Inhibiting Mitochondrial Fission Protects the Heart Against Ischemia/Reperfusion Injury

Sang-Bing Ong, MSc; Sapna Subrayan, PhD; Shiang Y. Lim, PhD; Derek M. Yellon, DSc; Sean M. Davidson, PhD; Derek J. Hausenloy, MRCP, PhD

Background—Whether alterations in mitochondrial morphology affect the susceptibility of the heart to ischemia/reperfusion injury is unknown. We hypothesized that modulating mitochondrial morphology protects the heart against ischemia/reperfusion injury.

Methods and Results—In response to ischemia, mitochondria in HL-1 cells (a cardiac-derived cell line) undergo fragmentation, a process that is dependent on the mitochondrial fission protein dynamin-related protein 1 (Drp1). Transfection of HL-1 cells with the mitochondrial fusion proteins mitofusin 1 or 2 or with Drp1K38A, a dominant-negative mutant form of Drp1, increased the percentage of cells containing elongated mitochondria (65±4%, 69±5%, and 63±6%, respectively, versus 46±6% in control; n=80 cells per group; P<0.05), decreased mitochondrial permeability transition pore sensitivity (by 2.4±0.5-, 2.3±0.7-, and 2.4±0.3-fold, respectively; n=80 cells per group; P<0.05), and reduced cell death after simulated ischemia/reperfusion injury (11.6±3.9%, 16.2±3.9%, and 12.1±2.9%, respectively, versus 41.8±4.1% in control; n=320 cells per group; P<0.05). Treatment of HL-1 cells with mitochondrial division inhibitor-1, a pharmacological inhibitor of Drp1, replicated these beneficial effects. Interestingly, elongated interfibrillar mitochondria were identified in the adult rodent heart with confocal and electron microscopy, and in vivo treatment with mitochondrial division inhibitor-1 increased the percentage of elongated mitochondria from 3.6±0.5% to 14.5±2.8% (P=0.023). Finally, treatment of adult murine cardiomyocytes with mitochondrial division inhibitor-1 reduced cell death and inhibited mitochondrial permeability transition pore opening after simulated ischemia/reperfusion injury, and in vivo treatment with mitochondrial division inhibitor-1 reduced myocardial infarct size in mice subject to coronary artery occlusion and reperfusion (21.0±2.2% with mitochondrial division inhibitor-1 versus 48.0±4.5% in control; n=6 animals per group; P<0.05).

Conclusion—Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury, suggesting a novel pharmacological strategy for cardioprotection. (Circulation. 2010;121:2012-2022.)

Key Words: cardiomyocytes ■ hypoxia ■ ischemia ■ myocardial infarction ■ reperfusion

Innovative treatment strategies for protecting the heart from ischemia/reperfusion injury (IRI) are needed to improve clinical outcomes in patients with coronary heart disease. Previous studies suggest that mitochondria are highly dynamic and that changes in mitochondrial shape can affect a variety of biological processes such as apoptosis, respiration, mitosis, and development.1,2 Mitochondria change their morphology by undergoing either fusion or fission, resulting in either elongated, tubular, interconnected mitochondrial networks or fragmented, discontinuous mitochondria, respectively.1,2 These 2 opposing processes are regulated by the mitochondrial fusion proteins mitofusin (Mfn) 1, Mfn2, and optic atrophy protein 1 and the mitochondrial fission proteins dynamin-related protein 1 (Drp1) and human mitochondrial fission protein 1 (hFis1).1,2 The fine balance between mitochondrial fusion and fission within a cell may be upset by a variety of factors, including oxidative stress3 and simulated ischemia,4 which can predispose the cell to apoptosis,5 and the opening of the mitochondrial permeability transition pore (mPTP),6 critical mediators of IRI.7 Therefore, on the basis that changes in mitochondrial morphology appear to affect biological processes that may be fundamental to cardioprotection, we hypothesized that modulating mitochondrial morphology may protect the heart against IRI.

Clinical Perspective on p 2022

Methods

HL-1 Cell Plasmid Transfection

The HL-1 cardiac cell line (derived from murine atrial cardiomyocytes) was cultured according to previously published methods,8 Using Fugene 6 (Roche Molecular Biochemicals, Basel, Switzerland).
land), we transfected cells with plasmids expressing Mfn1, Mfn2,\textsuperscript{9} Drp1\textsubscript{K38A}, the dominant-negative mutant form of the mitochondrial fission protein Drp1,\textsuperscript{5} hFis1, or empty vector.

Determining Mitochondrial Morphology in HL-1 Cells

Mitochondrial morphology was determined in HL-1 cells transfected with red fluorescent protein targeted to the mitochondrial matrix in addition to empty vector control (A), Mfn1 (B), Mfn2 (C), Drp1\textsubscript{K38A} (D) (all demonstrating predominantly [>50%] elongated mitochondria), and hFis1 (E) (demonstrating predominantly fragmented mitochondria). F, Overexpressing Mfn1, Mfn2, or Drp1\textsubscript{K38A} increased the percentage whereas overexpressing hFis1 decreased the percentage of HL-1 cells displaying elongated mitochondria. n=4 experiments with 80 cells per treatment group. \textsuperscript{*}P<0.05 vs vector control.

Real-Time Changes in Mitochondrial Morphology During Ischemia and Reperfusion

HL-1 cells containing predominantly (>50%) elongated mitochondria under basal conditions were identified and subjected to 120 minutes of simulated ischemia and 30 minutes of simulated reperfusion (SIRI) with a Warner PM-2 heated perfusion chamber (Harvard Apparatus, Holliston, Mass) mounted on the confocal microscope. The real-time effects of SIRI on changes in mitochondrial morphology were determined in HL-1 cells overexpressing red...
fluorescent protein targeted to the mitochondrial matrix and either empty vector or Drp1K38A. Over 3 independent experiments, 10 cells for each treatment group were analyzed.

**HL-1 Cell Death After SIRI**
To determine the effect of inducing mitochondrial elongation on the susceptibility to SIRI, HL-1 cells were subjected to 12 hours of simulated ischemia in an air-tight hypoxic chamber and 1 hour of simulated reperfusion, at the end of which cell death was assessed by propidium iodide staining. For each treatment group, 80 cells taken from 4 randomly selected fields of view were counted. This experiment was repeated on at least 4 separate occasions.

**Induction and Detection of mPTP Opening**
To determine the effect of inducing mitochondrial elongation on the susceptibility to mPTP opening in HL-1 cells, we used a well-characterized and validated model of oxidative stress to induce and detect mPTP opening in which confocal laser-induced activation of tetramethylrhodamine methyl ester (TMRM) generates oxidative stress within mitochondria and induces mPTP opening, which is indicated by mitochondrial membrane depolarization. In vivo mPTP opening was confirmed in this model through the use of the known mPTP inhibitors cyclosporin A (CsA) and sanglifehrin-A and investigation of the movement of mitochondrial-loaded calcein (see the online-only Data Supplement). Twenty transfected cells were randomly selected for each treatment group, and this was repeated in at least 4 independent experiments. For the adult cardiomyocytes, we used a different model to assess mPTP opening in which its opening is measured after 45 minutes of simulated ischemia and 30 minutes of simulated reperfusion by measuring the resultant TMRM fluorescence. Twenty cells were randomly selected for each treatment group, and this was repeated in at least 4 independent experiments.

