mitochondria interaction at reperfusion can protect cardiomyocytes against lethal reperfusion injury through the reduction of mitochondrial Ca\(^{2+}\) overload via the CypD/VDAC1/Grp75/IP3R1 complex. (Circulation. 2013;128:1555-1565.)

Key Words: calcium ■ ischemia ■ reperfusion ■ sarcoplasmic reticulum

Molecular Cardiology

Mitochondria and the endoplasmic reticulum (ER) are separately considered key players in cell death signaling.\(^1\) Mitochondria and ER are interconnected organelles and form an endomembrane network. The contact points through which ER communicates with mitochondria are referred to as mitochondria-associated membranes (MAM).\(^3\) MAM are enriched in phospholipid- and glycosphingolipid-synthesis enzymes, as well as chaperone proteins, which transport lipids and exchange calcium between these 2 organelles.\(^4\) 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.\(^3,4\) One of them has identified a macromolecular complex composed of VDAC1, Grp75, and IP3R1 that regulates direct Ca\(^{2+}\) transfer from ER to mitochondria.\(^3\) Indeed, ER-mitochondria junctions are aligned with mitochondrial contact points where VDAC1 is abundantly present, thus creating a hot spot for the Ca\(^{2+}\) transfer from the ER.\(^4\) Although the role of this organelle cross talk is beginning to be understood in cell physiology, MAM involvement in cardiac pathologies remains unknown.

Clinical Perspective on p 1565

Calcium signaling is central for heart function through its physiological role in excitation-contraction coupling and the detrimental impact of Ca\(^{2+}\) overload during heart failure and myocardial ischemia/reperfusion. During this latter condition, it is well accepted that the cytosolic accumulation of Ca\(^{2+}\) subsequently results in mitochondrial Ca\(^{2+}\) overload,
which activates the matrix chaperone cyclophilin D (CypD) and triggers the opening of the permeability transition pore (PTP), leading to cell death.\textsuperscript{7} 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 2 organelles is highly ordered.\textsuperscript{8,9} It has also been suggested that mitochondrial Ca\textsuperscript{2+} overload could be mediated through IP3R.\textsuperscript{10}

We therefore questioned whether Ca\textsuperscript{2+} transfer from ER to mitochondria via the VDAC1/Grp75/IP3R1 complex might play a role in mitochondrial Ca\textsuperscript{2+} overload and subsequent cardiomyocyte death observed during the reperfusion phase after a sustained ischemic insult. We further questioned the relationship between CypD and VDAC1/Grp75/IP3R1 complex in the regulation of the Ca\textsuperscript{2+} exchange during ischemia/reperfusion. We report a new physiological role of CypD in ER-mitochondria Ca\textsuperscript{2+} overload with major impact on cardiomyocyte death on hypoxia-reoxygenation (HR).

Methods
For further details, see Methods in the online-only Data Supplement.

Animals
Ppif\textsuperscript{−/−} mice under C57Bl6/SV129 background were a gift from S.J. Korsmeyer's laboratory (Dana Farber Cancer Institute, Boston, MA).\textsuperscript{11} Both wild-type (WT) and Ppif\textsuperscript{−/−} 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 September 22, 2010 Council on the Protection of Animals and were approved by the local institutional animal research committee (No. BH2012-64).

Duolink Proximity Ligation In Situ Assay
Cells were fixed with 4% paraformaldehyde (10 minutes at room temperature) and permeabilized with the use of 0.1% Triton X-100 (15 minutes at room temperature). 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 3 times with TBS-0.05% Tween 20. Briefly, after incubation with primary antibodies, we applied combinations of corresponding in situ proximity ligation assay (PLA) probes for 1 hour at 37°C. The cells were washed with TBS-0.05% Tween 20, incubated for 30 minutes with ligase, and finally washed with TBS-0.05% Tween 20. Then cells were incubated with polymerase for 100 minutes. Finally, the cells were washed once with saline–sodium citrate 1x and then with saline–sodium citrate 0.1x. All reactions were performed at 37°C in a humid chamber with 30 µL of reaction mixture per well. Preparations were mounted in Duolink II mounting medium containing DAPI (Eurogentec). Fluorescence was analyzed with a Zeiss inverted fluorescent microscope equipped with an ApoTome, with the use of the AxioVision program.

Quantification of signals was performed with BlobFinder software (Center for Image Analysis, Uppsala University) and expressed as interactions per cell relative to the nontreated group. Experiments were performed at least 3 times, with a minimum of 5 fields taken per condition.

