Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death

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Mitochondria play an important role in energy production, Ca2+ homeostasis and cell death. In recent years, the role of the mitochondria in apoptotic and necrotic cell death has attracted much attention. In apoptosis and necrosis, the mitochondrial permeability transition (mPT), which leads to disruption of the mitochondrial membranes and mitochondrial dysfunction, is considered to be one of the key events, although its exact role in cell death remains elusive. We therefore created mice lacking cyclophilin D (CypD), a protein considered to be involved in the mPT, to analyse its role in cell death. CypD-deficient mice showed a high level of resistance to ischaemia/reperfusion-induced cardiac injury. Our results indicate that the CypD-dependent mPT regulates some forms of necrotic death, but not apoptotic death.

The mitochondrial permeability transition (mPT) is a regulated Ca2+-dependent increase in the permeability of the mitochondrial membrane, which results in a loss of matrix potential (∆ψm), mitochondrial swelling and rupture of the outer membrane. The mPT is thought to occur after the opening of a channel, termed the permeability transition pore, which putatively consists of a voltage-dependent anion channel, an adenine nucleotide translocator, CypD, and some other molecule(s); however, an essential role for the adenine nucleotide translocator in the mPT is a matter of recent controversy.

CypD is a mitochondrial member of the cyclophilin family of peptidyl prolyl-cis, trans-isomerasers (PPIases) and has a crucial role in protein folding. It has been suggested that CypD is involved in regulating the mPT, on the basis of the observation that cyclophilin A (Csa), a specific inhibitor of cyclophilin family activity, blocks the mPT5. A Csa-insensitive mPT has also been suggested, although the molecular mechanism is completely unknown. It has been shown that some forms of apoptosis are significantly inhibited by CsA, suggesting a role for Csa-sensitive mPT in apoptosis. The mPT is also implicated in the remodelling of mitochondrial structure with mobilization of cytochrome c stores in cristae during apoptosis. To determine whether CypD has a crucial role in the Csa-sensitive mPT and to investigate whether the mPT is a key regulator of cell death, we created CypD-deficient mice by gene targeting (see Supplementary Fig. 1a–c). The absence of cyclophilin D protein in CypD-deficient mice was verified by western blotting (see Fig. 1a and Supplementary Fig. 1d). CypD-deficient mice were born at the

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an intradermal injection of 50 μg mBSA in Freund’s Complete Adjuvant at two sites near the base of the tail. Arthritis was induced on day 7, by intra-articular injection of 10 μl of 20 mg ml−1 mBSA directly into both knee joints. As a negative control, some mice received 10 μl mBSA in the right knee only, and 10 μl saline in the left knee; inflammation ensued in the mBSA-injected knee only. The mice were killed on day 10 (3 days after induction of arthritis, when argecin loss is maximal) and the knee joints were processed for histology. Toluidine blue stained slides, containing bone or cartilage from a single joint, were blinded and scored independently by five examiners. The slides were scored for argecin loss using a four-point scale, where 0 = no loss of staining intensity in non-calcified articular cartilage (NCAC) and calcified articular cartilage, 1 = localized decrease, but not total loss of staining in NCAC, 2 = widespread decrease in staining and localized complete loss of staining in NCAC, and 3 = widespread complete loss of staining in NCAC. The femur and the tibia were evaluated in each section. The sections were also assessed for evidence of cartilage fibrillation. The inflammatory and destructive parameters of synovitis, joint space exudates, and pannus-mediated erosion and bone destruction were scored on a scale from 0–3, with 3 being the maximum score. Total histological scores were analysed for significance using Mann-Whitney U-tests.

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expected mendelian frequency, developed normally, and did not have any detectable phenotypic anomalies.