**Pharmacological Inhibition of Drp1 to Induce Mitochondrial Fusion**
HL-1 cells were incubated with a small-molecule Drp1, inhibitor mitochondrial division inhibitor-1 (mdivi-1) (Key Organics Ltd, Camelford, Cornwall, UK), for 40 minutes (at 10 or 50 μmol/L) to investigate the effects of Drp1 inhibition on mitochondrial morphology, susceptibility to mPTP opening, and cell death in HL-1 cells after SIRI as described above.

**Confocal Microscopy of Adult Cardiomyocytes**
All animal experiments were carried out in accordance with the UK Home Office Guide on the Operation of Animal (Scientific Procedures) Act of 1986. Adenoviral vectors carrying the mitochondrial matrix-targeted photoactivatable green fluorescent protein (mPA-GFP) expression cassette were generated with the AdEasy XL Adenoviral Vector System (Stratagene, La Jolla, Calif). Ventricular cardiomyocytes were isolated from adult Sprague-Dawley rats by enzymatic digestion with collagenase and were cultured in 48-well plates. Twenty to 40 transfected cells expressing GFP were selected from each well and werephotoactivated with the 405-nm wavelength ultraviolet laser. The cell was immediately reimaged at 488 nm, and the difference in the intensity of green fluorescence between the 2 images before and after photoactivation was determined with Image J software (National Institutes of Health, Bethesda, Md). The spread of GFP beyond the ROI was expressed as a fold increase relative to the intensity within the ROI. Results were obtained from 30 randomly chosen cells isolated from 3 rats.

**Adult Cardiomyocyte Death After SIRI**
Ventricular cardiomyocytes were isolated from adult C57BL/6 male mice by perfusion and digestion of ventricles with collagenase according to a previously described method. The cells were incubated with phlebotomy medium containing an appropriate titer of virus for 4 hours and were imaged 72 hours later. mPA-GFP within a prespecified region of interest (ROI) was photoactivated by scanning adult rat cardiomyocytes with the 405-nm wavelength ultra violet laser. The cell was immediately reimaged at 488 nm, and the difference in the intensity of green fluorescence between the 2 images before and after photoactivation was determined with Image J software (National Institutes of Health, Bethesda, Md). The spread of GFP beyond the ROI was expressed as a fold increase relative to the intensity within the ROI. Results were obtained from 30 randomly chosen cells isolated from 3 rats.

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**In Vivo Model of IRI**
C57BL/6 male mice were anesthetized by intraperitoneal injection (0.01 mL/g) of a solution containing ketamine 10 mg/mL, xylazine 2 mg/mL, and atropine 0.06 mg/mL and were subjected to in vivo 30 minutes of regional myocardial ischemia followed by 120 minutes of myocardial reperfusion, at the end of which myocardial infarct size was determined by triphenyltetrazolium staining. Mice were randomly assigned to receive by intravenous injection either vehicle control (0.1 mL of 0.1% dimethyl sulfoxide) or mdivi-1 (at either 0.24 or 1.2 mg/kg, doses that were equivalent to the ex vivo concentrations of 10 and 50 μmol/L, respectively) 15 minutes before myocardial ischemia (n=6 mice per treatment group). The in vivo doses were calculated to reflect as closely as possible the in vitro concentrations used; however, the pharmacokinetic properties of the drug in vivo are unknown.

**Electron Microscopy**
Hearts were excised from C57BL/6 male mice after an intravenous injection of either vehicle control (0.1 mL of 0.1% dimethyl sulfoxide) or mdivi-1 (0.24 mg/kg) after 15 minutes of stabilization (n=4 mice) or after 15 minutes of stabilization followed by 20 minutes of regional ischemia (n=4 mice). The excised hearts were perfused with a fixative overnight, following which a 2-mm transverse slice, 3 mm from the apex, was obtained from each heart. Ultrathin sections were viewed with a Joel 1010 transition electron microscope (Joel Ltd, Warwickshire, UK). In 6 randomly selected electron micrographs of longitudinally arranged cardiomyocytes, the proportions of interfibrillar mitochondria with lengths were <2.2 or >2 μm (the length of a single sarcomere) were determined. For each heart, the lengths of >500 to 600 interfibrillar mitochondria were assessed.

**Statistical Analysis**
All values are expressed as mean±SEM. Data were analyzed by 1-way ANOVA followed by a Tukey multiple-comparison posthoc test. Differences were considered significant at values of P<0.05. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**
Inducing Mitochondrial Fusion in the HL-1 Cells
Transfecting HL-1 cells with Mfn1, Mfn2, or Drp1K38A increased the percentage of cells containing predominantly elongated mitochondria: 46±6% in the vector control versus 65±4% with Mfn1 (P<0.05), 69±5% with Mfn2 (P<0.01), and 63±6% with Drp1K38A (P<0.05; Figure 1F). Conversely, overexpressing hFis1 reduced the percentage of cells containing predominantly elongated mitochondria (46±6% in the vector control to 11±4% with hFis1; P<0.001; Figure 1F).

Mitochondrial Fission Is Induced by Ischemia and Is Dependent on Drp1
Mitochondria in the majority of HL-1 cells underwent fission in response to simulated ischemia (Figure 2A). However, this effect was largely prevented in cells overexpressing Drp1K38A (Figure 2A), suggesting that this fission process was dependent on Drp1. By the end of the simulated ischemia period, the percentage of cells displaying elongated mitochondria had fallen from 100% to 11.0±11.0% in control cells and to 91.0±5.6% in the Drp1K38A-transfected cells (P<0.05; Figure 2B), a difference that persisted into simulated reperfusion (Figure 2B). Of note, it would have been preferable to have performed this
experiment with an unselected population of HL-1 cells rather than choosing cells displaying only elongated mitochondria.

**Increased Mitochondrial Fusion Protects HL-1 Cells Against SIRI**

The overexpression of the mitochondrial fusion proteins (Mfn1 or Mfn2) or Drp1K38A significantly reduced the percentage cell death after SIRI (assessed after 1 hour of reperfusion): 41.8±4.1% in the vector control to 11.6±3.0% with Mfn1 ($P<0.0001$), 16.2±3.9% with Mfn2 ($P<0.0001$), and 12.1±2.9% with Drp1K38A ($P<0.001$; Figure 2C). In contrast, overexpression of hFis1 increased the percentage cell death (assessed after 1 hour of reperfusion): 41.8±4.1% in the vector control to 65.5±2.1% with hFis1 ($P<0.05$; Figure 2C). The percentage of dead cells under normoxic conditions was <5.0%, and this was not significantly altered by transgene expression.