Ca\textsuperscript{2+} Measurements in H9c2 Cells
Ca\textsuperscript{2+} transfer from ER to mitochondria was assessed by recording mitochondrial Ca\textsuperscript{2+} changes in a native cell environment in the presence of extracellular calcium (phenol-free Dulbecco’s modified Eagle medium [DMEM] with 1.8 mmol/L CaCl\textsubscript{2}, and 10% FBS). Cells were plated in an 8-well chamber glass slide (LabTek, Dutsher). To measure mitochondrial Ca\textsuperscript{2+}, cells were incubated with Rhod2-AM (2 µmol/L; Invitrogen) in phenol-free DMEM with 0.03% pluronic acid (Sigma) for 1 hour 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 biphoton confocal microscope (Zeiss; Le Pecq, France) equipped with a ×63 lens (oil immersion; numeric aperture=1.4). Rhod2 fluorescence signals were recorded at 30°C by excitation at 561 nm and measurement of the emitted light at 585 nm. 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 with the use of enzymatic digestion according to a previously described procedure.\textsuperscript{12} Cardiomyocytes were loaded for 20 minutes at 37°C with Fluo4-AM (5 µmol/L; Invitrogen). To measure Ca\textsuperscript{2+} transients, cardiomyocytes were field-stimulated at 1 Hz with a current pulse delivered via 2 platinum electrodes. Changes in Fluo4 fluorescence were recorded at 25°C with the use of an LSM510 Meta confocal microscope equipped with a ×63 water-immersion objective (numeric aperture=1.2; line-scan mode=1.5 ms per line; Zeiss). Scanning was performed along the long axis of the cell.

To measure mitochondrial Ca\textsuperscript{2+}, cardiomyocytes were loaded for 40 minutes at 37°C with Rhod2-AM (5 µmol/L; Tellabs, Austin, TX). Cardiomyocytes were stimulated under action potential–induced clamp conditions with the use of a whole-cell patch-clamp technique at room temperature with an Axopatch 200B (Axon Instruments), as described previously.\textsuperscript{13}

Cellular Model of HR and Analysis of Cell Death and Mitochondrial Ca\textsuperscript{2+} Concentration
H9c2 cardiomyoblasts were subjected to 3 hours of hypoxia followed by 2 hours of reoxygenation at 37°C. Cells were randomized to receive either no additional intervention (HR group), 1 µmol/L NIM811 (HR+NIM811 group), or 50 µmol/L 2-APB (HR+2-APB group), administered at the onset of reoxygenation. To simulate hypoxia, the cell culture medium was replaced with Tyrode solution containing the following (in mmol/L): 130 NaCl, 5 KCl, 10 HEPES, 1 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, at pH 7.4/37°C: 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 chamber at 37°C by replacing the ischemia medium for 2 hours with DMEM supplemented with 10% FBS. The time control group consisted of cells without the hypoxic stimulus kept in the reoxygenation buffer. Cell death was determined after loading with 1 µg/mL propidium iodide, which permeates only the damaged cells, and was measured by flow cytometry (FACSCalibur, BD) at the end of the 2-hour reoxygenation period. A similar protocol was used for isolated adult cardiomyocytes except that the duration of hypoxia was reduced to 45 minutes and cell death was measured by counting propidium iodide–positive cells with the use of microscopy after the 120-minute reoxygenation period. For mitochondrial Ca\textsuperscript{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 542 nm and an emission wavelength of 590 nm. Intensity was normalized by cell density measured with Crystal Violet staining.

Statistical Analysis
Data are expressed as mean±SEM. Experiments were performed at least 3 times, in duplicates or more. Statistical analysis was performed with GraphPad Prism software with the use of nonparametric tests. Comparisons among ≥2 groups were analyzed by the Kruskal-Wallis test. When the Kruskal-Wallis test was significant, the Dunn
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 Ca2+ Channeling Complex in the Heart

We first questioned whether CypD could be present at the heart MAM interface by adapting the protocol of MAM isolation from the liver to the heart.14 Absence of cross-contamination and purity of each fraction was assessed by both electron microscopy (Figure I in the online-only Data Supplement) and Western blot (Figure 1A). As expected, CypD was concentrated in pure mitochondria and absent in the ER. However, a small fraction of CypD was also detected in the MAM fraction, together with IP3R1, Grp75, and VDAC1. To determine whether these proteins could form a macromolecular complex, we performed 2-dimensional blue native polyacrylamide gel electrophoresis separation of heart homogenates. CypD was present in varying amounts in complexes that have a wide range of sizes but more particularly in a high-molecular-weight complex in which VDAC1, Grp75, and IP3R1, but not ANT, were detected (Figure 1B). A specific interaction of CypD with the IP3R1 channeling complex was also demonstrated by IP3R1 immunoprecipitation in mice heart homogenates (Figure 1C). To further confirm the involvement of CypD in the IP3R1 channeling complex, we monitored in situ protein-protein interactions at the 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 and 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 Ca2+ channeling proteins, such as Serca2 and RyR2, or 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 (Figure II in the online-only Data Supplement).

