Involvement of Cyclophilin D and Calcium in Isoflurane-induced Preconditioning

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ABSTRACT

Background: The mitochondrial permeability transition pore (PTP) has been established as an important mediator of ischemia–reperfusion–induced cell death. The matrix protein cyclophilin D (CypD) is the best known regulator of PTP opening. Therefore, the authors hypothesized that isoflurane, by inhibiting the respiratory chain complex I, another regulator of PTP, might reinforce the myocardial protection afforded by CypD inhibition.

Methods: Adult mouse or isolated cardiomyocytes from wild-type or CypD knockout (CypD-KO) mice were subjected to ischemia or hypoxia followed by reperfusion or reoxygennation. Infarct size was assessed in vivo. Mitochondrial membrane potential and PTP opening were assessed using tetrakis-methylene-diamine methyl ester perchlorate and calcine–cobalt fluorescence, respectively. Fluo-4 AM and rhod-2 AM staining allowed the measurement, by confocal microscopy, of Ca2+ transient and Ca2+ transfer from sarcoplasmic reticulum (SR) to mitochondria after caffeine stimulation.

Results: Both inhibition of CypD and isoflurane significantly reduced infarct size (−50 and −37%, respectively) and delayed PTP opening (+63% each). Their combination had no additive effect (n = 6/group). CypD-KO mice displayed endogenous protection against ischemia–reperfusion. Isoflurane depolarized the mitochondrial membrane (−28%, n = 5), decreased oxidative phosphorylation (−59%, n = 5), and blunted the caffeine-induced Ca2+ transfer from SR to mitochondria (−22%, n = 7) in the cardiomyocytes of wild-type mice. Importantly, this transfer was spontaneously decreased in the cardiomyocytes of CypD-KO mice (−25%, n = 4 to 5).

Conclusions: The results suggest that the partial inhibitory effect of isoflurane on respiratory complex I is insufficient to afford a synergy to CypD-induced protection. Isoflurane attenuates the Ca2+ transfer from SR to mitochondria, which is also the prominent role of CypD, and finally prevents PTP opening. (Anesthesiology 2015; 123:1374-84)

PERIOPERATIVE myocardial ischemia–reperfusion is associated with high morbidity and mortality during cardiac surgery and/or noncardiac surgery in patients at risk of coronary artery disease.1,2 Mitochondria are recognized as essential organelles involved in cell damage during ischemia and reperfusion, with the opening of the mitochondrial permeability transition pore (PTP) playing an important role in the process.3-5 Although the molecular structure of the PTP remains unclear, the mitochondrial matrix protein cyclophilin D (CypD) is known to be a critical regulatory component of the PTP.6,7 CypD is a mitochondrial chaperone that, on accumulation of Ca2+ in the matrix, binds to the inner mitochondrial membrane, triggers the opening of the PTP, releases proapoptotic factors, and finally promotes cardiomyocyte death.7 Genetic ablation or pharmacologic inhibition of this Ca2+-sensitive chaperone by cyclosporine A (CsA) or its analogs reduces the infarct size in animal models, as well as in acute myocardial infarction or in patients undergoing cardiac surgery.8-12 One of the main sources of Ca2+ in
cardiomyocytes is the sarcoplasmic reticulum (SR). SR and mitochondria are spatially and functionally organized as a network. This physical association plays a crucial role in Ca\(^{2+}\) signaling.\(^{13-15}\) Ca\(^{2+}\) crosstalk between SR and mitochondria has been involved in myocardial reperfusion injury.\(^{13}\)

The mitochondrial respiratory chain complex I is also one of the PTP regulators. Recently, we found that rotenone, a specific complex I inhibitor, reinforces the cardioprotective effect of CsA.\(^{16}\) The volatile anesthetic agent isoflurane affords both preconditioning and postconditioning and has been extensively studied in experimental and clinical situations.\(^{17-20}\) Among the several molecular targets, isoflurane modulates SR and mitochondrial and cytosolic Ca\(^{2+}\) contents.\(^{21-23}\) Isoflurane can also regulate mitochondrial respiration at the level of complex I.\(^{24,25}\) We previously demonstrated in a rat in vivo model that isoflurane prevents lethal reperfusion injury by modulation of complex I activity.\(^{26}\)

On the basis of these findings, we hypothesized that the protective effect of isoflurane could be synergistic with the protection afforded by CypD inhibition. We specifically examined the interaction between complex I and CypD in cardioprotection and studied the effects of isoflurane on Ca\(^{2+}\) transfer from SR to mitochondria.