**Inducing Mitochondrial Fusion in HL-1 Cells Decreases the Susceptibility to mPTP Opening**

Overexpression of Mfn1, Mfn2, or Drp1K38A delayed the time taken to induce mPTP opening compared with the vector control group by 2.4±0.5-fold with Mfn1 ($P<0.01$), 2.3±0.7-fold with Mfn2 ($P<0.05$), and 2.4±0.3-fold with Drp1K38A ($P<0.05$; Figure 3B). This was similar to the effect of the known mPTP inhibitor, CsA, which delayed the time taken to induce mPTP opening by 2.2±0.4-fold ($P<0.05$). The overexpression of hFis1 did not significantly influence the time taken to induce

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**Figure 2.** A, Representative confocal microscope images depicting HL-1 cells subjected to SIRI. The mitochondria in the control cell undergo fission; the mitochondria in the cell transfected with Drp1K38A are maintained in an elongated formation. B, HL-1 cells containing predominantly (>50%) elongated mitochondria under basal conditions were identified and subjected to SIRI. By 2 hours of simulated ischemia, the majority of control cells displayed fragmented mitochondria, whereas in those cells transfected with Drp1K38A, mitochondrial fission induced by simulated ischemia was largely prevented. n=3 experiments with 7 to 10 cells per treatment group. *$P<0.05$. C, Overexpression of HL-1 cells with Mfn1, Mfn2, or Drp1K38A decreased cell death after a period of SIRI, whereas overexpression with hFis1 increased cell death compared with control. n=4 experiments with 80 cells per treatment group. *$P<0.05$ vs control.
mPTP opening compared with the vector control group (0.89±0.70-fold with hFis1; \( P > 0.05 \)). To further verify that the observed mitochondrial membrane depolarization was due to mPTP opening, we demonstrated that its onset coincided with the redistribution into the cytosol of mitochondrion-loaded calcein (see the online-only Data Supplement).12,15

The Beneficial Effects of the Drp1 Inhibitor mdivi-1 in HL-1 Cells

The optimal treatment regimen for mdivi-1 was first determined in HL-1 cells identified as containing predominantly fragmented mitochondria only. At 50 \( \mu \text{mol/L} \) (the concentration previously demonstrated as having a maximal effect on mitochondrial elongation)13 but not at 10 \( \mu \text{mol/L} \), mdivi-1 significantly increased the percentage of HL-1 cells displaying elongated mitochondria after 40 minutes of drug incubation. After 20 minutes, the percentage of untreated cells containing elongated mitochondria rose from 0 to a new steady state of 19±11%, which was not significantly altered after a further 20-minute incubation (13±11%). After 20 minutes in 50 \( \mu \text{mol/L} \) mdivi-1, the percentage of elongated mitochondria was similar (19±3%), but by 40 minutes, this had increased significantly to 60±8% (Figure 4A). The presence of 10 \( \mu \text{mol/L} \) mdivi-1 had no significant effect (8±8% at 20 minutes and 19±3% at 40 minutes).

In a separate set of experiments, the incubation of HL-1 cells containing either elongated or fragmented mitochondria under basal conditions with 50 \( \mu \text{mol/L} \) mdivi-1 for 40 minutes resulted in an increase in the percentage of cells displaying elongated mitochondria from 26±7% at baseline to 67±4% after 40 minutes (\( P < 0.05 \)). There was no significant change in the percentage of cells displaying elongated mitochondria in cells treated with vehicle control (34±9% at baseline versus 38±8% after 40 minutes; \( P > 0.05 \)) or in cells treated with mdivi-1 at 10 \( \mu \text{mol/L} \) (33±1% at baseline versus 33±11% after 40 minutes; \( P > 0.05 \); Figure 4B).

Pretreatment of HL-1 cells with mdivi-1 for 40 minutes decreased the percentage cell death after subsequent exposure to SIRI at 50 \( \mu \text{mol/L} \) (42.1±4.8% in the vehicle control versus 20.0±2.6% with mdivi-1; \( P < 0.05 \)) but not at 10 \( \mu \text{mol/L} \) (42.1±4.8% in the vehicle control versus 45.5±3.5% with mdivi-1; \( P > 0.05 \); Figure 4C). Similarly, pretreatment of HL-1 cells with mdivi-1 for 40 minutes delayed the time taken to induce mPTP opening at 50 \( \mu \text{mol/L} \) (2.1±0.5-fold delay; \( P < 0.05 \)) but not at 10 \( \mu \text{mol/L} \) (0.6±0.1-fold delay; \( P > 0.05 \); Figure 4D). Again, we confirmed that the model was sensitive to CsA (2.3±0.5-fold delay; \( P < 0.05 \); Figure 4D).

Elongated Mitochondria Are Present in Adult Cardiomyocytes

Using confocal microscopy, we were able to visualize mitochondrial morphology in adult rat cardiomyocytes loaded with TMRM and the adenoviral construct expressing mTPA-GFP,
which is nonfluorescent but can be activated in a highly localized manner by scanning with the ultraviolet confocal laser line (Figure 5A). The photoactivated GFP diffuses rapidly within the mitochondrial matrix to the full extent of the inner mitochondrial membrane and can therefore be used to "tag" individual mitochondria. Using this experimental approach, we were able to identify elongated, interfibrillar mitochondria in primary adult cardiomyocytes. The length of these elongated mitochondria ranged from 2 to 6 μm (corresponding to 1 to 3 sarcomeres). In addition, we observed the spread of photoactivated mitochondrial GFP outside the initial area of photoactivation (2.17 ± 0.06-fold increase in the area of GFP; Figure 5A). This finding was confirmed by observing the changes in mitochondrial membrane potential taking place in individual mitochondria in response to low levels of oxidative stress generated by laser scanning of TMRM. This results in "flickering" of individual mitochondria as they depolarize and repolarize, with coincident loss and reaccumulation of TMRM dye. Both mitochondrial membrane depolarization and repolarization were found to occur in a synchronous fashion along the entire length of the elongated mitochondria (Movie I of the online-only Data Supplement), implying a single, elongated mitochondrion. After 72 hours of culture to allow expression of the adenoviral DNA constructs, there was some cell death and some rounding of cell ends, although the changes appear to be relatively minor and the healthy cells remain typically rod shaped (Figure 5A).

**The Beneficial Effects of Pharmacologically Inhibiting Drp1 in the Adult Heart**

Forty minutes of pretreatment of adult murine cardiomyocytes with 50 μmol/L mdivi-1 decreased the percentage cell death after SIRI (46.0 ± 1.1% in the vehicle control compared with 34.0 ± 1.9% with mdivi-1; P < 0.05), whereas pretreatment with 10 μmol/L mdivi-1 had no significant effect (46.0 ± 1.1% in the vehicle control to 48.0 ± 3.2% with mdivi-1; P > 0.05; Figure 6A). There was no significant effect of mdivi-1 on percentage cell death during normoxia (data not shown). Furthermore, 40 minutes of pretreatment with 50 μmol/L mdivi-1 decreased mPTP sensitivity after SIRI as evidenced by preservation of mitochondrial membrane potential detected with TMRM fluorescence (0.78 ± 0.06 SIRI control versus 1.04 ± 0.04 mdivi-1; P < 0.05; Figure 6B).