To examine the role of CypD in the mPT, mitochondria were isolated from the livers of CypD-deficient mice and control littermates. The mitochondria showed no significant change in respiration rate in the absence of CypD (see Supplementary Fig. 2). As shown in Fig. 1a, PPIase activity was absent in CypD-deficient mitochondria, but not in control mitochondria, indicating that CypD is the major PPIase in the mitochondria. When control mitochondria were treated with 50 μM Ca²⁺, the mPT occurred, as shown by mitochondrial swelling (Fig. 1b) and loss of ΔΨ (Fig. 1c). These phenomena were not observed in CypD-deficient mitochondria (Fig. 1b, c). To examine the extent of mPT inhibition as a consequence of CypD-deficiency, successive doses of Ca²⁺ were added to the mitochondria. ΔΨ was lost after two additions of 50 μM Ca²⁺ to control mitochondria, but it was still maintained after seven additions of Ca²⁺ to CypD-deficient mitochondria (Fig. 1d). We investigated whether the absence of Ca²⁺-induced

![Figure 1 Absence of mPT in CypD-deficient (CypD -/-) mitochondria.](image)

a. Absence of CypD protein and PPIase activity in CypD -/- mitochondria. WT, wild type.

b. Absence of mPT in CypD -/- mitochondria. Isolated wild-type (WT, left column) or CypD -/- (right column) mitochondria were incubated with 50 μM Ca²⁺ in the presence (pink) or absence (blue) of 1 μM CsA, and monitored for (b) swelling (by light scatter) or (c) ΔΨ (by Rh123 intensity), see Methods. Loss of ΔΨ causes release of Rh123 from the mitochondria, resulting in increased Rh123 intensity.

d. e. CypD deficiency prevents Ca²⁺-induced ΔΨ loss without altering Ca²⁺ uptake. Isolated mitochondria were successively treated with 50 μM Ca²⁺ (indicated by arrowheads) in the presence (pink) or absence (blue) of 1 μM CsA, and ΔΨ (d) and extra-mitochondrial Ca²⁺ (e) were monitored.

**Figure 1** Absence of mPT in CypD-deficient (CypD -/-) mitochondria. a. Absence of CypD protein and PPIase activity in CypD -/- mitochondria. WT, wild type. b. Absence of mPT in CypD -/- mitochondria. Isolated wild-type (WT, left column) or CypD -/- (right column) mitochondria were incubated with 50 μM Ca²⁺ in the presence (pink) or absence (blue) of 1 μM CsA, and monitored for (b) swelling (by light scatter) or (c) ΔΨ (by Rh123 intensity), see Methods. Loss of ΔΨ causes release of Rh123 from the mitochondria, resulting in increased Rh123 intensity. d. e. CypD deficiency prevents Ca²⁺-induced ΔΨ loss without altering Ca²⁺ uptake. Isolated mitochondria were successively treated with 50 μM Ca²⁺ (indicated by arrowheads) in the presence (pink) or absence (blue) of 1 μM CsA, and ΔΨ (d) and extra-mitochondrial Ca²⁺ (e) were monitored.

f. Accumulation of Ca²⁺ in the mitochondria as a result of CypD deficiency. Mitochondria were incubated with the indicated concentrations of 45Ca²⁺ for 25 min and intra-mitochondrial Ca²⁺ was measured. Data shown as mean ± s.e.m. g. Lack of Ca²⁺-induced, but not rBid- and rBax-induced, cytochrome c release in CypD -/- mitochondria. WT, Bak -/- and CypD -/- mitochondria were incubated with Ca²⁺, rBid, and rBax at the indicated concentrations. After 30 min, samples were centrifuged and aliquots of supernatants were subjected to western blot analysis for cytochrome c. 'Total' represents the total amount of cytochrome c found in an equivalent aliquot of the mitochondria.
mPT in CypD-deficient mitochondria was due to disturbance of Ca\(^{2+}\) uptake. As shown in Fig. 1e, the extra-mitochondrial Ca\(^{2+}\) concentration increased transiently, and rapidly returned to basal levels after each successive addition of Ca\(^{2+}\) to CypD-deficient mitochondria, indicating normal Ca\(^{2+}\) uptake by the mitochondria. Up to concentrations of 500 \(\mu\)M Ca\(^{2+}\), most of the Ca\(^{2+}\) was taken up by CypD-deficient mitochondria (Fig. 1e, f). Next, we analysed CypD-deficient mitochondria in the presence of much higher concentrations of Ca\(^{2+}\), which can induce CsA-insensitive mPT. Addition of more than 1 mM Ca\(^{2+}\) induced swelling, collapse of \(\Delta \Psi\), and impaired Ca\(^{2+}\) uptake even in CypD-deficient mitochondria (Fig. 1f and Supplementary Fig. 3), and all of these events were insensitive to CsA, even when it was added to CypD-deficient mitochondria (see Supplementary Fig. 3). Taken together, these results suggest that the mPT induced by low doses of Ca\(^{2+}\) is completely inhibited in CypD-deficient mitochondria, and that CypD is not involved in the CsA-insensitive increase in membrane permeability induced by high doses of Ca\(^{2+}\). Furthermore, the addition of other mPT inducers, such as H\(_2\)O\(_2\) and atractyloside, did not trigger the mPT in CypD-deficient mitochondria (see Supplementary Fig. 4).