**Materials and Methods**

**Animals**

CypD knockout (CypD-KO) mice under C57Bl6/SV129 background were obtained from the laboratory of the late Stanley Korsmeyer, M.D. (Dana Farber Cancer Institute, Boston, Massachusetts).\(^{27}\) Both wild-type (WT) and CypD-KO male mice (8 to 12 weeks, 20 to 30 g) were obtained through homozygous intercross in our laboratory.

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all the experimental procedures were approved by a local ethics committee (Université Claude Bernard Lyon 1, n BH2007-07, Lyon, France). WT and CypD-KO mice were anesthetized with sodium pentobarbital (70 mg/kg) and buprenorphine (50 μg/kg; Sanofi Santé Animale, France) administered intraperitoneally.

**Ischemia–Reperfusion In Vivo**

Once pedal pinch reflexes were completely inhibited, animals were intubated and mechanically ventilated with a rodent ventilator (MiniVent*, Harvard Apparatus, USA). A left thoracotomy was performed in the fourth intercostal space. A small curved needle was passed around the left anterior descending coronary artery to induce ischemia. (Myocardial ischemia was induced by occluding the artery, and reperfusion was initiated by loosening the suture.) Mice were subjected to 45 min of regional myocardial ischemia followed by 24 h of reperfusion (fig. 1A). In six separate experimental groups, WT mice were randomly assigned to receive saline (control), a pharmacologic preconditioning performed with CsA (10 mg/kg injected 10 min before ischemia; Novartis, Switzerland), isoflurane (two cycles of a 15-min administration at 1.4% [1 minimum alveolar concentration] per 5 min washout before ischemia; Abbott, USA), or the combination of isoflurane + CsA; CypD-KO mice were randomly assigned to receive saline (control) or isoflurane (fig. 1A). Infarct size was determined by triphenyltetrazolium chloride staining as described previously. Twenty-six experimenters were blinded to the condition groups during infarct size analyses. Animals with an area at risk (AR) less than 15% of total left ventricle (LV) mass were excluded from subsequent analysis.

**Isolation of Adult Murine Ventricular Cardiomyocytes and Mitochondria**

Once pedal pinch reflexes were completely inhibited, a thoracotomy was performed and the heart was collected. Ventricular cardiomyocytes were isolated using enzymatic digestion with 0.167 mg/ml liberase (Roche Diagnostic, France) and 0.14 mg/ml 2.5% trypsin (Sigma-Aldrich, USA), and mitochondria were isolated according to the previously described procedure.\(^{16,28}\) In each randomized group, five mice were used for the isolation of cardiomyocytes and five mice for the mitochondria isolation for oxidative phosphorylation and PTP opening measurements. Cardiomyocytes and mitochondria were used within 5 h of isolation.