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. To understand the role of CypD in the MAM, we performed at different time points (30 minutes, 2 hours, and 16 hours) 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 whether it might change this protein localization in the MAM of H9c2 cells. As shown in Figure 2A and 2B, NIM811 treatment significantly decreased the interactions between CypD and IP3R1 after 2 and 16 hours 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 30 minutes after NIM811 treatment (Figure 2C), whereas interactions between Grp75 and VDAC1 diminished only after 2 hours of NIM811 treatment (Figure 2D).

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

Alternatively, the inhibition of IP3R by either 2-APB or XestoC also decreased the interactions of IP3R1 with CypD and Grp75 (Figure III in the online-only Data Supplement).

**CypD Controls the Ca²⁺ Transfer From ER to Mitochondria Through IP3R1**

Given that the VDAC1/Grp75/IP3R1 complex has been shown to directly control the Ca²⁺ transfer from ER to mitochondria, we hypothesized that CypD would play a role in this Ca²⁺ exchange between the 2 organelles. Assessment of specific mitochondrial Ca²⁺ loading with Rhod2 was performed on H9c2 cells after histamine stimulation, which is known to induce Ca²⁺ release from ER stores (Figure 3A). In untreated H9c2, histamine rapidly increased mitochondrial Ca²⁺ levels, reflecting IP3R-mediated Ca²⁺ transfer from ER to mitochondria. Both the downregulation of CypD expression by specific short interfering RNA (siRNA) and the pharmacological inhibition of CypD by NIM811 led to a nonsignificant reduction of the amplitude of the histamine-stimulated increase of mitochondrial Ca²⁺ compared with control (Figure 3B through 3D). Inhibition of CypD also accelerated the decay time, as

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Cyclophilin D (CypD) removal from the endoplasmic reticulum–mitochondria interface decreases protein interactions within the VDAC1/IP3R1/IP3R1 complex. H9c2 cells were treated with NIM811 (NIM; 2 µmol/L) for 30 minutes, 2 hours, or 16 hours, and the interactions were analyzed by proximity ligation assay to quantify IP3R1/CypD (B), Grp75/IP3R1 (C), and Grp75/VDAC1 (D) interactions compared with vehicle (Veh). A shows typical images after 2 hours of treatment. Quantification of the Duolink proximity ligation assay red fluorescent dots was performed with the use of BlobFinder version 3.2. Nuclei were stained with DAPI. *P<0.05 vs respective vehicle; n=3 experiments.

E. Typical images of in situ interactions between CypD, VDAC1, Grp75, and IP3R1 with proximity ligation assay in isolated adult cardiomyocytes from wild-type (WT) and Ppif−/− mice. Quantification in F demonstrates a significant decrease in both VDAC1 and Grp75 interactions with IP3R1 in Ppif−/− cardiomyocytes. *P<0.05 vs WT; n=6.

G. Analysis of mitochondria-associated membrane composition after overnight treatment of mice with cyclosporin A (CsA) or NIM811 (10 mg/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 cyclosporin A or NIM811 treatment; n=4.
depicted by the decreased tau, but did not significantly modify the time of rise of mitochondrial Ca\textsuperscript{2+} (Figure 3E and 3F). Therefore, to rule out an effect on the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, we analyzed the effect of its inhibitor, CGP-37157, on the histamine-induced Ca\textsuperscript{2+} transfer after NIM811 treatment (Figure IVA in the online-only Data Supplement). In H9c2 cells treated with NIM811, we observed a similar decrease of Ca\textsuperscript{2+} transfer to mitochondria with prevention of the increase in tau after histamine stimulation when the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is inhibited (Figure IVB and IVC in the online-only Data Supplement). Importantly, neither the inhibition of CypD by NIM811 nor the inhibition of IP3R1 by 2-APB caused any significant change in the mitochondrial membrane potential, as assessed by tetramethylrhodamine methyl ester (Figure V in the online-only Data Supplement).