In many forms of apoptosis, BH3-domain-containing proteins of the Bcl-2 family (‘BH3-only’ proteins) transduce apoptotic signals to the mitochondria, and induce cytochrome c release in a Bax/Bak-dependent manner\(^{11}\). Whether the CsA-sensitive mPT is involved in apoptotic cytochrome c release is controversial\(^{12-15}\). To address this question, we added recombinantBid (rBid), one of the BH3-only proteins, to CypD-deficient and control mitochondria. As shown in Fig. 1g (top panel), CypD deficiency had no effect on rBid-induced cytochrome c release, which was different from the result in Bak-deficient mitochondria (Fig. 1g, bottom panel). Bad (another BH3-only protein, data not shown) and Bax (Fig. 1g; second panel), were also found to induce cytochrome c release equally in both CypD-deficient and control mitochondria. In contrast, Ca\(^{2+}\)-induced cytochrome c release was markedly reduced in CypD-deficient mitochondria (Fig. 1g; third panel). Interestingly, Bak deficiency did not have any effect on Ca\(^{2+}\)-induced cytochrome c release (Fig. 1g, bottom panel). Together, these data indicate that the mPT is involved in cytochrome c release induced by Ca\(^{2+}\), but not by pro-apoptotic BH3-only proteins or Bax.

The results described above raise the possibility that the mPT might be involved in cell death due to mPT inducers (including Ca\(^{2+}\) overload and reactive oxygen species), but is not involved in the common apoptotic pathway to which BH3-only proteins and Bax/Bak are committed. We first examined the responses of CypD-deficient cells to these death stimuli. As shown in Fig. 2a, control and CypD-deficient thymocytes underwent comparable levels of apoptosis when exposed to various apoptotic stimuli. Similar findings were also obtained when murine embryonic fibroblast cells (MEFs) and hepatocytes from CypD-deficient mice were treated with various apoptotic reagents (Fig. 2b, c), and when these cells were transfected with DNA encoding Bax or a truncated BH3-only protein, data not shown) and rBax (Fig. 1g; second panel). Bad (another BH3-only protein, data not shown) and Bax (Fig. 1g; second panel), were also found to induce cytochrome c release equally in both CypD-deficient and control mitochondria. In contrast, Ca\(^{2+}\)-induced cytochrome c release was markedly reduced in CypD-deficient mitochondria (Fig. 1g, third panel). Interestingly, Bak deficiency did not have any effect on Ca\(^{2+}\)-induced cytochrome c release (Fig. 1g, bottom panel). Together, these data indicate that the mPT is involved in cytochrome c release induced by Ca\(^{2+}\), but not by pro-apoptotic BH3-only proteins or Bax.