**Hypoxia–Reoxygenation In Vitro**

Cardiomyocytes from WT or CypD-KO hearts were placed at 37°C in a thermostated chamber mounted on the stage of an IX50 Olympus microscope (Olympus, Japan), as described previously.\(^{16,28}\) Cells were perfused with a Tyrode solution containing (in mM) 130 NaCl, 5 KCl, 10 HEPES, 1 MgCl\(_2\), 1 CaCl\(_2\), and 10 glucose, pH 7.4, at 37°C for a 10-min stabilization period. The chamber was connected to a constant stream of 21% O\(_2\), 74% N\(_2\), and 5% CO\(_2\). Oxygen in the solution was measured using a fiber optic sensor system (Ocean Optics, Inc., USA). For the hypoxia challenge, WT or CypD-KO cardiomyocytes were bathed for 30 min in a glucose-free Tyrode solution bubbled with 100% N\(_2\) and under a constant stream of N\(_2\) (100%) in the chamber. At the end of the hypoxia period, a 60-min reoxygenation phase was completed by rapidly restoring the Tyrode flow and oxygen fraction to 21%. Pharmacologic preconditioning was performed with CsA (1 μM; Novartis) and/or isoflurane (0.5 mM; Abbott) 10 min before hypoxia (fig. 1B). Isoflurane diluted in dimethylsulfoxide (Sigma-Aldrich) was added to experimental buffers to achieve a desired concentration of 0.5 mM, confirmed by high-performance liquid chromatography, according to the method previously described by Janicki et al.\(^{29}\) The final percentage of dimethylsulfoxide was 0.2% in all groups.
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PTP Opening in Isolated Cardiomyocytes
Cardiomyocytes were loaded for 30 min at 37°C with 1 μM calcein-AM and 1 mM CoCl₂ to selectively load the mitochondria with calcein by quenching the cytosolic calcein with CoCl₂. After loading, cardiomyocytes were submitted to 30-min hypoxia followed by 60-min reoxygenation. Calcein fluorescence was detected at reperfusion by using a 460- to 490-nm excitation filter and a 510-nm emission filter. Images were acquired every 2 min after an illumination time of 100 ms per image. Fluorescence was integrated over a region of interest (approximately 80 μm²) for each cardiomyocyte, and a fluorescence background corresponding to an area without cells was removed. For comparison, the fluorescence intensity minus background (ΔF) was normalized according to the initial fluorescence value (F₀). For each treatment, we calculated the global response by averaging the fluorescence changes obtained from all of the cardiomyocytes contained in a field (at least 10 cells). This measure was repeated in four to five separate experiments per treatment group. The time delay to 50% PTP opening (t_{PTP50}) was measured as the average reoxygenation time necessary to induce a 50% decrease in calcein fluorescence in the same field. Image analysis was performed using Image J v.1.44 (NIH, USA).

Mitochondrial Membrane Potential (ΔΨₘ)
Cardiomyocytes were loaded with 10 nM tetramethylrhodamine methyl ester perchlorate (TMRM) for 40 min at 37°C, followed by a washout. TMRM fluorescence intensity was recorded with a 560-nm longpass filter after excitation at 543 nm. Images were taken every minute, and fluorescence signals were normalized to the fluorescence measured at the start of the experiment. To minimize the impact of ΔΨₘ variability, TMRM fluorescence was measured in five different areas in each cell. At the end of each experiment, cells were exposed to the mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (1 μM) to determine the dynamic range of the dye.

Oxidative Phosphorylation
Mitochondrial oxygen consumption was measured at 25°C using a Clark-type oxygen electrode. Permeabilized cardiomyocytes (300 µg protein) with digitonin (40 µM) or cardiac mitochondria (250 µg protein) were incubated in...
2 ml respiration buffer containing (in mM) 100 KCl, 50 MOPS, 1 EGTA, 5 KH₂PO₄, and 1 mg/ml defatted bovine serum albumin. Glutamate, malate, and pyruvate (5 mM each) were used as electron donors to complex I in the electron transport chain in the absence (control) or presence of 0.5 mM isoflurane. Respiration state 2 was measured without ADP, and respiration state 3 was initiated with addition of ADP (1 mM for cardiomyocytes or 200 µM for mitochondria) and expressed as nmol O₂ min⁻¹ mg protein⁻¹. The respiratory control index was calculated as the ratio of state 3/state 2 (for cardiomyocytes) or state 3/state 4 (for mitochondria). To characterize isoflurane-induced complex I inhibition and to compare this effect with that of rotenone, the specific complex I inhibitor, another set of experiments was performed using increasing doses of rotenone (0 to 1,000 nM) on isolated heart mitochondria.

**Calcium Retention Capacity**
Calcium retention capacity (CRC) measurement was performed with a spectrofluorometer (excitation, 506 nm; emission, 530 nm). The incubation medium of heart mitochondria contained (in mM) 150 sucrose, 50 KCl, 2 KH₂PO₄, 2 Tris/HCl, 5 succinate-Tris, and 0.25 µM Calcium Green-5N. Isolated mitochondria (250 µg protein) were added to 2 ml of this incubation medium. Rotenone (0 to 1000 nM) and/or CsA (1 µM) was added to the whole preparation. After 2 min of incubation, the CRC was measured by adding Ca²⁺ pulses every minute until mitochondrial PTP opening. CRC was expressed as nmol Ca²⁺ mg protein⁻¹.

