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Inhibition of the Mitochondrial Fission Protein Drp1 Improves Survival in a Murine Cardiac Arrest Model

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Abstract

Objectives—Survival following sudden cardiac arrest is poor despite advances in cardiopulmonary resuscitation (CPR) and the use of therapeutic hypothermia. Dynamin related protein 1 (Drp1), a regulator of mitochondrial fission, is an important determinant of reactive oxygen species generation, myocardial necrosis, and left ventricular function following ischemia/reperfusion injury, but its role in cardiac arrest is unknown. We hypothesized that Drp1 inhibition would improve survival, cardiac hemodynamics, and mitochondrial function in an *in vivo* model of cardiac arrest.

Design—Laboratory investigation.

Setting—University laboratory

Interventions—Anesthetized and ventilated adult female C57BL/6 wild-type mice underwent an 8-min KCl induced cardiac arrest followed by 90 seconds of CPR. Mice were then blindly randomized to a single intravenous injection of Mdivi-1 (0.24 mg/kg), a small molecule Drp1 inhibitor or vehicle (DMSO).

Measurements and Main Results—Following resuscitation from cardiac arrest, mitochondrial fission was evidenced by Drp1 translocation to the mitochondrial membrane and a decrease in mitochondrial size. Mitochondrial fission was associated with increased lactate and evidence of oxidative damage. Mdivi-1 administration during CPR inhibited Drp1 activation, preserved mitochondrial morphology, and decreased oxidative damage. Mdivi-1 also reduced the time to return of spontaneous circulation (ROSC) 116±4 vs. 143±7 sec (p<.001) during CPR and enhanced myocardial performance post-ROSC. These improvements were associated with significant increases in survival (65% vs. 33%) and improved neurological scores up to 72 hours post cardiac arrest.

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Disclosures: None.

Conclusions—Post cardiac arrest inhibition of Drp1 improves time to ROSC and myocardial hemodynamics resulting in improved survival and neurological outcomes in a murine model of cardiac arrest. Pharmacological targeting of mitochondrial fission may be a promising therapy for cardiac arrest.

Keywords

Cardiac Arrest; Therapeutic Hypothermia; Mdivi-1; Cardiopulmonary Resuscitation (CPR); cardioprotection; neuroprotection; ischemia/reperfusion injury; resuscitation; mitochondrial fission

Introduction

Sudden cardiac arrest is a leading cause of death in the United States and worldwide(1, 2). Rapid utilization of defibrillation and an emphasis on high quality cardiopulmonary resuscitation (CPR) have improved survival, but mortality remains high. Most victims fail to ever achieve return of spontaneous circulation (ROSC), and if achieved, suffer from post-cardiac arrest syndrome (3). This syndrome is characterized by cardiovascular instability and neurological injury resulting in a survival rate of 10% for out of hospital cardiac arrest (3). Global ischemia-reperfusion (IR) injury of the myocardium during cardiac arrest resuscitation is a known contributor to post cardiac arrest myocardial dysfunction and a likely contributor to the failure to achieve ROSC (3, 4). Mild hypothermia attenuates IR injury in cellular and animal models of cardiac arrest (5–7) and is now used clinically to improve human survival and neurological outcomes (8,). Unfortunately, hypothermia as a clinical therapy is limited by the time required for implementation as well as potential side effects (10). Pharmacological strategies to improve myocardial function post cardiac arrest are needed to improve rates of spontaneous circulation as well as limit cardiovascular and neurological reperfusion injury immediately following resuscitation. One promising strategy is the targeting of mitochondrial fission following IR injury (11, 12).

Mitochondria are essential to myocardial function based on their pivotal role in energy production. The myocardium has one of the highest densities of mitochondria of any tissue (12). However, in addition to generating ATP, mitochondria are important regulators of calcium, reactive oxygen species (ROS), and metabolic substrates. These processes are regulated in part by mitochondrial changes in morphology. Mitochondria are dynamic organelles that undergo cycles of fission (division) and fusion (joining). These cycles serve to remove damaged mitochondria (mitophagy), distribute mitochondria between dividing cells, and to help regulate cellular response to stress (13, 14). Mitochondrial fission occurs in the heart following IR injury and has been demonstrated to be responsible for increased ROS generation and infarct generation. Inhibiting mitochondrial fission even after the heart is ischemic, reduces calcium concentrations in cardiac myocytes and improves myocardial function (15, 16).