Next, we tested control and CypD-deficient MEFs for cell death after exposure to H\(_2\)O\(_2\). CypD-deficient MEFs were more resistant to H\(_2\)O\(_2\)-induced cell death than control cells as assessed by a CTB (cell titre blue) assay, which measures the metabolic activity of viable cells (Fig. 3a), and by Annexin-V staining (data not shown). H\(_2\)O\(_2\)-induced cell death is predominantly due to necrosis, based on the following observations: a lack of caspase activation (Fig. 3b), no effect of a caspase inhibitor (Fig. 3a), early disruption of the plasma membrane (Fig. 3c), and finally, no nuclear or oligonucleosomal DNA fragmentation (Fig. 3c and data not shown). Similar results for H\(_2\)O\(_2\)-induced cell death were also observed using CypD-deficient hepatocytes (Fig. 3d and data not shown).

We also examined the effect of CypD on cell death induced by A23187, a Ca\(^{2+}\) ionophore. CypD-deficient hepatocytes showed significant resistance to A23187-induced cell death (Fig. 3e). Like H\(_2\)O\(_2\)-induced cell death, A23187-induced cell death was not accompanied by caspase activation (Fig. 3f), so this type of...
Figure 3 Resistance of CypD<sup>−/−</sup> cells to necrosis induced by reactive oxygen species and Ca<sup>2+ </sup>overload. a–d, Reduction of H<sub>2</sub>O<sub>2</sub>-induced necrotic cell death by CypD deficiency. Wild-type (WT) and CypD<sup>−/−</sup> MEFS (a–c) and hepatocytes (d) were exposed to H<sub>2</sub>O<sub>2</sub> (a–c, 0.75 mM for 24 h; d, 0.5 mM for 4 h), and the extent of cell death was assessed by Ctb assay (a, d). zVAD is a pan-caspase inhibitor. e, Caspase activation in wild-type (black) and CypD<sup>−/−</sup> (grey) hepatocytes after treatment with 0.75 mM H<sub>2</sub>O<sub>2</sub> was assessed at 16 h. Treatment with VP16 (100 μM) for 16 h was used as a positive control for caspase activation. Data shown as mean ± s.e.m. f, Assessment of caspase activation in response to 2 μM A23187 (upper panel); treatment with 40 ng ml<sup>−1</sup> TNF-α and 10 μg ml<sup>−1</sup> CHX was used as a positive control for caspase activation (lower panel). Data shown as mean ± s.e.m. g–i, Reduced Δψ<sub>0</sub> loss in CypD<sup>−/−</sup> hepatocytes (preloaded with TMRM) treated with 10 μM A23187. TMRM fluorescence intensity was monitored by laser scanning confocal microscopy. Representative real-time images (g), average TMRM intensity of individual WT (blue) and CypD<sup>−/−</sup> (pink) cells (h), and the half-life time of the fluorescence intensity of individual cells (i) are shown. Data shown as mean ± s.d. The arrow in (h) indicates the addition of 5 μM CCCP, which completely dissipated Δψ<sub>0</sub>. j–l, Increased A23187-induced mitochondrial Ca<sup>2+ </sup>uptake by CypD<sup>−/−</sup> hepatocytes. Cells were loaded with Rhod2-AM and treated with 10 μM A23187. Rhod2 fluorescence intensity was monitored by laser scanning confocal microscopy. Representative images of baseline and peak fluorescence are shown (j). Average (k) and peak (l) fluorescence intensities of cells are shown (mean ± s.d.). In (i), hepatocytes were also loaded with Rhod2-AM in the presence of 1 μM Ru360.
death was also considered to represent necrotic cell death. These results indicate that CypD (and the CsA-sensitive mPT) is involved in necrotic cell death induced by reactive oxygen species or Ca\(^{2+}\) overload.