We examined longitudinal sections of adult murine hearts by electron microscopy to determine the arrangement and morphology of the interfibrillar subpopulation of mitochondria (Figure 5B). Interestingly, we observed a significant number of elongated interfibrillar mitochondria with lengths that extended beyond 2 μm and in some cases were up to 6 μm, allowing them
to align next to up to 3 adjacent sarcomeres (each \(\approx 2 \mu m\) in length; Figure 5C and 5D). We found that the in vivo treatment of adult murine hearts with mdivi-1 (50 \(\mu mol/L\)) for 15 minutes had no effect on mitochondrial length before ischemia (Figure 6C), although it significantly increased the proportion of elongated mitochondria (>2 \(\mu m\) or 1 sarcomere in length) after 15 minutes of stabilization plus 20 minutes of myocardial ischemia from 3.6\(\pm\)0.5% in control to 14.5\(\pm\)2.8% with mdivi-1.
Finally, the effect of pretreatment with mdivi-1 was investigated in vivo in the whole murine heart subjected to myocardial infarction using 2 doses, 0.24 mg/kg (dose 1) or 1.2 mg/kg (dose 2), which were equivalent to the in vitro concentrations of 10 and 50 μM, respectively. The hemodynamic profiles during coronary artery occlusion and reperfusion (in terms of heart rate and blood pressure) were comparable between the treatment groups (Figure 7A and 7B).

Figure 6. A, Pretreatment of adult rat cardiomyocytes with mdivi-1 at 50 but not 10 μmol/L reduced cell death after an episode of SIRI. n=4 experiments with 20 cells per treatment group. *P<0.05. B, Pretreatment of adult rat cardiomyocytes with mdivi-1 at 50 μmol/L inhibited mPTP opening as evidenced by the preserved TMRM fluorescence after SIRI. n=4 experiments with 20 cells per treatment group. *P<0.05. The percentages of mitochondria of lengths <1 sarcomere, equal to 1 sarcomere (1:1), or >1 sarcomere were determined by electron microscopy in hearts treated with placebo and mdivi-1 in vivo (C) before ischemia and (D) after 20 minutes of ischemia. After ischemia, mdivi-1 pretreatment increased the percentage of mitochondria >1 sarcomere compared with placebo. n=4 animals per treatment group. *P<0.05. E, Representative electron micrographs depicting (left) relatively fragmented mitochondria in a placebo-treated ischemic adult murine heart and (right) elongated mitochondria (marked with an asterisk) in an mdivi-1–treated ischemic adult murine heart.
However, the observed basal heart rates of 400 bpm in the control and treated groups were depressed to a similar extent by the anesthetic protocol used in this study. The area at risk, expressed as a percentage of the left ventricular volume, was also comparable between treatment groups: 53.6±5.6% in vehicle control versus 54.5±6.5% with mdivi-1 (dose 1) and 55.8±3.9% with mdivi-1 (dose 2; Figure 7C). A single intravenous bolus of mdivi-1 (dose 1) administered 10 minutes before acute coronary occlusion of the left main coronary artery failed to significantly reduce myocardial infarct size, expressed as a percentage of the area at risk (42.0±4.9% with mdivi-1 versus 48.0±4.5% in vehicle control; $P>0.05$), whereas a single intravenous bolus of mdivi-1 (dose 2) significantly reduced myocardial infarct size in the in vivo murine heart (21.0±2.2% with mdivi-1 versus 48.0±4.5% in vehicle control; $P<0.05$; Figure 7D and 7E).

**Discussion**

The present study has shown that modulating mitochondrial morphology protects the heart against IRI. We found that inducing mitochondrial elongation with either genetic or pharmacological manipulation decreased mPTP sensitivity and reduced cell death after SIRI in HL-1 cells. Crucially, in the adult rodent heart in which the spatial organization of interfibrillar mitochondria differs from HL-1 cells, elongated
mitochondria were identified with the use of confocal and electron microscopy. Furthermore, in vivo treatment with the Drp1 inhibitor mdivi-1 significantly increased the proportion of elongated interfibrillar mitochondria in the ischemic adult murine heart. Finally, pharmacological Drp1 inhibition with mdivi-1 protected adult cardiomyocytes against SIRI, inhibited mPTP opening in cardiomyocytes, and reduced myocardial infarct size in an in vivo murine model.

Changes in mitochondrial morphology may affect the susceptibility of the cell to apoptotic cell death, a contributory cause for the cell death incurred during IRI. In the kidney, renal injury has been reported to occur via the induction of Drp1-dependent mitochondrial fragmentation and apoptosis, and prevention of this process was found to be beneficial.

The limited number of studies that have examined mitochondrial morphology in cardiovascular cells have, on the whole, been confined to either cell lines or neonatal cardiomyocytes and have tended to focus on the effect on apoptosis (reviewed elsewhere). Mfn2 has been reported to exert a diverse range of effects that appear to be unrelated to its ability to elongate mitochondria (reviewed elsewhere). Therefore, we used the dominant-negative mutant form of the mitochondrial fission protein Drp1 (Drp1K38A) to induce mitochondrial elongation; this had the same effects as Mfn2, suggesting that the observed cardioprotection and mPTP inhibition were due to mitochondrial elongation.

The cardioprotective effect elicited by mitochondrial elongation was linked to the inhibition of mPTP opening in the present study. The mPTP is a critical mediator of cell death induced by IRI. Overexpressing Drp1 or hFis1 has been reported to increase the susceptibility to calcium-induced mPTP opening in COS epithelial cells, whereas in our study, the overexpression of hFis1 in HL-1 cells did not influence mPTP opening sensitivity. This may be due to a difference in models used, ie, a limitation in the resolution of our model to detect increased mPTP opening sensitivity. An alternative explanation may be that hFis1 increased death without a detectable effect on mPTP opening, suggesting that it may have damaging effects that are independent of the mPTP. Inhibiting Drp1 has been reported to increase mPTP resistance in response to hyperglycemia. Neuspiel and coworkers also found that the overexpression of Mfn2 prevented mPTP opening induced by free radicals in COS-7 cells. The mechanism through which increased mitochondrial elongation prevents mPTP opening is not clear, although one may speculate that elongated mitochondria may accommodate a greater burden of mitochondrial calcium load and oxidative stress before undergoing mPTP opening compared with fragmented mitochondria and that mitochondrial elongation may generate mitochondria with greater respiratory capacity that are better equipped to withstand the metabolic and biochemical stresses associated with IRI. In this regard, it has previously been demonstrated that altering mitochondrial fusion protein expression similarly alters oxygen consumption, mitochondrial membrane potential, and mitochondrial respiration in myotubes and other cells. It must be noted that the model of mPTP opening used in the present study may not accurately reflect the conditions of ischemia and reperfusion. Another limitation of the present study is that we used a number of different experimental models to investigate the effect of modulating mitochondrial morphology on the susceptibility to IRI when a single model may have been preferable.