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

To confirm the involvement of CypD in vivo in the Ca\textsuperscript{2+} transfer from SR to mitochondria, we measured mitochondrial Ca\textsuperscript{2+} load by Rhod2 in isolated cardiomyocytes from WT and Ppif\textsuperscript{−/−} mice under patch-clamp conditions (Figure 4F). Ppif\textsuperscript{−/−} cardiomyocytes exhibited a significant reduction of mitochondrial Ca\textsuperscript{2+} loading after both action potential–induced Ca\textsuperscript{2+} transients and caffeine-induced SR Ca\textsuperscript{2+} release (Figure 4G and 4H). Altogether, these data strongly suggest that CypD is involved in the SR-mitochondria Ca\textsuperscript{2+} coupling in the heart through the VDAC1/Grp75/IP3R1 complex. This SR-mitochondria Ca\textsuperscript{2+} coupling may also contribute to modulate the global Ca\textsuperscript{2+} homeostasis and excitation-contraction coupling in cardiomyocytes.

**Inhibiting the CypD/VDAC1/Grp75/IP3R1 Complex Protects From HR Injury**

On the basis of these results, we hypothesized that the IP3R1-dependent Ca\textsuperscript{2+} transfer could also play a role in the mitochondrial Ca\textsuperscript{2+} overload observed during ischemia/reperfusion.
and thus may be a new pharmacological target to prevent ischemia/reperfusion injury. 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 4B and Figure VI in the online-only Data Supplement). These increased protein-protein interactions were associated with a higher mitochondrial Ca\(^{2+}\) load (Figure 5C) and a significant increase of cell death compared with time control cells (Figure 5D).

Interestingly, treatment at reoxygenation with either 2 \(\mu\)mol/L NIM811 or 50 \(\mu\)mol/L 2-APB reduced CypD/IP3R1 interactions and inhibited HR-induced mitochondrial Ca\(^{2+}\) overload and cell death (Figure 5A through 5D). Similar observations were made after genetic downregulation of CypD (Figure VII in the online-only Data Supplement) or treatment with xestospongin C, another inhibitor of IP3R (Figure VIII in the online-only Data Supplement). To confirm the involvement of CypD and the VDAC1/Grp75/IP3R1 complex during HR injury, we downregulated another partner of this complex, Grp75.
Under baseline conditions (time control), the downregulation 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 through 6C). 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 compared with control (Figure 6A through 6C). 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 attenuates mitochondrial Ca\(^{2+}\) loading and subsequent lethal cell injury.

**Downregulation of Mfn2 Protects H9c2 Cells From Lethal HR 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.\(^3\,8\) Downregulation of Mfn2 reduced the interactions between both CypD and VDAC1 with IP3R1 at baseline but also after HR (Figure 7A). In addition, downregulation 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 determined 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 between CypD and VDAC1/Grp75/IP3R1 by in situ PLA showed that HR significantly increased the interaction between the partners of the IP3R1 Ca\(^{2+}\) channeling complex, concomitant with 45\% of cell death as assessed by propidium iodide staining (Figure 8A through 8C), in agreement with our data in H9c2 cells. Treatment at reoxygenation with 1 \(\mu\)mol/L NIM811 significantly reduced the interaction between VDAC1 and IP3R1 and tended nonsignificantly to reduce the CypD/IP3R1 interactions while significantly decreasing HR-induced cell death (Figure 8A through 8C). Importantly, \(Ppif^{-/-}\) cardiomyocytes displayed a significant reduction of both VDAC1/IP3R1 and Grp75/IP3R1 interactions after HR, concomitant with reduced cell death (Figure 8D and 8E).