Mitochondrial fission is primarily regulated by the GTPase dynamin related protein 1 (Drp1) and the Drp1 targeting molecule (Fis1) (11, 17). Drp1 is a cytosolic protein that is targeted to the outer mitochondrial membrane to initiate fission by interaction with binding partners (as Fis1) (18, 19). Changes in Drp1 expression can initiate fission but regulation occurs

mainly by post-translational modification of Drp1. Drp1 can be modified post-translationally via proteases, sumoylation, or altered phosphorylation states. During IR injury of the myocardium, Drp1 dephosphorylation at Serine 637 (S637) is mediated by the calcium-activated phosphatase calcineurin resulting in mitochondrial fission (20, 21). These effects can be attenuated using the small molecule inhibitor of Drp1 known as Mdivi-1 which has been demonstrated to be an effective pre-conditioning agent(22, 23). Recently, we have demonstrated that Mdivi-1 improves myocardial function when administered at the time of reperfusion by inhibiting calcineurin induced dephosphorylation of Drp1 in a Langendorff model of global cardiac ischemia (15). However, it unknown whether Drp1 is activated following cardiac arrest *in vivo* and whether Drp1 inhibition *at the time of resuscitation from cardiac arrest* improves outcomes.

In this study, we hypothesized that global IR injury resulting from asystolic cardiac arrest, in an *in vivo* murine model, would result in Drp1-S637 dephosphorylation and its subsequent translocation to the mitochondria resulting in mitochondrial fission. We further hypothesized that post cardiac arrest Drp1 activation would contribute to myocardial dysfunction and that an inhibitor of Drp1 would improve myocardial hemodynamics, survival, and neurological outcomes in an established murine model of cardiac arrest.

Materials and Methods

Reagents

All reagents used were purchased from Sigma (St Louis, MO) unless otherwise specified. Mdivi-1 was obtained from Enzo life sciences (Farmingdale, NY). Mdivi-1 was dissolved in Dimethyl sulfoxide (DMSO) and used at a concentration of 0.24 mg/kg Mdivi-1 for most experiments. DMSO vehicle was used as a control for all experiments involving Mdivi-1 or for Sham treated mice.

Mouse Cardiac Surgery and Anesthetization and mode of Cardiac Arrest

In this study, we used a previously published murine model of asystolic cardiac arrest (24–26). This model of potassium induced cardiac arrest was chosen because of it allows a reproducible method of precisely timing cardiac arrest and because electrical defibrillation of the murine heart is damaging and impractical (24). The Institutional Animal Care and Use Committee (IACUC) of the University of Chicago, in accordance with National Institutes of Health guidelines, approved of all animal procedures. Adult (age 6–8 months, 20–30g) retired breeder female C57BL/6 mice were used for the study and were allowed free access to food and water prior to experiments. Mice (25–30g) were anesthetized with ketamine/xylazine, intubated/ventilated and surgically prepared as previously described for this model, except for echocardiography experiments in which mice received 1% isoflurane for anesthesia. In all experiments, a 0.4mm O.D. heparinized micro-PE Cannula (BioTime Inc., Berkeley, CA) was placed in the left jugular vein for fluid administration. For animals undergoing invasive hemodynamic monitoring, a 1.0 F pressure–volume (P–V) catheter (PVR-1030, Millar Instruments, Houston, TX) was advanced into the left ventricle via the carotid artery to determine baseline measurements but was withdrawn into the ascending aorta during cardiac arrest and CPR. Asystolic cardiac arrest was induced by an intravenous

bolus of 0.08mg/g KCl via the jugular catheter and the ventilator was disconnected. Following 8 minutes of asystolic cardiac arrest, the ventilator was reconnected and manual chest compression were performed at a rate 350~400bpm. After 90 seconds of CPR, 1.5 µg epinephrine combined with 0.24 mg/kg Mdivi-1 dissolved in Dimethyl sulfoxide (DMSO) or epinephrine + DMSO only was injected in a blinded fashion. In either case the intervention was followed by saline flush in a total volume of 400 µl. CPR was terminated when ROSC was achieved (defined by a sinus rhythm with a mean arterial pressure (MAP) greater than 40 mmHg lasting at least 5 minutes) or after 5 minutes of unsuccessful CPR. Resuscitated animals received intravenous 0.9% saline at a rate of 100 µl/h and were monitored on mechanical ventilation for up to 120 minutes. Thereafter, they were extubated and allowed free access to water and food. Animals for survival studies were returned to the animal facility and observed for 72 hours post cardiac arrest. An illustration of the protocol used is provided (Figure 1).