Whether the spatial organization of interfibrillar mitochondria in adult cardiomyocytes restricts their movement and prevents them from undergoing fusion or fission has been the subject of recent debate. The machinery required for regulating mitochondrial morphology in terms of the mitochondrial fusion proteins and fission proteins is present in the adult heart. It has recently been demonstrated that interfibrillar mitochondria in adult rat cardiomyocytes are dynamic structures that are capable of undergoing rapid low-amplitude fluctuations, although they appear isolated with respect to electric activity, and it is generally assumed that mitochondria are restricted to 2-mum lengths alongside each sarcomere in adult cardiomyocytes. However, using both confocal and electron microscopy, we were readily able to identify elongated mitochondria extending up to 2 to 3 sarcomeres (4 to 6 mum) in length. The synchronous depolarization and repolarization of mitochondrial membrane potential in response to low levels of oxidative stress occurred throughout the whole length of the elongated mitochondrion, providing strong confirmation that we were visualizing a single functional unit. Interestingly, previous electron microscopy studies have observed individual mitochondria >2 to 3 sarcomeres in length, and as far back as 1969, Sun and coworkers demonstrated elongated mitochondria ranging from 3 to 7 sarcomeres in length in isolated perfused adult rat hearts in response to short periods of hypoxia (3 to 7 minutes). This raises the interesting, although at this point speculative, possibility that short nonlethal periods of hypoxia may have induced mitochondrial elongation as an endogenous protective mechanism against further hypoxic or ischemic insult. In this regard, a recently published study suggests that mitochondrial elongation may be an initial response to a low level of stress, which provides a protective response against a future insult.

Conclusions

We report here that inducing mitochondrial elongation protects the HL-1 cardiac cell line from IRI by inhibiting mPTP opening. We have been able to detect changes in mitochondrial morphology in the adult heart despite their distinctive arrangement. Crucially, we have demonstrated that pharmacological inhibition of the mitochondrial fission protein Drp1 protected adult murine cardiomyocytes against SIRI, inhibited mPTP opening, and more important, reduced myocardial infarct size in the in vivo murine heart. These findings suggest that manipulating mitochondrial morphology may provide a novel therapeutic strategy for cardioprotection.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Despite optimal therapy, clinical outcomes in patients with coronary heart disease can be further improved by the discovery of new therapeutic strategies for protecting the heart from ischemia/reperfusion injury. In this respect, in the present study, we have demonstrated that the modulation of mitochondrial morphology in the heart may provide a novel therapeutic strategy for cardioprotection. Specifically, we have demonstrated that inhibiting mitochondrial fission protected the heart by inhibiting the opening of the mitochondrial permeability transition pore, a critical mediator of ischemia/reperfusion injury. As a cardioprotective strategy, inhibition of mitochondrial fission may be induced through the pharmacological manipulation of mitochondrial morphology. In the present study, the in vivo pharmacological inhibition of the mitochondrial fission protein dynamin-related protein 1 with the small molecule inhibitor mdivi-1 reduced myocardial infarct size in the adult murine heart. The discovery of other novel compounds capable of inhibiting mitochondrial fission proteins such as dynamin-related protein 1 or activating mitochondrial fusion proteins such as mitofusin 1, mitofusin 2 or optic atrophy protein 1 may in the future be used to block mitochondrial fission in the clinical settings of acute ischemia/reperfusion injury to reduce myocardial injury and infarct size in patients undergoing cardiac bypass surgery or patients presenting with a myocardial infarction, respectively, and to improve clinical outcomes in patients with coronary heart disease.

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Inhibiting mitochondrial fission protects the heart against ischemia reperfusion injury

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Supplemental Methods

HL-1 cell plasmid transfection

The HL-1 cardiac cell line (derived from murine atrial cardiomyocytes) was cultured according to published methods (1). Upon reaching 50-60% confluency, the cells were seeded onto fibronectin-coated cover-slips and transfected, using Fugene 6 (Roche Molecular Biochemicals) according to standard protocols, with one of the following plasmids: an empty plasmid expression vector (RcCMV); one expressing mitofusin 1 (pCB6-MYC-Mfn1); one expressing mitofusin 2 (pCB6-MYC-Mfn2) (2); one containing Drp1\textsubscript{K38A} (pcDNA3.1-HA-K38A-DRP1), the dominant negative mutant form of the mitochondrial fission protein Drp1\textsuperscript{3}; and one containing hFis1. Drp1\textsubscript{K38A} has a mutation in the GTPase domain that results in replacement of lysine 38 with alanine (designated as Drp1\textsubscript{K38A}), disabling its ability to induce mitochondrial fission (3). For the mitochondrial morphology studies a ratio of 2:1 mitochondria-targeted red fluorescent protein (mtRFP: Mitochondria-targeted dsRED) expression plasmid was included in order to permit visual assessment of mitochondrial morphology. All plasmids were a generous gift of Dr Luca Scorrano (Padova, Italy).

For the simulated ischemia-reperfusion injury and mPTP experiments, a 1:2 ratio of pEGFP expression plasmid (Clontech) was included in order to identify those cells which had been successfully transfected. After transfection for 24 hours, the buffer was replaced with fresh culture medium and the cells were left in an incubator overnight.

Determining mitochondrial morphology in HL-1 cells
Changes in mitochondrial morphology in HL-1 cells transfected with mtRFP were determined using a Zeiss 510 CLSM confocal microscope equipped with 63x oil immersion, quartz objective lens (Plan Apochromat, NA 1.3). The cells were illuminated using the 543-nm emission line of a HeNe laser. The fluorescence of mtRFP was collected using a 560-nm long pass filter. Images were analyzed using the Zeiss software (LSM Image Browser v3.5 or 4.0). The Claycomb culture medium was removed and replaced with Krebs imaging buffer comprising (in mM): NaCl 118.0, NaHCO₃ 25.0, d-Glucose 11.0, KCl 4.7, MgSO₄·7H₂O 1.2, KH₂PO₄ 1.2, CaCl₂·2H₂O 1.8, and HEPES 10.0 (pH 7.4). Images of twenty randomly chosen cells were taken and this was repeated for each group in at least four independent transfection experiments giving a total number of approximately 80 cells per treatment group. Three investigators, blinded to the initial treatment, independently assigned the cells as displaying either predominantly (>50%) elongated or (>50%) fragmented mitochondria, indicating that either mitochondrial fusion or fission, respectively, was the predominant process in that cell at that particular time, a method which has been adapted from a previously published study (2). Figures 1a and 1b depict representative HL-1 cells displaying fragmented mitochondria and elongated mitochondria, respectively.

Real-time changes in mitochondrial morphology during ischemia and reperfusion

In order to determine the effect of simulated ischemia-reperfusion injury on changes in mitochondrial morphology, HL-1 cells were imaged in real-time using an air-tight hypoxic chamber mounted on the confocal microscope. HL-1 cells transfected with
mtRFP and either RcCMV or Drp1K38A were seeded onto coverslips, and placed into a Warner PM-2 heated perfusion chamber (Harvard Apparatus, USA). The HL-1 cells were perfused with normoxic buffer specific for the real-time imaging experiments (in mM: NaCl 110, KCl 4.7, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.25, CaCl$_2$ 1.2, NaHCO$_3$ 25.0, glucose 15.0, HEPES 20.0, pH 7.4) equilibrated with 95% O$_2$-5% CO$_2$. HL-1 cells with predominantly (>50%) elongated mitochondria were then located and imaged using the 63x objective lens. To simulate ischemia, the cells were perfused with hypoxic ischemic buffer specific for the real-time imaging experiments (in mM: NaCl 125, KCl 8, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.25, CaCl$_2$ 1.2, NaHCO$_3$ 6.25, Na-lactate 5.0, HEPES 20.0, 2-deoxyglucose 2.5, pH 6.6) equilibrated with 95% N$_2$-5% CO$_2$, at a rate of 1 ml/min, for 120 minutes. After this, to simulate reperfusion, the HL-1 cells were reoxygenated with normoxic buffer. Images of the same HL-1 cells were acquired prior to simulated ischemia, after 120 minutes simulated ischemia, at the immediate onset of simulated reperfusion and after 30 minutes of simulated reperfusion. For each treatment group 7-10 cells were counted. This experiment was repeated on three independent occasions giving a total of 21-30 cells per treatment group.