**Discussion**

We report that the mitochondrial chaperone CypD modulates ER-mitochondria Ca\(^{2+}\) crosstalk via the VDAC1/Grp75/IP3R1 complex. This modulation plays an important role in HR injury. Pharmacological or genetic inhibition of each partner of the complex modified the Ca\(^{2+}\) fluxes and blunted lethal reoxygenation injury. Decreasing the tethering of ER to mitochondria by downregulation of Mfn2 also prevented mitochondrial Ca\(^{2+}\) overload and reduced cell death after HR injury. These findings provide 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, α-1 receptor, and promyelocytic leukemia.\(^3\,4\,16\,17\) Most of these proteins are ER proteins, with only a few (eg, 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\(^{2+}\)-sensitive chaperones. Interestingly, CypD is a Ca\(^{2+}\)-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\(^{2+}\) signaling 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 complex, 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, as a result of its ability to interact with the VDAC1/Grp75/IP3R1 complex. The mechanism by which CypD physically and functionally interacts with the VDAC1/Grp75/IP3R1 Ca\(^{2+}\) channeling 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 agreement 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 because both genetic and pharmacological 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 3 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 IP3R isoform 2 is recognized as the predominant isoform in the cardiac myocyte. However, most of the functions of IP3Rs are attributed to the type-1 isoform, and our results show that CypD preferentially interacts with IP3R isoform 1 because no interaction with IP3R isoform 2 was observed. One can wonder whether the impact of IP3R signaling plays an important role in the heart compared with the cardiac RyR2, which is the major Ca\(^{2+}\) release channel on the SR 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 excitation-transcription coupling in the normal heart but also in some pathologies such as 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 on 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 2 organelles. After 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 and 4B) because the RyR2-dependent release process does not seem to be affected (Figure 4D). One may question whether the reduced SR
Cross talk between ER and mitochondria has been involved in myocardial reperfusion injury. 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 for the challenge of HR injury. The interactions among all of 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 and mitochondria and the subsequent increased transfer of Ca2+ through the VDAC1/Grp75/IP3R1 complex during reoxygenation after a prolonged hypoxic insult may contribute to lethal cardiomyocyte injury. Obviously, one may object that the reduction of cell death after inhibition of CypD was attributable to the sole inhibition of PTP opening within mitochondria. Although this point is difficult to clarify because CypD-independent inhibitors of PTP would also reduce mitochondrial Ca2+ overload and cell death, different observations are against this proposal. First, the pharmacological inhibition or genetic ablation of CypD caused disturbances in ER Ca2+ homeostasis even under normoxic conditions (ie, when the PTP is not formed). Second, the mitochondrial CypD is located at the matrix under normoxic conditions and needs to be translocated to the inner membrane to trigger PTP opening on HR. In contrast, it appears that the subtraction 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 Ca2+ in the vicinity of the mitochondrial side of the VDAC1/Grp75/IP3R1 channel favors 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 HR and significantly attenuated Ca2+ 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 comparable effects to inhibition of CypD in terms of reduction of Ca2+ flux from ER to mitochondria and cell death. There is also no evidence that either protein is linked to the PTP. This strongly

Figure 7. Decreasing endoplasmic reticulum–mitochondria interactions by downregulation of Mfn2 protects from cell death after hypoxia-reoxygenation (HR). A, Quantification of cyclophilin D (CypD)/IP3R1 and VDAC1/IP3R1 interactions after Mfn2 downregulation and HR. At baseline (time control [TC]), downregulation of Mfn2 tended (nonsignificantly) to decrease the interactions of both CypD and VDAC1 with IP3R1. After HR, siMfn2 significantly prevented the increased interactions between CypD, VDAC1, and IP3R1 compared with control (siCTL). Insert shows Mfn2 level after 24 hours of transfection. B, Measurement of mitochondrial calcium by Rhod2 loading after 2 hours of reoxygenation. Downregulation of Mfn2 significantly decreased mitochondrial Ca2+ accumulation after HR compared with control (siCTL). C, Assessment of cell death after 3 hours of hypoxia followed by 2 hours of reoxygenation. Downregulation of Mfn2 decreased cell death after HR compared with control (siCTL); n=6. PI indicates propidium iodide.
suggests that the VDAC1/IP3R1 complex plays a major role in HR 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 HR. With this paradigm, inhibition of CypD would protect against ischemia/reperfusion injury via 2 synergistic actions (Figure IX in the online-only Data Supplement): (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/IP3R1 complex at the contact sites between ER and mitochondria, contributing to the Ca\(^{2+}\) cross talk between the 2 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.

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**Disclosures**

None.

**References**


**Figure 8.** NIM811 (NIM) decreases the interaction between the partners of the cyclophilin D (CypD)/VDAC1/Grp75/IP3R1 complex and protects adult mice cardiomyocytes from cell death. A and B, Interactions between CypD/IP3R1 and VDAC1/IP3R1 were studied after hypoxia-reoxygenation (HR) by in situ proximity ligation assay. 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 compared with time control cells (TC). NIM811 prevented this increase. C, Assessment of cell death after 45 minutes of hypoxia followed by 2 hours 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 wild-type (WT) and Ppif\(^{-/-}\) mice cardiomyocytes is shown. Ppif\(^{-/-}\) cardiomyocytes displayed decreased interactions between VDAC1/Grp75/IP3R1 together with significantly reduced cell death; n=6. PI indicates propidium iodide.
in the heart. In the present study, we demonstrated a new role for cyclophilin D, a well-known regulator of the mitochondrial and sarcoplasmic reticulum at the level of the IP3 receptor Ca2+ channeling complex protecting cardiomyocytes from lethal reperfusion injury.