Mouse Echocardiography

Two-dimensional M mode echocardiography in 1% vaporized isoflurane anesthetized mice was performed two hours post-cardiac arrest. Mice were secured to a Vevo 2100 (VisualSonics, Toronto, Ontario Canada) platform and were monitored for temperature, heart rate, and ECG. Transthoracic echocardiography was performed using a parasternal long-axis approach to obtain 2-dimensional left ventricular images. M-mode images were used to measure left ventricular end-diastolic, end-systolic, ventricular size, and to calculate the percent fractional shortening (%FS).

Neurological Scoring of Mice

Neurological scoring of the mice undergoing survival analysis was performed in a blinded fashion at 6, 18, 24, 48, and 72 hours following cardiac arrest. Scoring was performed by an observer blinded to the treatment group as previously described (24, 27). Scoring was performed using 6 criteria (response to tail pinch, corneal reaction, respirations, righting reflex, coordination and activity). Each criterion was scored on a scale of 0–2, with a score of 0 reflecting absent function, and 2 reflecting normal function. A maximal aggregate score of 12 was possible, reflecting normal behavior. Deceased animals received a score of 0. Neurological scores were grouped from 0–3.9, 4–5.9, 6–7.9, 8–9.9, and 10–12 and analyzed as described in the statistical methods.

Immunoblotting

Protein was extracted from harvested tissue and placed in lysis buffer to measure global Drp1 expression, as described⁴. In other experiments compartmental expression of Drp1 was measured on mitochondrial versus cytosolic fractions, as separated using a cell fractionation kit (Thermo Fisher Scientific, Rockford, IL). The purity of each fraction was confirmed by measuring expression of reporters, including the mitochondrial reporter, voltage dependent anion channel (VDAC) and the cytosolic reporter Rho GDP dissociation inhibitor (GDI). The following antibodies were used: anti-Drp1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-Drp1 (1:1000; Ser-616 Cat. 3455S and Ser-637 Cat. 4867S), anti-VDAC (1:2000, Cat 4661S), and anti Rho-GDI (1:2000) Cell Signaling Technology,

Danvers, MA, USA). Tubulin was used for total protein loading (Neo-Markers, Fremont, CA). Experiments were repeated in 4–5 hearts/group.

ATP/ADP/Lactate Assay

Adenosine 5'-triphosphate (ATP), adenosine diphosphate, and lactate were analyzed from frozen ventricular heart tissue obtained 15 minutes following resuscitation from cardiac arrest using a bioluminescent assay kit (BioVision Inc., Milpitas, CA.). The assay was performed according to manufacturer's instructions using a bench top plate reader (BioTek Winooski, VT). Data was normalized to control fluorescence.

Aconitase Assay

Aconitase is an iron-sulfur protein that is a catalyst for the conversion of citrate to isocitrate. The loss of aconitase activity in tissue is interpreted as an indicator of damage by oxidative activity (reactive oxygen species) and has been used in studies of cardiac arrest (28). Tissue aconitase activity was assayed using a kit obtained from Abcam (Cat #ab83459). The kit detects aconitase activity by the conversion of aconitase (colorless) into isocitrate, which was then further processed into a colored product at $\lambda_{\max}$ at 450nm. Measurements were performed using a BioTek plate reader.

Transmission Electron Microscopy (TEM)

Ventricular sections isolated from the anterior left ventricular myocardium were obtained from hearts 2 hours post cardiac arrest resuscitation. They were fixed overnight in 2.5% glutaraldehyde / 4% paraformaldehyde in 0.1 M sodium cacodylate buffer and maleate buffer (pH 5.1) and processed as previously described (n=3 hearts/group) (29). Images were collected using a scanning transmission electron microscope (TEM) at 300 KV (Tecnai F30; FEI) with a Gatan charge-coupled device camera. Blinded observers using Image J software analyzed Mitochondrial images. A minimum of 600 mitochondria per group underwent morphometric analysis.

Statistics

Data are presented as mean $\pm$ SEM or as 95% Confidence Intervals (CI). Statistical analyses were performed using Prism software (Graph Pad Inc., La Jolla, CA, USA) as well as by R statistical software (Vienna, Austria). Comparison between >2 groups or multiple groups over time were performed using simple or repeated measures ANOVA, as appropriate. All pairwise comparisons were performed by pairwise t test with p values adjusted for multiple comparisons using Benjamini-Hochberg method. A $p < .05$ was considered statistically significant. Kaplan-Meier survival curves were compared using a Log Rank (Mantel Cox) test. Neurological scores were represented as ordinal outcomes and modeled using a cumulative link mixed model.