**Measurement of Ca²⁺ Transient**
Isolated cardiomyocytes were loaded for 20 min at 37°C with fluo-4 AM (5 µM) assessing the cytosolic Ca²⁺. To measure Ca²⁺ transients, cardiomyocytes were field stimulated at 1 Hz with a current pulse delivered through two platinum electrodes. To enable comparisons between cells, the normalized change fluorescence (ΔF/F₀) was measured, where ΔF is the fluorescence signal (F) subtracted by the fluorescence background detected immediately before the 1-Hz stimulation pulse (F₀). Analysis included amplitude of Ca²⁺ transients, rising phase of Ca²⁺ transients (normalizing the peak amplitude over the time to peak), and the decay of electrically stimulated Ca²⁺ transients.

**Measurement of SR to Mitochondrial Ca²⁺ Transfer after Caffeine Stimulation**
Cardiomyocytes were loaded for 40 min at 37°C with rhod-2 AM (5 µM; Teflabs, USA). To eliminate cytosolic Rhod-2 and to record mitochondrial Ca²⁺ movements, whole cell patch clamp technique was used to dialyze cytosolic Rhod-2 as described by Maack et al. Cells were maintained under clamp conditions using an Axopatch 200B (Axon Instruments, USA), as described previously. Pipettes (2 to 3 MΩ) were filled with recording solution containing (in mM) 130 KCl, 25 HEPES, 3 ATP (Mg), 0.4 GTP (Na), 0.5 EGTA, pH adjusted to 7.2 with KOH. The cardiomyocytes were perfused with Tyrode solution. Mitochondrial Ca²⁺ was measured during the release of SR Ca²⁺ induced by 10 mM caffeine application. Rhod-2 signal was analyzed by dividing fluorescence values (F) by the background fluorescence (F₀) detected immediately before caffeine application.

**Confocal Imaging Acquisition**
Fluo-4 was excited at 488 nm, and emission was collected through a 505-nm longpass filter. Rhod-2 was excited at 550 nm, and emitted fluorescence was collected at 590 nm on excitation with argon laser. The changes in dye fluorescence were recorded using an LSM510 Meta Zeiss confocal microscope (Carl Zeiss, Germany) equipped with a 63X water-immersion objective (numerical aperture: 1.2). All measurements were performed in line-scan mode (1.54 ms/line), and scanning was carried out along the long axis (Ca²⁺ transients) or along the short axis of the cell, closed to the patch pipette (mitochondrial Ca²⁺).

**Statistical Analysis**
All values are expressed as mean ± SD. The number of animals per group was chosen according to the previous studies of our group. Data were analyzed using two-tailed Student t test when only two groups are being compared or one-way ANOVA followed by Bonferroni post hoc test to analyze differences between more than two groups. P ≤ 0.05 was considered significant. Statistical calculation was performed using GraphPad Prism® version 6 software (GraphPad Software, USA).

**Results**

**Effect of Isoflurane and CypD Inhibition on Infarct Size**
Forty mice were instrumented to obtain 36 successful experiments. Two mice were excluded because of technical problems during surgery. Two mice were excluded because AR was too small. The ratio of AR to LV was similar between groups (fig. 2A, P > 0.9). Area of necrosis was expressed as percent of LV (in fig. 2B). We confirmed in our in vivo ischemia–reperfusion mice model that CypD deletion or inhibition had a cardioprotective effect, and we investigated whether combining administration of isoflurane could have an additive effect. Our results showed that CsA, isoflurane, and CsA + isoflurane significantly and similarly reduced infarct size, which averaged 30.5 ± 6.4% (P < 0.0001), 24.5 ± 6.0% (P < 0.0001), and 32.3 ± 6.9% (P = 0.0004) of AR, respectively, versus 48.6 ± 5.7% in control WT mice (fig. 2C). As expected, infarct size was reduced in CypD-KO control mice to 32.8 ± 3.5% (P = 0.0004) of AR compared with that of WT control (fig. 2C). Finally, isoflurane did not afford any additional protection to CypD-KO (32.3 ± 4.2% of AR, P > 0.9; fig. 2C).