Suppression of A23187-induced cell death by CypD deficiency seemed likely to be due to inhibition of the mPT. To determine whether A23187-induced mPT is suppressed by CypD deficiency in cells, we monitored mitochondrial \(\Delta \Psi\) and \(\text{Ca}^{2+}\) accumulation in the presence of A23187. Hepatocytes were loaded with the \(\Delta \Psi\) markerstetramethylrhodamine methylester (TMRM) or Mito Tracker Orange CMTM Ros, and treated with A23187, after which the fluorescence intensity was monitored by real-time imaging. Addition of A23187 caused rapid loss of \(\Delta \Psi\) in control (wild-type) hepatocytes, whereas CypD-deficient hepatocytes maintained \(\Delta \Psi\) for much longer periods of time (Fig. 3g–i and Supplementary Fig. 5). The addition of CCCP (carbonyl cyanide m-chlorophenyl-hydrazone), a protonophore, completely dissipated \(\Delta \Psi\). Mitochondrial accumulation of \(\text{Ca}^{2+}\) was investigated using Rhod2-AM\(^{16}\). After A23187 treatment, CypD-deficient hepatocytes showed a rapid increase in Rhod2 fluorescence intensity, but wild-type hepatocytes showed only a marginal increase (Fig. 3j–l). The validity of using Rhod2 as an indicator of mitochondrial \(\text{Ca}^{2+}\) under these conditions was confirmed using Ru360, an inhibitor of the mitochondrial calcium uniporter (Fig. 3l). These results indicate that compared to control hepatocytes, CypD-deficient hepatocytes absorb a larger amount of cytosolic \(\text{Ca}^{2+}\) into their mitochondria without loss of mitochondrial \(\Delta \Psi\); this is consistent with the results obtained using isolated mitochondria, and suggests that A23187 induces CypD-dependent mPT in cells.

**Figure 4** Prevention of cardiac ischaemia/reperfusion injury in CypD\(^{-/-}\) mice. 

a–d, Absence of mitochondrial damage induced by anoxia/reoxygenation (A/R) in CypD\(^{-/-}\) mitochondria. Isolated mitochondria were treated without (Pre) or with anoxia for 30 min followed by reoxygenation for the indicated times. The \(\Delta \Psi\) (a), swelling (b), mAST release (c), and respiratory control ratio (state 3/state 4) (d) were measured. Data shown as mean ± s.e.m. e–h, Reduction of cardiac I/R injury in CypD\(^{-/-}\) mice.
Finally, we investigated the role of CypD in ischaemia/reperfusion (I/R) injury, in which disturbance of Ca\(^{2+}\) homeostasis and generation of reactive oxygen species have been implicated\(^2\). Many reports have described a protective effect of CsA against I/R injury\(^3\)–\(^8\). First, we investigated whether CypD-deficient mitochondria showed resistance to anoxia/reoxygenation-induced injury, which simulates I/R-induced injury in vivo. Isolated mitochondria from control and CypD-deficient mice were subjected to anoxia for 30 min, followed by reoxygenation. Control mitochondria, but not CypD-deficient mitochondria, showed loss of ΔΨ (Fig. 4a), swelling (Fig. 4b), leakage of mitochondrial aspartate aminotransferase (MAST) (Fig. 4c), and a severe decrease in respiratory control rate (Fig. 4d), indicating that CypD-deficient mitochondria are more resistant to anoxia/reoxygenation injury than control mitochondria.

We next examined the effect of CypD on cardiac I/R injury, because the heart has high levels of Cypd (see Supplementary Fig. 1d). Several functional parameters assessed by echocardiography showed no differences between the resting hearts of control and CypD-deficient mice (see Supplementary Table). Mice were subjected to 30 min of left coronary artery occlusion followed by 2 h of reperfusion. The size of the area at risk, identified by the absence of Evans blue staining, was not significantly different between control and CypD-deficient hearts (Fig. 4f). In control hearts, I/R injury caused significant necrotic damage, as evidenced by a large area of myocardium that was negative for triphenyltetrazolium chloride (TTC) staining (Fig. 4e, g). In CypD-deficient hearts, however, the infarct area was dramatically reduced (Fig. 4e, g). Consistently, lactate dehydrogenase (LDH) release due to disruption of the plasma membrane was almost completely inhibited in CypD-deficient hearts (Fig. 4h). These results indicate that lack of CypD can markedly reduce cardiac I/R injury.