Results

Pre Cardiac Arrest Baseline Characteristics of Mice and Resuscitation Characteristics

In this study, 17 mice underwent Sham surgery (no cardiac arrest) and a total of 91 mice underwent cardiac arrest following a standardized protocol using ketamine/xylazine as an anesthetic. 49 of the cardiac arrest mice received a DMSO/epinephrine injection during CPR while 42 mice received an Mdivi-1/epinephrine injection in blinded fashion. There were no differences in the characteristics of the mice studied and hemodynamic measurements of the groups prior to cardiac arrest were similar (Table 1). Resuscitation quality as assessed by chest compressions and aortic diastolic pressure was similar between both cardiac arrest groups (Table 2). There was a non-significant trend ($p=0.1$) towards increased ROSC in the Mdivi-1 treatment group. However time to ROSC was significantly improved in the Mdivi-1 treated group ($p<0.001$). We also confirmed that mean blood potassium concentrations in both cardiac arrest control and Mdivi-1 treated cardiac arrest mice were similar 30 minutes after ROSC, $p=0.7$ (supplemental figure 1).

Mdivi-1 inhibits Drp1 dephosphorylation and translocation to the mitochondria following cardiac arrest

A major regulator of mitochondrial fission following IR injury is the cytoplasmic protein Drp1. During IR, Drp1 is dephosphorylated at Serine 637 (S637) by the calcium activated phosphatase calcineurin, thereby regulating its translocation from the cytosol to the mitochondria to initiate fission (20). The cytoplasmic pool of Drp1 is large and decreases in the total Drp1 pool are not always reflective of changes of Drp1 occurring at the mitochondrial surface so purification of mitochondrial fractions is often necessary. In isolated langendorff perfused hearts, IR injury activates calcineurin resulting in Drp1 dephosphorylation (15). Here, we first sought to establish whether Drp1 was activated by *in vivo* cardiac arrest. Total cellular lysate was collected from Sham operated, cardiac arrest control (DMSO), and cardiac arrest + Mdivi-1 treated hearts for Western blotting. As expected, cardiac arrest was associated with Drp1-S637 dephosphorylation. (Figure 2a). In contrast, Mdivi-1 preserved Drp1-S637 phosphorylation following cardiac arrest compared to cardiac arrest alone (One Way ANOVA, $p=0.05$ $n=5$, pairwise t-test of CA and CA+Mdivi-1 $p=0.028$, Wilcoxon $p=0.048$). Changes in the total cytoplasmic pool of Drp1 were not observed. We next sought to determine if Drp1-S637 dephosphorylation was associated with Drp1 translocation to the mitochondria by performing westerns on purified mitochondrial fraction from treated hearts. Confirming the hypothesis, increased Drp1 expression was found in the mitochondrial fraction of cardiac arrest control hearts compared to Mdivi-1 treated or Sham operated hearts (Figure 2b), (One Way ANOVA $p<0.001$, pairwise t-test, CA vs. Sham $p=0.002$, CA vs. Mdivi-1 $p=0.0014$, Sham vs. CA + Mdivi-1 $p=0.034$).

Drp1 inhibition preserves mitochondrial morphology following cardiac arrest

To determine if changes in mitochondrial morphology were associated with cardiac arrest and Drp1 activation, we isolated and prepared tissue sections of the left ventricle for transmission electron microscopy (TEM). Mitochondria imaged from hearts 2 hours post cardiac arrest appeared smaller and had less dense cristae compared to mitochondria imaged

from Sham operated and Mdivi-1 treated mice (Figure 3). Sham mitochondria were not morphologically distinct from CA+Mdivi-1 treated hearts (One Way ANOVA, pairwise t test, CA vs. Sham $p<0.001$, CA vs. Mdivi-1 $p<0.001$, Sham vs. CA+Mdivi-1 $p=0.18$). These findings are consistent with the occurrence of mitochondrial fission following cardiac arrest resuscitation and its prevention by Mdivi-1.