**HL-1 cell death following simulated ischemia-reperfusion injury**

In order to determine the effect of inducing mitochondrial fusion on the susceptibility to simulated ischemia-reperfusion injury, HL-1 cells were subjected to a lethal episode of simulated ischemia and reperfusion. The culture medium was removed and replaced by hypoxic ischemic buffer specific for the cell-death experiments (comprising in mM:
SUPPLEMENTAL MATERIAL

KH₂PO₄ 1.0, NaHCO₃ 10.0, MgCl₂.6H₂O 1.2, NaHEPES 25.0, NaCl 74.0, KCl 16, CaCl₂ 1.2 and NaLactate 20 at pH 6.2, bubbled with 100% nitrogen) and then placed in an airtight custom-built hypoxic chamber kept at 37°C for 12 hours to simulate ischemia. A shorter period of simulated ischemia (i.e. 2 hours) was used for the real-time studies described above, based on a previously published study (4) in which the hypoxic ischemia buffer contained deoxyglucose in addition to the other constituents. Following the period of simulated ischemia, the cells were removed from the hypoxic chamber and placed in normoxic Claycomb medium (containing 3 µM propidium iodide) and returned to a tissue culture incubator, to simulate reperfusion. After 1 hour of simulated reperfusion at 37°C, the percentage of GFP-transfected cells stained with propidium iodide was determined using a Nikon Eclipse TE200 fluorescent microscope in order to calculate the percentage cell death in each treatment group. For each treatment group 80 cells were counted, taken from four randomly-selected fields of view. This experiment was repeated on at least four separate occasions giving a total of 320 cells per treatment group. For a time-matched normoxic control group, HL-1 cells were placed in normoxic buffer specific for cell-survival experiments (5) (comprising in mM: KH₂PO₄ 1.0, NaHCO₃ 10.0, MgCl₂.6H₂O 1.2, NaHEPES 25.0, NaCl 98.0, KCl 3, CaCl₂ 1.2, d-glucose 10.0, Na pyruvate 2.0 at pH 7.4, bubbled with 5% CO₂/95% O₂) for the total 13 hours duration of the experiment and the percentage cell death was determined.

*Induction and detection of mPTP opening*
In order to determine the effect of inducing mitochondrial fusion on the susceptibility to mPTP opening, we used a well-characterized and validated model of oxidative stress to induce and detect mPTP opening \(^{(6-9)}\). The culture medium was removed and replaced with Krebs imaging buffer. The HL-1 cells were then loaded with the fluorescent dye tetramethylrhodamine methyl ester (TMRM, 3 µM) for 15 min at 37°C and then washed with Krebs imaging buffer. Confocal laser-stimulation of TMRM generates reactive oxygen species (ROS) within the mitochondria \(^{(10)}\) thereby simulating mitochondrial ROS production during reperfusion, which induces mPTP opening. The opening of the mPTP is visualized as mitochondria membrane depolarization and the dequenching of TMRM fluorescence as it moves into the cytoplasm. The time taken to induce mitochondrial membrane depolarization is recorded as a measure of susceptibility to mPTP opening. This was defined as the time taken to reach half the maximum TMRM fluorescence intensity. Twenty transfected cells were randomly selected for the induction and detection of mPTP opening from each treatment group, and this was repeated in at least four independent experiments giving a total of 80 cells per treatment group. As a positive control and in order to confirm that mitochondrial membrane depolarization was indicative of mPTP opening, following TMRM loading, a group of cells were pre-treated for 10 minutes with the mPTP inhibitor, cyclosporin A (0.2 µM) \(^{(11)}\). In order to confirm that this model was actually measuring mPTP opening we used the mPTP inhibitor, we pre-treated cells for 10 minutes with Sanglifehrin A (SfA, 1.0 µM), which does not inhibit calcineurin \(^{(12)}\). In a previous study, we have also confirmed that the mitochondrial membrane depolarization induced by oxidative stress in this
experimental model reflects mPTP opening as evidenced by the redistribution of mitochondrial-loaded calcein (6;13).

To confirm the efficacy of the experimental protocol used for inducing and detecting mPTP opening, we also examined the effect of laser-induced mitochondrial oxidative stress on the redistribution of the fluorescent dye, calcein, from the mitochondrial matrix. We used an established method for detecting mPTP opening in the intact cell (13-15). HL-1 cells were loaded for 10 minutes at room temperature with 1µM calcein-AM + 1 mM CoCl2. In this model, calcein-AM, which is membrane permeable, enters the cytosol and mitochondria, where on de-esterification the calcein becomes entrapped within the cytosol and the mitochondria. The CoCl2 quenches the calcein signal within the cytosol only, leaving calcein fluorescence selectively visible within the mitochondria. Because of its relatively large size (620 Da), the only way calcein can exit the mitochondria is if the mPTP opens. Therefore, the extent of mPTP opening can be measured by the loss of mitochondrial calcein fluorescence (13;14;16).

HL-1 cells were visualized using a Zeiss 510 CLSM confocal microscope equipped with 40x oil immersion, quartz objective lens (Plan-Neofluar, NA 5 1.3) using the 488-nm of an Argon laser and the 543-nm emission line of a HeNe laser. Time scans were recorded with simultaneous excitation at 488 nm (for GFP and calcein) and 543 nm (for TMRM), collecting fluorescence emission at 505–530 nm and >560 nm, respectively. For these mPTP experiments, all conditions of the confocal imaging system (laser power, confocal pinhole - set to give an optical slice of 1 micron - pixel dwell time, and detector sensitivity) were identical to ensure comparability between
experiments. Images were analyzed using the Zeiss software (LSM Image Browser v3.5 or 4.0).

In adult cardiomyocytes a different model of mPTP opening was used in which the cells were subjected to 45 minutes of simulated ischemia then 30 minutes of simulated reperfusion to simulate ischemia-reperfusion injury, a model which we have previously established in our laboratory. Simulated ischemia was induced in a custom-made airtight hypoxic chamber, using a hypoxic ischemic buffer specific for cell-survival experiments (5) (in mM: KH2PO4 1, NaHCO3 10, MgCl2.6H2O 1.2, NaHEPES 25, NaCl 74, KCl 16.0, CaCl2 1.2 and Na lactate 20.0, pH 6.2), bubbled with 100% nitrogen. Simulated reperfusion was achieved by replacing the buffer with M199 culture medium containing 100 nM TMRM, or a combination of M199 containing 100 nM TMRM with either 50µM mdivi-1 or 1 µM Sanglifehrin A. At the end of the simulated reperfusion, the M199 reperfusion medium was replaced with fresh M199 medium alone and images of the cells were obtained to determine differences in fluorescent intensities. Therefore, in this model of the resultant TMRM fluorescence at reperfusion provides a measure of mPTP sensitivity.