**Effect of Isoflurane and CypD Inhibition on the Time to PTP Opening**
We then examined whether this protection might be related to a direct effect on PTP opening (fig. 3). In WT cardiomyocytes,
CsA and isoflurane significantly delayed PTP opening, with $t_{PTP50}$ averaging $44 \pm 9$ min and $44 \pm 6$ min, respectively, versus $27 \pm 3$ min in untreated WT (control; $P < 0.0001$; fig. 3, A and C). However, simultaneous administration of isoflurane and CsA did not increase $t_{PTP50}$ when compared with each treatment alone in WT cardiomyocytes. In control CypD-KO cardiomyocytes, $t_{PTP50}$ was significantly prolonged to $42 \pm 5$ min versus $27 \pm 3$ min compared with that of Control WT ($P < 0.0001$; fig. 3C). Again, isoflurane treatment did not further increase $t_{PTP50}$ in CypD-KO cardiomyocytes (fig. 3, B and C).

**Effects of Isoflurane and CypD Inhibition on Oxidative Phosphorylation and Mitochondrial Membrane Potential**

WT and CypD-KO cardiomyocytes exhibited comparable oxygen consumption in control condition (fig. 4A). In both WT and CypD-KO cardiomyocytes, isoflurane (0.5 mM) significantly decreased state 3 respiration by approximately 50 to 60% ($P < 0.0001$), and the average mean values were $21 \pm 4$ and $21 \pm 3$ nmol O$_2$ min$^{-1}$ mg protein$^{-1}$ after administration of isoflurane versus $51 \pm 4$ and $43 \pm 9$ nmol O$_2$ min$^{-1}$ mg protein$^{-1}$ without isoflurane, respectively (fig. 4A). Similar results were obtained using isolated cardiac mitochondria instead of cardiomyocytes (fig. 4B). State 2 and respiratory control index in all groups are presented as insets in figure 4, A and B. A comparable mitochondrial depolarization was observed after the administration of isoflurane, reaching $72 \pm 9\%$ and $78 \pm 7\%$ of control values for WT and CypD-KO, respectively ($P = 0.043$; fig. 4C).

**A Complete Inhibition of Complex I Is Required for a Synergistic Protection to CypD Inhibition**

Given that isoflurane inhibits complex I and based on our previous results demonstrating the synergistic protective effect of rotenone when added to CsA, we were surprised to observe the absence of isoflurane effect (on infarct size reduction and PTP opening) combined with CsA or in the absence of CypD.$^{16}$ To clarify these results, we tested the dose–response effect of rotenone (0 to 1,000 nM), a specific complex I inhibitor, on complex I oxygen consumption and on CRC (fig. 5). As shown by figure 5A, CsA had no effect on the state 3 of complex I oxygen consumption, whereas rotenone inhibited it in a dose-dependent manner. The IC$_{50}$ value for the rotenone was between 2.5 and 5 nM. The same results were observed in CypD-KO heart mitochondria (data not shown). As shown in figure 5B, we confirmed that CsA increased the value of CRC in WT mitochondria ($P = 0.005$) and that this effect was amplified by rotenone at 1,000 nM ($P = 0.02$), a dose that totally inhibited complex I activity. Importantly, rotenone at doses less than or equal to 50 nM did not modify CRC ($P > 0.9$). The same results were observed in CypD-KO heart mitochondria (fig. 5C).

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**Fig. 2. In vivo myocardial infarct size results.** (A) AR expressed as % of LV. (B) AN expressed as percent of LV. (C) AN expressed as percent of AR. Each value was expressed as mean ± SD, one-way ANOVA, Bonferroni post hoc test, *$P = 0.038$, **$P = 0.0004$, ***$P < 0.0001$ versus Ctrl WT, n = 6/group. AN = area of necrosis; AR = area at risk; CsA = cyclosporine A; Ctrl = control; CypD-KO = cyclophilin D knockout; Iso = isoflurane; LV = left ventricle; NS = not significantly different; WT = wild type.
Isoflurane and CypD Deletion Decreased Ca\textsuperscript{2+} Transfer from SR to Mitochondria

Because isoflurane might act through different mechanism(s) that may indirectly protect against hypoxia–reoxygenation injury, we specifically examined Ca\textsuperscript{2+} transfer between SR and mitochondria, which has been proposed as potential contributor to hypoxia–reoxygenation injury. We measured Ca\textsuperscript{2+} transient (amplitude, velocity, and decay) in WT and CypD-KO cardiomyocytes with and without isoflurane (fig. 6). We also determined mitochondrial Ca\textsuperscript{2+} concentration after application of caffeine (to stimulate Ca\textsuperscript{2+} release from SR).