ATP is unchanged following resuscitation from cardiac arrest

Decreased ATP following prolonged anoxia and myocardial IR injury is well established. The effects of brief (<10 minutes) asystolic cardiac arrest in an *in vivo* model of cardiac arrest are not as well known. We next sought to determine tissue ATP, ADP, and ATP/ADP levels following cardiac arrest in our model and to determine if Drp1 inhibition via Mdivi-1 had any restorative effects (Figure 4a). ATP and ADP levels were measured from tissue collected 15 minutes after cardiac arrest resuscitation as well as from Sham operated mice. At this time point, ATP and ADP concentrations were not significantly different between Sham operated, post-cardiac arrest controls, or post-cardiac arrest Mdivi-1 treated groups, (ATP One Way ANOVA $p=0.39$, ADP One Way ANOVA $p=0.15$, ADP/ATP One Way ANOVA $p=0.40$). Since changes in ATP are relatively insensitive to acute changes in cellular bioenergetics, we also measured myocardial tissue lactate following cardiac arrest (Figure 4b). In contrast to ATP and ADP, myocardial lactate was markedly raised following cardiac arrest compared to Sham and Mdivi-1 treated mice (One Way ANOVA, $p<0.001$, pairwise t test CA vs. Sham $p=0.009$, CA vs. CA+Mdivi-1, $p=0.009$, Sham vs. CA+Mdivi-1, $p=0.057$). Sham and Mdivi-1 treated hearts had differences in lactate that approached significance.

Drp1 inhibition decreases oxidative damage following cardiac arrest

Oxidative damage is a known consequence of cardiac arrest and a likely contributor to myocardial dysfunction. Mitochondrial fission is also a contributor to ROS production (16, 30). We next sought to confirm increased oxidant production in our model of cardiac arrest and to determine the effects of Drp1 inhibition by Mdivi-1 (Figure 4b). Aconitase is an enzyme whose activity is easily diminished by increased oxidant production and is a useful marker for oxidant damage. In previous studies of murine cardiac arrest, aconitase activity has been observed to be depressed within 5–15 minutes of resuscitation from cardiac arrest (28). 15 minutes following resuscitation from cardiac arrest, aconitase activity was found to be depressed compared to Sham and Mdivi-1 treated hearts (One Way ANOVA, $p=0.001$, pairwise t test Sham vs. CA $p<0.001$, CA vs. CA+Mdivi-1 $p<0.001$, Sham vs. CA+Mdivi-1 $p<0.001$). Although Mdivi-1 preserved aconitase activity compared to CA alone, it did not preserve aconitase entirely as compared to Sham treated animals. These data confirm increased oxidant damage following cardiac arrest in this model and demonstrate that Drp1 inhibition reduces oxidant injury.

Drp1 inhibition improves cardiovascular hemodynamics following cardiac arrest

We next sought to determine if Mdivi-1 administration would improve myocardial hemodynamics following cardiac arrest by measuring cardiac function with Millar catheter (Figure 5). Left ventricular maximal pressure (LVP max), dP/dT max, and cardiac output

(CO) were all significantly depressed following cardiac arrest compared to cardiac arrest mice treated with Mdivi-1 (Repeated measures ANOVA, LVP max $p=0.001$, dP/dT max $p=0.003$, CO $p=0.015$), while LVP min was increased ($p=0.05$, data not shown). Heart rates and stroke volumes in Mdivi-1 treated mice were also higher than the cardiac arrest alone animals (Repeated Measures ANOVA, $p<0.05$). Mean arterial pressure was also significantly depressed following cardiac arrest compared to Sham and Mdivi-1 treated mice at all time points examined (data not shown, Repeated Measures ANOVA, $p=0.001$). To further confirm Mdivi-1's myocardial effects following cardiac arrest, we next performed ventricular transthoracic echocardiography on an additional group of mice. As seen in Figure 6, 8 minutes of cardiac arrest was associated with significant depression of cardiac fractional shortening (%FS) at 2 hours post resuscitation. Fractional shortening of Mdivi-1 treated mice 2 hours post cardiac arrest was preserved similar to Sham operated mice (One way ANOVA, $p<0.05$, CA vs. CA+Mdivi-1 and CA vs. Sham). Sham vs. CA+Mdivi-1 was not significantly different.

Drp1 inhibition following cardiac arrest improves survival

Mdivi-1 inhibition of Drp1 following cardiac arrest was not only associated with improved myocardial performance but with improved survival as well (Figure 7). The greatest difference in survival between treatment groups was seen in the first 24 hours post cardiac arrest. 12/21 (57%) of cardiac arrest control mice died in the first 24 hours compared to 3/20 (15%) of cardiac arrest+Mdivi-1 treated mice. Between 24 and 72 hours post cardiac arrest, 4/17 (24%) of Mdivi-1 treated mice died compared to only 1/9 cardiac arrest control mice (11%). As expected, mortality in the Sham surgery group of mice was low with 1/17 (6%) of the Sham treated mice dying between day 2 and 3 post surgery. All three groups had survival curves that were significantly different from one another, $p<0.01$.