Pharmacological inhibition of Drp1 to induce mitochondrial fusion

The recently described pharmacological inhibitor of Drp1, called mdivi-1 (mitochondrial division inhibitor-1)(Key Organics Ltd, UK) (17), was used in the current study to investigate the effects of pharmacologically inducing mitochondrial fusion on cardioprotection. Using the HL-1 cardiac cells we determined the optimum time required
for mdivi-1 treatment in order to induce maximal mitochondrial fusion. HL-1 cells were transfected with mtRFP and were treated with mdivi-1 dissolved in Krebs imaging buffer at two different concentrations: at 50µM, the dose shown previously to induce maximum mitochondrial fusion (17), and at 10µM. HL-1 cells with predominantly fragmented mitochondria were identified and the subsequent changes in mitochondrial morphology monitored over a period of 40 minutes. Twelve HL-1 cardiac cells from each treatment group were imaged for mitochondrial morphological analysis and this was repeated for each group in at least four independent experiments giving a total number of approximately 50 cells per treatment group. To further validate the results in a population of cells, a separate group of HL-1 cardiac cells were incubated in mdivi-1 at 50µM or 10µM dissolved in Krebs imaging buffer for forty minutes, and mitochondrial morphology determined. Twenty cells from each treatment group were imaged for subsequent mitochondrial morphological analysis and this was repeated for each group in at least four independent experiments giving a total number of 80 cells per treatment group.

In order to examine the effects of pharmacological inhibition of Drp1 on cell survival following a period of simulated ischemia-reperfusion injury, HL-1 cells were incubated with 0.01% DMSO (vehicle control), 10µM mdivi-1 or 50µM mdivi-1 for 40 minutes and were then subjected to 12 hours of simulated ischemia followed by 1 hour of simulated reperfusion in the presence of the mdivi-1. For each treatment group 80 cells were counted, taken from four randomly-selected fields of view. This experiment was repeated in at least four independent experiments giving a total of 320 cells per
In order to examine the effects of pharmacological inhibition of Drp1 on the susceptibility to mPTP opening in response to oxidative stress, HL-1 cells were treated with either 0.01% DMSO (vehicle control), 10µM mdivi-1 or 50µM mdivi-1 for 40 minutes. The HL-1 cells were then loaded with TMRM; 3 µM with either 0.01% DMSO, 10µM 10µM mdivi-1 or 50µM mdivi-1 for 15 min at 37ºC and then washed with either Krebs imaging buffer containing 0.01% DMSO, 10µM mdivi-1 or 50µM mdivi-1. Twenty cells were randomly selected for the induction and detection of mPTP opening from each treatment group, and this was repeated in at least four independent experiments giving a total of 80 cells per treatment group.

Construction of mitochondrial matrix targeted PA-GFP adenoviral vectors

The mitochondrial matrix-targeted photo-activatable green fluorescent protein (mtPA-GFP) plasmid encodes the photo-activatable green fluorescent protein as well as the mitochondrial targeting sequence for the subunit VIII of Cytochrome C oxidase (kind gift from Dr Luca Scorrano). Adenoviral vectors carrying the mtPA-GFP expression cassette were generated using AdEasy XL Adenoviral Vector System (Stratagene). A KpnI restriction site was introduced upstream of the COX VIII encoding sequence by PCR using mutagenic oligonucleotide primer pair (Left primer 5’-GCTGGTTTAGGGTACCGTCAG-3’; Right primer 5’-GGAGGTGTGGGGAGGTTTT-3’). Using the restriction sites KpnI and NotI, the PCR product was cloned into the multiple cloning site of shuttle vector pShuttle-CMV provided in the kit. The modified shuttle
vector was then used to prepare the adenovirus vector as per the instructions. An adenovirus stock solution of 3 to 3.5x10^6 pfu/ml was used for the experiments. The multiplicity of infection used for the adenovirus was 1000.

Adenovirus-mediated transduction of mtPA-GFP in adult rat cardiomyocytes

All animal experiments were carried out in accordance with the United Kingdom Home Office Guide on the Operation of Animal (Scientific Procedures) Act of 1986. Adult rat ventricular cardiomyocytes were isolated from adult Sprague Dawley rats by perfusion and digestion of ventricles with collagenase, according to a previously described method (6). The cells were then seeded onto laminin-coated cell-culture dishes. After 1 hour of incubation in the cardiomyocytes growth medium (M199 buffer: BSA 2 mg/ml, creatine 5 mM, taurine 5 mM, carnitine hydrochloride 1.6 mM, Pencillin-Streptomycin 1%), the cells were incubated with plating medium containing an appropriate titre of virus for 4 hours, after which the medium was replaced by fresh virus-free plating medium. The cells were imaged using confocal microscopy for the expression of mtPA-GFP after 72 hours. After excitation of the cells with the ultraviolet laser to activate mtPA-GFP, fluorescing mtPA-GFP localized solely to mitochondria, as confirmed by co-localization of TMRM, a cell-permeable red-fluorescent dye that is sequestered by mitochondria according to the mitochondrial membrane potential.

Detecting mitochondrial fusion in adult cardiomyocytes using mtPA-GFP

We investigated mitochondrial morphology in adult rat cardiomyocytes expressing
mtPA-GFP using confocal microscopy. Initially, a background image of the cardiomyocyte was obtained using the 488nm laser. Next PA-GFP within a specified Region of Interest (ROI) was photo-activated by scanning with the 405 nm wavelength ultraviolet laser. The cell was immediately re-imaged at 488nm and the difference in the intensity of green fluorescence between the two images – prior to and after photo-activation, was determined using Image J software (NIH, US). In the situation where all mitochondria are fragmented and disconnected, one would expect the photo-activated GFP to remain within the boundaries of the ROI. However, the fact that photo-activated GFP was able to spread outside the ROI would imply that it has diffused throughout an elongated mitochondria or alternatively fusion had occurred between neighboring mitochondria. The spread of GFP beyond the ROI was expressed as a fold increase relative to the intensity within the ROI, to account for different efficiency of activation in different transfectants. Results were obtained from 30 randomly chosen cells isolated from 3 rats (N=3 experiments with 30 cells). The imaging parameters were identical the same for all the experiments.