Thus, there is still an obvious need for new therapeutics to prevent myocardial damage in acute ischemic syndromes. Accumulating evidence suggests that mitochondria–sarcoplasmic reticulum interactions are relevant to the adult heart despite the complex cellular localization of inositol 1,4,5-trisphosphate receptors that may modulate excitation-contraction coupling in the heart.

In ischemia/reperfusion, there is strong evidence for intracellular signaling of structurally diverse Ca2+ homeostasis proteins. However, the molecular mechanisms that mediate these responses have not been fully elucidated. One critical step in the cardioprotective response is the inhibition of apoptosis by agents such as cyclosporine. The cyclophilin D (CyP-D) member of the CyPs family, like other CyPs, contains a highly conserved peptidebinding domain (PBD) and a unique amino-terminal domain (ATD) that are important for regulating cell survival.

In the heart, inositol 1,4,5-trisphosphate receptors (IP3Rs) play a central role in mediating the release of Ca2+ from the sarcoplasmic reticulum (SR) into the cytosol. These receptors are composed of three subunits (α, β, γ) in a 1:1:1 stoichiometry and are expressed in different isoforms that vary in tissue distribution and function. The α-subunit is ubiquitous and is involved in receptor activation, while the β-subunit modulates receptor sensitivity, and the γ-subunit regulates receptor desensitization.

The IP3Rs are situated adjacent to mitochondria, suggesting a role in mitochondrial modulation. The opening of mitochondrial Ca2+ channels is a key event in the regulation of mitochondrial permeability transition (MPT) pore, which may lead to cell death. Thus, the MPT pore is a critical determinant of cell survival during ischemia/reperfusion.

Mitochondria and sarcoplasmic reticulum interact to regulate Ca2+ homeostasis, which is crucial for cell survival and function. However, the precise mechanisms underlying these interactions are not fully understood. The current understanding is based on several key observations:

1. **Mitochondria-SR Interaction:** The close proximity and physical tethering between mitochondria and SR are facilitated by the ER-mitochondrion (MAM) domain, which contains Mfn2 and Opa1 proteins.
2. **Mitochondrial Ca2+ Uptake:** Mitochondria take up Ca2+ from the cytosol to maintain their Ca2+ stores and protect from the cell death induced by cell Ca2+ overload.
3. **SR Ca2+ Uptake:** The SR pumps Ca2+ into the extracellular space, maintaining intracellular Ca2+ levels below toxic levels.
4. **Mitochondrial Ca2+ Release:** Mitochondria release Ca2+ during the MPT, which is an important mechanism for cell death.

Overall, the interplay between mitochondria and SR is a dynamic and complex process that is crucial for cell survival during ischemia/reperfusion. Further research is needed to fully understand the regulatory mechanisms and signaling pathways involved in these interactions, which may lead to the development of novel therapeutic strategies for myocardial infarction.
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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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 30min at 4°C. To remove unsolubilized material, the lysates were centrifuged at 10000 for 10min. 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 15min at RT. Then, cysteines alkylation was done with N, N-Dimethylacrylamide for 15min at RT. Finally, the reaction was quenched for 15min 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 13000–16000 cells/cm². Depending of 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 Xestospongicyn (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 ºC 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 ºC, postfixed in 1% osmium tetroxide for 1h at 4 ºC, 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 1h 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 Ca^{2+} measurements**
Briefly, WT and Ppi/- 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 KH2PO4, 0.6 Na2HPO4, 1.2 MgSO4, 12 NaHCO3, 10 KHCO3, 10 Heps, 30 Taurine, pH 7.4) containing 10mM 2,3-Butanedione, 5.5mM Glucose, 12.5µM CaCl2, 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%CO2–95% air at 37°C and then washed once to remove unattached cells before being used.

Analysis of Ca2+ transients was performed on different parameters: amplitude of Ca2+ transients (change in fluorescence ΔF was divided by the fluorescence F0 detected before the electrical stimulation pulses), rising phase of Ca2+ transients (normalizing the peak amplitude over the time to peak) as an index of ryanodine receptor (RyR) function and the decay of electrically stimulated Ca2+ transients as an index of sarco/endoplasmic reticulum Ca2+ 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 IP3RI, 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.