Drp1 inhibition improves neurological outcomes following cardiac arrest

Neurological injury post cardiac arrest is a major contributor to post cardiac arrest syndrome morbidity and mortality. Using a previously published neurological scoring system (see Methods) mice in all groups were evaluated by an observer blinded to the treatment groups at 6, 18, 24, 48, and 72 hours post cardiac arrest (Figure 8). Deceased subjects were scored as 0. Cardiac arrest controls had significantly depressed neurological scores compared to Sham operated and Mdivi-1 treated mice at all time points (CA vs. CA+Mdivi $p=0.007$, CA vs. Sham $p<0.001$). These data demonstrate that Mdivi-1 not only improved survival following cardiac arrest, but also was associated with improved neurological outcomes.

Discussion

This study demonstrates for the first time that Drp1 activity associated with mitochondrial fission is increased following *in vivo* cardiac arrest and that its inhibition with Mdivi-1 improves resuscitation outcomes. These findings, coupled with Mdivi-1's preservation of mitochondrial morphology and reduction of oxidant damage, indicate an important pathological role of Drp1 and mitochondrial fission following cardiac arrest. Our results are particularly promising when considering the fact that Mdivi-1 was administered during CPR, a highly clinically relevant period for pharmacological intervention in cardiac arrest.

The therapeutic benefits of Drp1 inhibition were evident not only during CPR, resulting in decreased times to ROSC, but extended over the next 72 hours as improved survival and neurological outcomes. Thus, Drp1 inhibition represents a promising therapy with true therapeutic potential and clinical relevancy.

Mitochondrial fission has recently been recognized as a promising therapeutic target for many different diseases, in particular, IR injury (11, 13). Following IR injury, mitochondrial fission occurs and its inhibition has been demonstrated to be protective against injury in the kidney and in the heart when administered as a preconditioning agent (31) (22). Recently, work by our group extended these findings demonstrating that Drp1 inhibition with the pharmacological agent Mdivi-1 can be cardio-protective when administered following the IR injury resulting in improved myocardial performance (15). Targeting other molecular partners of Drp1 may also have beneficial effects. Inhibition of the interaction between Drp1 and its mitochondrial targeting partner, Fis1 using the disruptive peptide, P110 has also been demonstrated to reduce infarct size following IR injury(32) and could be a promising target for future resuscitation studies.

The molecular mechanisms whereby IR injury initiates mitochondrial fission and how this then contributes to myocardial dysfunction are not clear. Evidence suggests that IR induced increases in cytosolic calcium result in activation of the phosphatase calcineurin, a known regulator of Drp1(20, 21). Calcineurin activation then results in causes Drp1-S637 dephosphorylation and its concomitant mitochondrial translocation. The calcineurin inhibitor FK506 has previously demonstrated to preserve Drp1-S637 phosphorylation following IR injury, thereby inhibiting Drp1 mitochondrial translocation and preserving myocardial function (15). Increased mitochondrial fission then results in increased ROS generation, additional calcium overload, and impaired ATP generation.

Oxidative damage following myocardial IR injury is a known contributor to myocardial dysfunction and stunning (33). In this study, we assessed aconitase activity as a surrogate marker of oxidative damage and demonstrated aconitase depletion following cardiac arrest. The implied increased ROS following IR injury and cardiac arrest is widely known and similar to other studies (28) but its amelioration with a Drp1 inhibitor following cardiac arrest is novel. These finding are consistent with the observation that Mdivi-1 inhibits ROS generation following IR injury in the isolated heart (15). In our study, we were unable to detect changes in myocardial tissue ATP, ADP, or ATP/ADP levels. ATP however is know to change relatively late in relation to changes in cellular bioenergetics. We therefore also measured myocardial tissue lactate levels. In contrast to our finding with ATP and ADP, myocardial lactate levels were elevated following cardiac arrest. Furthermore, Mdivi-1 inhibited lactate increases following cardiac arrest, implying improved cellular function.