An increase in the permeability of the outer mitochondrial membrane is central to apoptotic signalling, and is directly regulated by the Bcl-2 family of proteins\(^1\). It has been suggested that the mPT plays a role in apoptotic mitochondrial membrane permeabilization\(^2\). However, we show here that cytochrome c release induced by Bid and Bax is not blocked by CypD deficiency and that CypD deficiency does not affect many forms of apoptotic cell death, indicating that the CypD-dependent mPT does not play a significant role in apoptosis in general; however, this does not exclude the possibility that certain forms of apoptosis are mediated by the mPT, and thereby inhibited by CsA. On the other hand, CypD deficiency blocks Ca\(^{2+}\)-induced and oxidative stress-induced cytochrome c release from isolated mitochondria and also prevents necrotic cell death induced by these stimuli; this indicates that the CypD-dependent mPT is a critical event in some forms of cellular necrosis. Notably, overexpressed CypD can induce necrosis\(^3\).

We showed that lack of CypD markedly suppresses cardiac I/R injury (which mimics cardiac infarction). In I/R injury, production of reactive oxygen species and Ca\(^{2+}\) overload are known to be key events\(^2\). Our results indicate that CypD-deficient mitochondria can accumulate excess Ca\(^{2+}\) without loss of ΔΨ and that they also tolerate reactive oxygen species-induced damage. Consistently, CypD-deficient hepatocytes accumulated more Ca\(^{2+}\) than wild-type hepatocytes, and were significantly more resistant to A23187- and H\(_2\)O\(_2\)-induced death. Thus, the CypD-dependent mPT is a critical event in I/R injury, suggesting that CypD and the mPT may be important therapeutic targets for preventing myocardial infarction.

Methods

Generation of cyclophilin D-deficient mice

A genomic clone (pKOS 63) containing the CypD locus was isolated from a 129/J mouse genomic library. Lox 1 embryonic stem (ES) cells (derived from the 129sv/Fv strain) were electroporated with the targeting vector and selected by G418. ES clones with the targeted CypD allele were screened by polymerase chain reaction (PCR) and Southern blotting analysis. Heterozygous mutant ES cells were injected into C57BL/6J blastocysts. Germline transmission of mutant alleles to F\(_1\) offspring was confirmed by PCR and Southern blotting, and F\(_2\) offspring from heterozygous intercrosses were genotyped by PCR and Southern blotting.

Genotyping by PCR

The CypD locus was genotyped by PCR. The wild-type allele (553 base pairs, bp) was detected using a forward primer (5′-GACATCCAGACCCGACACTG-3′) and a reverse primer (5′-ACCTTGGGAAGCGGACCTGC-3′). To detect the mutant allele (206 bp), a neomycin-specific reverse primer (5′-GACCGGATCGCCCTTCTATC-3′) was used in combination with the wild-type reverse primer described above.

Antibodies

Anti-mouse CypD antibody was produced using a peptide (amino acids 43–57) of CypD. Anti-cytochrome c, anti-cyclophilin A and anti-GAPDH antibodies were purchased from Pharmingen, UpState and Biogenesis, respectively.

Mitochondrial biochemical parameters

Mitochondria were isolated from mouse livers as described previously\(^2\). In all experiments, except for assessment of respiration, mitochondria were in medium containing 0.3 mM mannitol, 10 mM potassium HEPES (pH 7.4), 0.1% fatty acid-free BSA, 300 μM potassium phosphate (pH 7.4) and 4.2 mM succinate.