Ca\textsuperscript{2+} transient amplitude, evoked by electrical stimulation (1 Hz), was spontaneously significantly attenuated in CypD-KO cardiomyocytes compared with that of WT cardiomyocytes (2.1 ± 0.2 vs. 2.6 ± 0.2, respectively, \(P \leq 0.03\); fig. 6A). Velocity of Ca\textsuperscript{2+} transient was also significantly decreased in CypD-KO when compared with WT cardiomyocytes (0.13 ± 0.02 vs. 0.16 ± 0.02, \(P \leq 0.03\); fig. 6B). Ca\textsuperscript{2+} transient decay, mostly dependent on the activity of Ca\textsuperscript{2+} reuptake by the SR, was significantly increased in CypD-KO cardiomyocytes compared with WT cardiomyocytes (280 ± 19 ms vs. 213 ± 26 ms, \(P = 0.0048\); fig. 6C).

In WT cardiomyocytes, isoflurane significantly decreased the amplitude of Ca\textsuperscript{2+} transients (1.6 ± 0.3 vs. 2.6 ± 0.2, \(P = 0.009\); fig. 6A) and attenuated the velocity of Ca\textsuperscript{2+} transients (0.12 ± 0.02 vs. 0.16 ± 0.02, \(P = 0.03\); fig. 6B) similar to that observed in CypD-KO cardiomyocytes. Isoflurane tended (although not significantly) to increase Ca\textsuperscript{2+} transient speed of decay in WT cardiomyocytes (fig. 6C). In CypD-KO cardiomyocytes, isoflurane further decreased the amplitude of Ca\textsuperscript{2+} transients (1.6 ± 0.2 vs. 2.1 ± 0.3, \(P = 0.042\); fig. 6A), the velocity of Ca\textsuperscript{2+} transients (0.10 ± 0.03 vs. 0.13 ± 0.02, \(P = 0.04\); fig. 6B), and the speed of Ca\textsuperscript{2+} reuptake by the SR (164 ± 34 ms vs. 280 ± 19 ms, \(P = 0.0038\); fig. 6C). Figure 6, D and E represents Ca\textsuperscript{2+} transients and averaged Ca\textsuperscript{2+} transient signals, respectively.

In WT cardiomyocytes, a 10 mM challenge with caffeine, an SR Ca\textsuperscript{2+} release stimulator, caused a significant increase in mitochondrial Ca\textsuperscript{2+} accumulation, as measured by rhod-2 fluorescence (fig. 7A). When exposed to the same dose of caffeine, mitochondrial Ca\textsuperscript{2+} uptake was lowered by 25 ± 8% in CypD-KO when compared with WT (\(P = 0.0038\); fig. 7B). Similar reduction in mitochondrial Ca\textsuperscript{2+} uptake (33 ± 16%, \(P = 0.0168\) vs. WT) was observed after transient exposure of WT cardiomyocytes to isoflurane. Isoflurane did not further decrease the mitochondrial Ca\textsuperscript{2+} uptake after application of caffeine to CypD-KO cells.