It is noteworthy that Drp1 inhibition improved neurological outcomes in addition to the positive effects on myocardial hemodynamics observed in our model. These neurological benefits could be indirect, reflecting the more rapid ROSC and improved hemodynamics following Mdivi-1 administration, although it is possible that Drp1 inhibition had direct neuro-protective effects in addition to its cardio-protective ones. This would not be surprising given Drp1's role in other neurological pathologies(14). Zhang et al have recently

demonstrated that Mdivi-1 is protective against apoptotic neuronal cell death in a cerebral artery occlusion model (34). The concept that disorders of mitochondrial dynamics is a common and therapeutically tractable abnormality in common cardiovascular and neurologic diseases has recently been reviewed (11).

Limitations

This study has several limitations. *First*, our model investigated the effects of Drp1 inhibition in a murine model of asystolic arrest. The efficacy of Mdivi-1 in a larger animal model of cardiac arrest or in a model of cardiac arrest using ventricular fibrillation remains to be studied. *Second*, our manipulations of Drp1 activity were pharmacologically based with the use of Mdivi-1. Confirmation of our studies is needed through the generation and use of Drp1 conditional knockout mice and/or the development of other novel inhibitors of Drp1. *Third*, we did not employ advanced neurological imaging and it is possible that the differences in neurological outcome between groups might have been greater than suggested by the neurological scoring system employed. *Fourth*, therapeutic hypothermia is used clinically post cardiac arrest and assessment of whether Mdivi-1's effects are additive to therapeutic hypothermia should be tested in the future. *Fifth*, as with most basic science animal studies, the numbers of subjects in this study is limited (less than 100 per group) resulting in some data variability and the possibility for statistical error particularly where no difference between groups was observed.

Conclusion

We conclude that Drp1 plays an important role in both myocardial and neurological dysfunction following cardiac arrest. Pharmacological inhibition of Drp1 during and following cardiac arrest merits evaluation as a tool to augment post cardiac arrest CPR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Model adapted from:

- Abella et al, Circulation 109:2786-91 2004
- Beiser et al, Am J. Physiol Heart Circ

Figure 1. Schematic of Experimental Protocol

6-month-old female C57B6 were subjected to an 8-minute asystolic, non-ventilated cardiac arrest induced by KCL. Ventilations and chest compressions were then performed for 90 seconds followed by intravenous epinephrine and Mdivi-1 or DMSO control. CPR was then continued until ROSC or terminated after 5 minutes. Mice achieving ROSC were monitored and ventilated for 2 hours before extubation. Survival and Neurological outcomes were then monitored over 72 hours.

Figure 2. Drp1 dephosphorylation and translocation to the mitochondria following cardiac arrest are prevented by Mdivi-1

A. Mdivi-1, an inhibitor of Drp1 was administered following 8 minutes of cardiac arrest IV. Mdivi-1 dephosphorylation at Serine 637 following cardiac arrest was prevented by Mdivi-1. Relative densitometry to the right expressed as box plots. * (One Way ANOVA $p=0.054$, pairwise t test $p,0.03$, $N=5$ CA vs. CA+Mdivi-1). **B.** Mitochondrial Protein fractions were probed for Drp1. Increased Drp1 accumulation in the mitochondrial fraction 2 hours post arrest was observed compared to Sham and Mdivi-1 treated mice. VDAC demonstrated mitochondrial purity and equal loading. Rho GDI demonstrates lack of cytosolic protein contaminants (One Way ANOVA $p<0.001$, $N=4$, all groups significantly different from one another by pairwise t-test and/or Wilcoxon rank sum test). Error Bars 95% confidence intervals.

Figure 3. Drp1 inhibition preserves mitochondrial morphology following cardiac arrest
 Representative TEM images and mean values for mitochondria area from mouse hearts two hours post cardiac arrest resuscitation. 2 hours post cardiac resuscitation mitochondria were significantly smaller compared to Sham and Mdivi-1 treated mice suggesting mitochondrial fission. Mdivi-1 and Sham groups were not significantly different from one another ($p < 0.001$, One Way ANOVA, CA different from Sham and CA+Mdivi-1 by pairwise t test, $n > 600$ /group from 3 hearts). Scale bar = 1μM. Error Bars $\pm$ SEM.