Mitochondrial PPlase activity was measured as described previously\(^2\), except that the final concentration of the synthetic peptide substrate (sucinyl-Ala-Ala-Pro-Phe-4-nitromandelamide) was 75 μM. The activity was expressed as E\(_{1/2}\), where K and E\(_{1/2}\) are the first-order rate constants in the presence and absence of the mitochondrial lysate, respectively. The mitochondrial membrane potential (ΔΨ) was assessed by measuring the ΔΨ-dependent uptake of rhodamine 123 (Rh123, ref. 24). Mitochondrial swelling was monitored by the decrease of 90° light scatter at 520 nm, which was determined using a spectrophotometer (Hitachi F-4500). Mitochondrial respiration was measured with an O\(_2\) electrode (Rank Brothers) in respiration buffer\(^2\). The external mitochondrial Ca\(^{2+}\) concentration was monitored using a Ca\(^{2+}\)-specific electrode (Orion). The amount of intra-mitochondrial Ca\(^{2+}\) was measured using 45Ca\(^{2+}\). Isolated mitochondria were incubated with 45Ca\(^{2+}\) for 25 min, and then the mitochondria (1 mg protein) were centrifuged at 12,000g for 3 min. The pellet and supernatant were counted and their radioactivity measured using a liquid scintillation counter (Wallac 1409).

Cytochrome c release was determined as previously described\(^1\).

In anoxia/reoxygenation experiments, mitochondria were suspended in a tube and treated with 100% N\(_2\) gas. The gas-saturated tube was sealed with parafilm and left for 30 min, and the mitochondria were then reoxygenated by shaking in a 12-well plate at room temperature for 30 min.

Cell death assay

Primary cultures of MEFs were obtained from CypD-deficient and control littermate embryos at embryonic day 14.5. MEFs immortalized by SV40 T antigen were also used. Hepatocytes were isolated from CypD-deficient mice and their wild-type littermates at 5–4 months of age using the retrograde two-step collagenase perfusion technique\(^2\). Cell viability was assessed by propidium iodide (PI) staining, Annexin-V staining or the cell titre blue (CTB) assay. Briefly, cells were stained with 1 μM PI or 1 μM Cy3-conjugated Annexin-V for 5 min at room temperature and were analysed using a flow cytometer (Becton–Dickinson). The CTB assay, which measures the metabolic activity of viable cells, was carried out using Cell Titer Blue reagent (Promega). The nuclear morphology was observed with 10 μM Hoechst 33342 and 1 μM PI staining as described previously\(^2\). DEVDase activity (cleavage of the caspase-3 substrate DEVD-ams) was measured as described elsewhere\(^2\).

Laser scanning confocal microscopy

Hepatocytes were cultured in covered glass-bottomed 24-well dishes coated with type I collagen (Iwaki glass) for 16–20 h. To monitor mitochondrial ΔΨ, hepatocytes were loaded with 0.25 μM TMRM (Molecular Probes) as described previously\(^2\). To monitor the mitochondrial Ca\(^{2+}\) level, hepatocytes were loaded with 5 μM Rhod2-AM (Molecular Probes) in culture medium containing 0.085% Pluronic F-127 (Molecular Probes) and 0.1 mM sulfipyrazole for 2 h at 4°C for mitochondria-selective loading as described previously\(^2\).

Ischaemia/reperfusion experiments

Using CypD-deficient and wild-type littermate mice at 10–12 weeks of age, ischaemia/reperfusion of the heart was performed as described previously\(^4\). Briefly, under general anesthesia with mechanical ventilation, a silk thread (7–0) was passed around the left coronary artery (LCA) about 1 mm distal to the LCA origin to make a snare. After 30 min ligation of the LCA, the snare was released for 2 h. The infarct size was evaluated by double staining using Evans blue dye and triphenyltetrazolium chloride (TTC). The area at risk was defined as the ratio of the area of the ischaemic region to that of the left ventricle, and the infarct size was defined as the ratio of the area of the infarct region to that of the ischaemic region.