**Fig. 3.** Cyclophilin D inhibition and isoflurane preconditioning delay permeability transition pore (PTP) opening. (A) Wild-type (WT) and (B) cyclophilin D knockout (CypD-KO) cardiomyocytes were loaded with calcein-AM and CoCl\textsubscript{2} and submitted to 30-min hypoxia followed by 60-min reoxygenation (Control). Preconditioning with 1 mM cyclosporine A (CsA) and/or 0.5 mM isoflurane (Iso) was performed 10 min before hypoxia. (C) \(t_{\text{PTP50}}\) is the average reoxygenation time necessary to induce a 50% decrease in calcein fluorescence in the same field. Each value was expressed as the mean ± SD, one-way ANOVA, Bonferroni post hoc test, ***\(P < 0.0001\) versus Control WT, n = 4 to 5 separate experiments. NS = not significantly different.
PTP opening plays a key role in myocardial ischemia–reperfusion injury.\(^5,33,34\) Mitochondrial Ca\(^{2+}\) overload is the primary inducer of PTP opening by triggering the translocation of the matrix chaperone CypD to the inner mitochondrial membrane.\(^35,36\) Indeed, the pharmacologic inhibition of CypD by CsA or genetic CypD ablation reduces myocardial infarct size and death of cardiomyocytes after ischemia–reperfusion.\(^8–10\) Moreover, CsA has been shown as an effective strategy to prevent lethal reperfusion injury in acute myocardial infarction patients.\(^11\) Similarly, we recently found that administration of CsA before aortic cross-unclamping reduced the postoperative cardiac troponin I in patients undergoing aortic valve surgery.\(^12\) Our present study confirms, using an \textit{in vivo} model, that pharmacologic inhibition or genetic loss of function of CypD protects cardiomyocytes from lethal reperfusion injury.

Cardioprotective properties of volatile anesthetic agents such as isoflurane are supported by numerous preclinical and clinical studies.\(^19,20\) Isoflurane is one of the few agents producing both preconditioning and postconditioning.\(^17,18,26\) We confirm here that isoflurane alone is as powerful as CsA in reducing infarct size \textit{in vivo} in mice. Volatile anesthetics in general act on multiple molecular targets and are implicated in several cardioprotective pathways.\(^37\) Among them, isoflurane has an effect on targets implicated in cardioprotective pathways like complex I.\(^25,38,39\) We recently found that isoflurane reduces infarct size, \textit{in vivo} in a rat model, by a modulation of complex I activity.\(^26\)

In two other studies, we pointed out the link between CypD and complex I in PTP opening and mortality reduction, demonstrating the synergistic protective effect of CsA and rotenone, the reference complex I inhibitor.\(^16,40\) On the basis of these results, we first hypothesized that isoflurane, by its effect on complex I, could reinforce the cardioprotection induced by CypD inhibition. Our infarct size and PTP opening data (figs. 2 and 3) demonstrated an absence of synergy. Isoflurane was not able to afford any additive protection after CsA administration or after genetic CypD deletion.

Second, we verified that isoflurane affected mitochondrial respiration and inhibited complex I in our model. As expected, 0.5 mM isoflurane decreased approximately 50% mitochondrial respiration in WT cardiomyocytes (fig. 4).
Hanley et al. demonstrated that isoflurane inhibits complex I in a dose-dependent fashion. This has been recently confirmed by Hirata et al. The impact of isoflurane on activities of other complexes, such as complex III, is still debated. Interestingly, isoflurane also decreased complex I state 3 respiration in CypD-KO cardiomyocytes and in isolated mitochondria. Isoflurane reduced mitochondrial membrane polarization in WT and CypD-KO cardiomyocytes. Importantly, in the absence of isoflurane, the loss of CypD function neither influenced mitochondrial respiration activity nor altered mitochondrial membrane polarization.

We then questioned why, in contrast to our previous studies, complex I inhibition by isoflurane was not able to reinforce the protection afforded by CypD inhibition. Therefore, we conducted a set of experiments, using rotenone, the reference complex I inhibitor, to assess the impact of complex I inhibition on PTP modulation. As displayed in figure 5, the synergistic effect of rotenone and CsA (fig. 5, B and C) was only found when 1 μM rotenone was administered, a dose that totally inhibits state 3 of complex I respiration (fig. 5A). However, when complex I was partially blocked by lower doses of rotenone, the synergistic effect of rotenone on CsA was lost. In accordance with these results, isoflurane reduced mitochondrial membrane polarization by approximately 50% (fig. 4B), suggesting that a partial complex I inhibition is not sufficient to afford an additional protection when CypD is inhibited or absent.