Figure 4. Drp1 inhibition reduces myocardial lactate and preserves aconitase activity 15 minutes post cardiac arrest

A–B. Myocardial measurements of ATP, ADP, and ATP/ADP were not significantly different between Sham, cardiac arrest, and cardiac arrest + Mdivi-1 groups 15 minutes after resuscitation from cardiac arrest. Myocardial tissue Lactate following cardiac arrest (CA) however, was significantly increased compared to Sham and Mdivi-1 treated mice (***One Way ANOVA, $p < 0.001$, pairwise t test Sham vs. CA $p = 0.009$, CA vs. CA+Mdivi-1 $p = 0.009$, CA+Mdivi-1 vs. Sham $p = 0.057$, $N = 4$). Values for Sham and CA+Mdivi-1 mice were not significantly different **C.** Decreased activity of aconitase is a marker for increased ROS generation and was assessed 15 minutes after resuscitation from cardiac arrest. Aconitase activity following cardiac arrest was significantly decreased compared to Mdivi-1 and Sham treated hearts. Mdivi-1 preserved aconitase activity following resuscitation from cardiac arrest. Mdivi-1, however was not completely preserved aconitase levels and was significantly depressed from Sham (***One Way ANOVA, $p < 0.001$, all pairs significantly different from one another by pairwise t-test $p < 0.001$, $N = 5$). Error bars $\pm$ SEM.

Figure 5. Drp1 inhibition improves cardiovascular hemodynamics following cardiac arrest
Myocardial performance was assessed with a Millar catheter. Maximum Left ventricular maximum systolic pressure (LVP max), dp/dt max, dp/dt min, and stroke volume were depressed at all time points examined following cardiac arrest compared to Sham operated controls and cardiac arrest + Mdivi-1 treated mice. Cardiac output was also significantly worse following cardiac arrest compared to the Mdivi-1 treated group at 90 and 120 minutes following resuscitation from cardiac arrest. Heart rates were significantly higher in Mdivi-1 treated mice compared to cardiac arrest alone animals. N=13, Error bars show 95% confidence intervals. * $p < 0.05$.

Figure 6. Drp1 Inhibition improves fractional shortening following cardiac arrest

Left ventricular transthoracic echocardiography was used to measure the percent fractional shortening (%FS) in Sham operated mice, 8 min cardiac arrest (CA), and 8 min cardiac arrest+Mdivi-1 mice. 2 hours following resuscitation, %FS was depressed in CA mice (20%FS), compared to CA+Mdivi-1 (38%FS), and Sham operated mice (37%FS).* (One Way ANOVA, $p=0.003$, pairwise t test Sham vs. CA $p=0.004$, Sham vs. CA+ Mdivi $p=0.81$, CA vs. CA+Mdivi-1 $p=0.004$. Representative echocardiograms are shown to the left.

Figure 7. Drp1 inhibition increases survival following cardiac arrest

24 hours following cardiac arrest survival in the control group was only 43% (9/21) compared to 85% (17/20) in the Mdivi-1 group and 100% of the sham animals. Survival decreased to 38% (8/21) in the controls, 65% (13/20) in the Mdivi-1, and 94% (16/17) of sham animals 72 hours following cardiac arrest. All 3 groups were significantly different. Log Rank (Mantel Cox) Test. $p < 0.001$. N= number of animals at each time point.

Figure 8. Drp1 inhibition improves neurological outcomes following cardiac arrest. Mice were scored using a previously published neurological scoring system ranging from 0–12

At 6, 18, 24, 48, and 72 hour post cardiac arrest, Mdivi-1 treated mice had significantly better scores than control cardiac arrest animals. Sham mice scored better than Mdivi-1 treated mice at only 6 and 18 hours post cardiac arrest. Ordinal regression analysis demonstrated significant differences between CA and CA+Mdivi-1 and CA and Sham.

* $p < 0.001$

Table 1

Pre Cardiac Arrest Baseline Characteristics of Mice

Baseline	Cardiac Arrest + DMSO	Cardiac Arrest + Mdivi-1	P value
Sex	Female	Female	NA
Body weight	27.6±0.3	27.1±0.3	0.3
Left ventricular systolic pressure (mmHg)	92 ± 2	90 ± 3	0.7
Cardiac output (ml/min)	5.0 ± 0.1	5.0 ± 0.2	0.7

Table 2

Resuscitation Characteristics

CPR Characteristics	Cardiac Arrest + DMSO (N=21)	Cardiac Arrest + Mdivi-1 (N=20)	P value
Chest compression rate (bpm)	357 ± 2	350 ± 2	0.06
Aortic diastolic pressure (mmHg)	61 ± 3	62 ± 1	0.87
Return of spontaneous circulation (ROSC) (%)	76	90	0.1
Time to ROSC (seconds)	143 ± 7	116 ± 4	<0.001

Return of Spontaneous Circulation (ROSC) (%) $p=0.1$, Fisher Exact Test. Time to ROSC (seconds) $p<0.00001$ 2-tailed t test.