Detecting mitochondrial fusion in adult cardiomyocytes using electron microscopy

C57BL/6 male mice were anesthetised by intraperitoneal injection with a combination of ketamine, xylazine and atropine (0.5-0.7 ml of solution containing: final concentration of ketamine, xylazine and atropine were 10 mg/ml, 2 mg/ml and 0.06 mg/ml respectively), and the hearts rapidly excised and perfused with a fixative containing fresh paraformaldehyde 1%, glutaraldehyde 1%, CaCl₂ 0.5 mM), glucose 0.031% in
phosphate buffer 0.1 M (pH 7.3), and left in fixative overnight before sampling. A 2 mm transverse slice through the whole heart, 3 mm from the apex, was obtained from each heart. These heart slices were then post-fixed in OsO₄ (1%) in phosphate buffer (0.1 M) pH 7.3 at 3.0°C for 1.5 hrs. They were then washed in phosphate buffer (0.1 M) pH 7.4 and Enbloc stained with 0.5% uranyl acetate in distilled water at 3.0°C for 30 minutes. After rinsing with distilled water, the specimens were dehydrated in a graded ethanol-water series and infiltrated with Agar-100 resin overnight (Agar Scientific, UK). Semi-thin sections were cut at 1µm, and mounted on glass slides and stained with toluidine blue (1%) in distilled water for light microscopy. Ultra-thin sections were then cut at 70-80 nm using a diamond knife on a Reichert Ultracut E microtome (Reichert Microscope Services, USA). Sections were collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate. The heart specimens were then viewed and recorded with a Joel 1010 transition electron microscope (Joel Ltd, UK).

The assessment of mitochondrial morphology was accomplished by randomly selecting 4 random electron micrographs of longitudinally-arranged cardiomyocytes from each adult heart (N=4 hearts). The arrangement and morphology of the interfibrillar mitochondria were noted and the number of mitochondria whose length was greater than 2 µm (the length of a single sarcomere) was determined.

*Experimental protocol for adult murine ventricular cardiomyocyte isolation*

We used a model of simulated ischemia-reperfusion injury to determine the effect of inducing mitochondrial fusion using mdivi-1 on the resistance of isolated murine
ventricular cardiomyocytes to cell death. Adult murine ventricular cardiomyocytes were isolated from adult C57BL/6 male mice by perfusion and digestion of ventricles with collagenase, according to a previously described method (18).

All cells were subjected to 45 minutes of simulated ischemia then 30 minutes of simulated reperfusion to simulate ischemia-reperfusion injury, a model which we have previously established in our laboratory (18). Simulated ischemia was induced in a custom-made airtight hypoxic chamber, using a hypoxic ischemic buffer specific for cell-survival experiments (5) (in mM: KH₂PO₄ 1, NaHCO₃ 10, MgCl₂.6H₂O 1.2, NaHEPES 25, NaCl 74, KCl 16.0, CaCl₂ 1.2 and Na lactate 20.0, pH 6.2), bubbled with 100% nitrogen. Simulated reperfusion was achieved by replacing the buffer with M199 culture medium. Cells were seeded onto laminin-coated cover-slips and randomized to the following treatment groups: (1) vehicle control, and (2) mdivi-1 treatment at either 10µM or 50µM (N>250 cells per experiment for 4 experiments).

At the end of the simulated reperfusion, 5 µl of propidium iodide (PI, 1 µg/ml) was added to the cells for 5 minutes. The percentage of dead cells (as indicated by red fluorescence, PI positive) was calculated by fluorescence microscopy and was expressed as a percentage of the total number of cardiomyocytes (PI positive and PI negative) (18).

**In vivo murine model of acute myocardial infarction**

We used an in vivo murine model of myocardial infarction to determine the effect of inducing mitochondrial fusion on the susceptibility of the heart to myocardial infarction. C57BL/6 male mice (8-12 weeks of age and weighing 25-30g) were anesthetised by...
intraperitoneal injection with a combination of ketamine, xylazine and atropine (0.01 ml/g of a final solution of, final concentration of ketamine, xylazine and atropine were 10 mg/ml, 2 mg/ml and 0.06 mg/ml respectively) and body temperature was maintained at 37°C. The external jugular vein and carotid artery were isolated and cannulated for drug administration and mean arterial blood pressure (MABP) measurement, respectively. A tracheotomy was performed for artificial respiration at 120 strokes/min and 200 µl stroke volume using a rodent Minivent (type 845, Harvard Apparatus, Kent, UK) and supplemental oxygen was supplied. A limb lead I electrocardiogram (ECG) was recorded. A left anterior thoracotomy and a chest retractor were used to expose the heart. Ligation of the left anterior descending (LAD) coronary artery was performed ~2 mm below the tip of the left atrium using a 8/0 prolene monofilament polypropylene suture. Successful LAD coronary artery occlusion was confirmed by the presence of ST-segment elevation and a reduction in arterial blood pressure. At the end of reperfusion, the heart was isolated and the aortic root was cannulated and used to inject TTC (5 ml of 1%) in order to demarcate the infarcted tissue. The LAD coronary artery was then re-ligated and Evans blue dye (2 ml of 0.5%) was perfused to delineate the area at risk (AAR). The heart was frozen and sectioned perpendicularly to the long axis (1-2 mm thick). The slices were then transferred to 10 % neutral buffer formalin for 2 hours at room temperature to stabilise the staining. AAR and infarct size were determined by computerised planimetry using the NIH software Image. AAR was expressed as a percentage of the left ventricle and infarct size was expressed as a percentage of the AAR (18).
C57BL/6 male mice were randomly assigned to one of three treatment groups: (1) Vehicle control (n=6): an intravenous bolus of DMSO (0.1ml of 0.1%) was given 15 minutes prior to the index myocardial ischemic episode, (2) mdivi-1 (n=6): an intravenous bolus of mdivi-1 (0.24 mg/kg) was given 15 minutes prior to the index myocardial ischemic episode, and (3) mdivi-1 (n=6): an intravenous bolus of mdivi-1 (1.2 mg/kg) was given 15 minutes prior to the index myocardial ischemic episode. These mdivi-1 concentrations were estimated from the ex-vivo concentrations of 10µM and 50µM used in the isolated adult murine cardiomyocytes experiments. Hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion at the end of which infarct size was determined by triphenyl-tetrazolium staining.

**Supplemental Data**

*Redistribution of mitochondrial calcein into the cytosol on mPTP opening*

In HL-1 cells, mPTP opening was indicated by an increase in TMRM fluorescence which coincided with the redistribution of mitochondrial calcein into the cytosol.

**Figure 1**

This graph demonstrates that the increase in TMRM fluorescence (in arbitrary units, a.u.) coincides with the redistribution of mitochondrial calcein fluorescence from the mitochondria into the cytosol, indicated by a reduction in standard deviation (SD)/mean calcein fluorescence intensity (a.u.).
The cell model of mPTP opening is sensitive to Sanglifehrin A

In HL-1 cells the TMRM model of mPTP opening was demonstrated to be sensitive by Sanglifehrin A (SfA), an mPTP inhibitor which does not inhibit calcineurin.

Figure 2

This graph demonstrates both ciclosporin A (CsA) and sanglifehrin A (SfA) delayed the time taken to induce mPTP opening. N=4 experiments with 20 cells per treatment group.*P<0.05.
Supplemental Reference List


**Video legend**

Representative movie of an adult rat cardiomyocyte loaded with the fluorescent dye TMRM. This movie is a close-up of two elongated mitochondria (extending up to 2-3 sarcomeres in length) which display exactly synchronous depolarization and repolarization in mitochondrial membrane potential as indicated by ‘flickering’ in TMRM fluorescence in response to minimal oxidative stress.