Statistical evaluation

Statistical evaluation was performed by unpaired t-tests. Data are presented as mean ± s.e.m. or mean ± s.d.
Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death

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Mitochondria play a critical role in mediating both apoptotic and necrotic cell death. The mitochondrial permeability transition (mPT) leads to mitochondrial swelling, outer membrane rupture and the release of apoptotic mediators. The mPT pore is thought to consist of the adenine nucleotide translocator, a voltage-dependent anion channel, and cyclophilin D (the Ppif gene product), a prolyl isomerase located within the mitochondrial matrix. Here we generated mice lacking Ppif and mice overexpressing cyclophilin D in the heart. Ppif null mice are protected from ischemia/reperfusion-induced cell death in vivo, whereas cyclophilin D-overexpressing mice show mitochondrial swelling and spontaneous cell death. Mitochondria isolated from the livers, hearts and brains of Ppif null mice are resistant to mitochondrial swelling and permeability transition in vitro. Moreover, primary hepatocytes and fibroblasts isolated from Ppif null mice are largely protected from Ca2+-overload and oxidative stress-induced cell death. However, Bcl-2 family member-induced cell death does not depend on cyclophilin D, and Ppif null fibroblasts are not protected from staurosporine or tumour-necrosis factor-α-induced death. Thus, cyclophilin D and the mitochondrial permeability transition are required for mediating Ca2+- and oxidative damage-induced cell death, but not Bcl-2 family member-regulated death. Disagreement persists as to the function of the mitochondrial permeability transition pore in mediating apoptotic and/or necrotic cell death. To address this issue, we created Ppif knockout mice (Ppif−/−) using homologous recombination in embryonic stem cells (Fig. 1a). Western blotting of cardiac protein extracts from Ppif and Ppif null mice showed the expected 50% reduction or complete absence of cyclophilin D protein, respectively, without a change in the protein levels of the voltage-dependent anion channel VDAC1 or the adenine nucleotide translocator ANT1/2 (Fig. 1b). Remarkably, mitochondria isolated from heart, liver and brain of Ppif null mice were resistant to swelling and permeability transition induced by Ca2+ and/or atracyloside compared with mitochondria isolated from wild-type mice (Fig. 1c–f). However, Bcl-2 family member-induced cell death does not depend on cyclophilin D, and Ppif null fibroblasts are not protected from staurosporine or tumour-necrosis factor-α-induced death. Thus, cyclophilin D and the mitochondrial permeability transition are required for mediating Ca2+- and oxidative damage-induced cell death, but not Bcl-2 family member-regulated death. Disagreement persists as to the function of the mitochondrial permeability transition pore in mediating apoptotic and/or necrotic cell death. To address this issue, we created Ppif knockout mice (Ppif−/−) using homologous recombination in embryonic stem cells (Fig. 1a). Western blotting of cardiac protein extracts from Ppif and Ppif null mice showed the expected 50% reduction or complete absence of cyclophilin D protein, respectively, without a change in the protein levels of the voltage-dependent anion channel VDAC1 or the adenine nucleotide translocator ANT1/2 (Fig. 1b). Remarkably, mitochondria isolated from heart, liver and brain of Ppif null mice were resistant to swelling and permeability transition induced by Ca2+ and/or atracyloside compared with mitochondria isolated from wild-type mice (Fig. 1c–f). However, this protective effect was lost at very high Ca2+ levels (Supplementary Fig. 1), as previously reported with the mPT inhibitor cyclosporin A (CsA). Ppif null mitochondria also showed a greater overall capacity for Ca2+ uptake before undergoing permeability transition (Supplementary Fig. 2). The baseline morphology of mitochondria and cristae organization in Ppif null hearts was otherwise unaffected compared to wild-type hearts (Fig. 1g, h). Indeed, the heart, which contains approximately 30% mitochondria by volume, showed no signs of cardiomyopathy in the absence of Ppif (Supplementary Fig. 3).

Primary embryonic fibroblast cultures were generated to more carefully evaluate mitochondria and cell death in the absence of Ppif (Fig. 2a). Wild-type and Ppif null fibroblasts were loaded with tetramethylrhodamine ethyl ester (TMRE) to measure inner mitochondrial membrane potential (ΔΨm) and permeability...