Finally, our results did not find any additive effect of isoflurane on CypD inhibition, suggesting that mechanism other than the complex I inhibition was involved in isoflurane-induced cardioprotection. We then assessed the role of Ca2+ exchange. When administered to WT cardiomyocytes, isoflurane alone had an effect comparable to that of CypD genetic ablation on Ca2+ transient amplitude and velocity as well as on Ca2+ accumulation in mitochondria after caffeine stimulation. Isoflurane enhanced the reduction of both the amplitude and the Ca2+ transient velocity in CypD-KO cardiomyocytes. After caffeine-induced SR Ca2+ release, isoflurane did not further reduce Ca2+ accumulation in mitochondria when applied to CypD-KO cardiomyocytes. This is in agreement with Ljubkovic et al. who have suggested that mitochondrial depolarization induced by isoflurane could attenuate mitochondrial Ca2+ overload by diminishing the driving force for Ca2+ influx via the mitochondrial uniporter. Isoflurane is also known to inhibit L-type Ca2+ channel and to decrease action potential duration, thus decreasing SR Ca2+ release. One cannot rule out that the modification of Ca2+ transient seen after the administration of isoflurane might be partly due to a reduced Ca2+-induced Ca2+ release.
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In summary, this study demonstrated that protection afforded by isoflurane and CypD inhibition is not additive. The partial inhibitory effect of isoflurane on respiratory matrix-free Ca\(^{2+}\) was higher in CypD-KO than in WT isolated mitochondria. Additional studies are required to address this issue in detail.

Fig. 6. Genetic cyclophilin D ablation and isoflurane affect sarcoplasmic reticulum (SR) Ca\(^{2+}\) regulation. Ca\(^{2+}\) transients were recorded in fluo4-AM–loaded intact cardiomyocytes electrically stimulated at 1 Hz in the absence (Control) or 10 min after 0.5 mM isoflurane (Iso) incubation. (A) Ca\(^{2+}\) transients amplitude. (B) Ca\(^{2+}\) transients velocity (calculated from the relative amplitude and time to peak of the electrical induced Ca\(^{2+}\) transient). (C) Ca\(^{2+}\) transients decays as an index of sarcoendoplasmic reticulum Ca\(^{2+}\) transport ATPase function. (D) Representative Ca\(^{2+}\) transients. (E) Averaged Ca\(^{2+}\) transient signals. Each value was expressed as the mean ± SD, one-way ANOVA, Bonferroni post hoc test, *P = 0.03, #P = 0.044, **P = 0.009, ***P = 0.0048 versus Control wild type (WT). Iso CypD-KO versus Control CypD-KO were compared using two-tailed Student t test, ##P = 0.0038, n = 7 separate experiments. CypD-KO = cyclophilin D knockout; ΔF/F0 = normalized change fluorescence.

Fig. 7. Genetic cyclophilin D ablation and isoflurane reduce mitochondrial Ca\(^{2+}\) uptake. To assess sarcoplasmic reticulum Ca\(^{2+}\) load (A), cytosolic fluo4-AM fluorescence was recorded by laser scanning confocal microscopy after caffeine (10mM) stimulation in the absence of isoflurane (Iso; Control) or 10 min after 0.5 mM Iso incubation. Cardiomyocytes were loaded with rhod-2 AM and patch clamped in whole cell configuration. Perfusion of an internal cytosolic solution removes cytosolic remains of the dye and allows the detection of mitochondrial specific fluorescence. Rhod-2 AM fluorescence was recorded by laser scanning confocal microscopy after caffeine (10mM) stimulation in the absence of Iso (Control) or 10 min after 0.5 mM Iso incubation. (A) Representative rhod-2 AM fluorescence signals. (B) Summarized data for mitochondrial Ca\(^{2+}\) measurements. Each value was expressed as the mean ± SD, one-way ANOVA, Bonferroni post hoc test, *P = 0.0168, **P = 0.0038, ***P = 0.002 versus Control WT, n = 4 to 5 separate experiments. CypD-KO = cyclophilin D knockout; NS = no significantly different; WT = wild type; ΔF/F0 = normalized change fluorescence.

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complex I is insufficient to afford a synergy to CypD-induced protection. Our results suggest that part of the prominent role of CypD in cardioprotection is mediated by its impact on Ca\textsuperscript{2+} transfer and finally PTP opening. Consequently, inhibition of Ca\textsuperscript{2+} transfer by isoflurane treatment cannot have an additional inhibitory effect on PTP opening and cardioprotection in the absence of CypD or after inhibition of CypD.

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Competing Interests
The authors declare no competing interests.

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