Dynasore protects mitochondria and improves cardiac lusitropy in Langendorff perfused mouse heart.
Dynasore Protects Mitochondria and Improves Cardiac Lusitropy in Langendorff Perfused Mouse Heart

Dan Chen Gao1, Li Zhang1, Ranvir Dhillon2, Ting-Ting Hong2, Robin M. Shaw2,3, Jianhua Zhu1*

1 Department of Cardiology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang, China, 2 Cardiovascular Research Institute, University of California San Francisco, San Francisco, California, United States of America, 3 Department of Medicine, Division of Cardiology, University of California San Francisco, San Francisco, California, United States of America

Abstract

Background: Heart failure due to diastolic dysfunction exacts a major economic, morbidity and mortality burden in the United States. Therapeutic agents to improve diastolic dysfunction are limited. It was recently found that Dynamin related protein 1 (Drp1) mediates mitochondrial fission during ischemia/reperfusion (I/R) injury, whereas inhibition of Drp1 decreases myocardial infarct size. We hypothesized that Dynasore, a small noncompetitive dynamin GTPase inhibitor, could have beneficial effects on cardiac physiology during I/R injury.

Methods and Results: In Langendorff perfused mouse hearts subjected to I/R (30 minutes of global ischemia followed by 1 hour of reperfusion), pretreatment with 1 μM Dynasore prevented I/R induced elevation of left ventricular end diastolic pressure (LVEDP), indicating a significant and specific lusitropic effect. Dynasore also decreased cardiac troponin I efflux during reperfusion and reduced infarct size. In cultured adult mouse cardiomyocytes subjected to oxidative stress, Dynasore increased cardiomyocyte survival and viability identified by trypan blue exclusion assay and reduced cellular Adenosine triphosphate (ATP) depletion. Moreover, in cultured cells, Dynasore pretreatment protected mitochondrial fragmentation induced by oxidative stress.

Conclusion: Dynasore protects cardiac lusitropy and limits cell damage through a mechanism that maintains mitochondrial morphology and intracellular ATP in stressed cells. Mitochondrial protection through an agent such as Dynasore can have clinical benefit by positively influencing the energetics of diastolic dysfunction.

Introduction

Heart failure is a major cause of morbidity and mortality in the United States [1], of which diastolic heart failure (DHF) is an important entity with rising prevalence. Of the 6 million patients with heart failure, as many as half had diastolic dysfunction [2,3]. The one year mortality associated with hospitalization due to diastolic dysfunction is between 22 and 29% [4]. Myocardial ischemia is a major contributor to DHF. Acute ischemia can result in DHF due to rapid myocardial changes including edema, calcium accumulation, and inflammation [5,6] and the severity of diastolic dysfunction depends on the duration of ischemia [7]. Hearts subjected to chronic microvascular or untreatable chronic ischemia also have diastolic dysfunction [4].

Current therapeutic approaches to DHF due to ischemia focus on relieving the ischemia with reperfusion [8]. Ironically, ischemia/reperfusion (I/R) can result in direct myocardial injury [9] and negatively affect diastolic function. In recent decades, the mechanisms involved in I/R injury have started to be identified. Cellular death and damage pathways involve subcellular organelles such as mitochondria which are critical mediators due to their ability to generate Adenosine triphosphate (ATP) and reactive oxygen species (ROS). During ischemia, progressive ATP depletion inhibits ion pump function which leads to intracellular accumulation of calcium [10,11]. Also, reintroduction of oxygen during reperfusion will regenerate ATP, however, it will also damage the electron transport chain resulting in increased mitochondrial generation of ROS [12,13]. Mitochondrial Ca2+ overload [14] and increased ROS result in opening of the mitochondrial permeability transition pore (MPT) [15], which initiates apoptosis and cell death by causing mitochondrial swelling and rupture. Interestingly, inhibition of MPT is reported to reduce infarct size [16].

More recently, the integrity and morphology of mitochondrial network has been recognized as critical to cell fate. In a healthy non-stressed intact cell, mitochondria consist of a continuous mitochondrial reticulum, which undergoes constant fusion and fission, two opposing processes controlled by local GTP gradients and mitochondrial energetics [17]. Dynamin-related GTPases such as mitofusins (MFN1, MFN2) and the mitochondrial inner membrane optic atrophy protein 1 (OPA1) isoforms are profusion. Alternatively, scission requires the pro-fission multimers...
containing mitochondrial fission protein 1 (FIS1), mitochondrial fission factor (MFF), and dynamin related protein 1 (Drp1). The fine balance between mitochondria fusion and fission determines cell fate [17]. Cardiac I/R injury results in significant mitochondrial fission, which induces apoptotic cell death. Inhibition of mitochondrial fission mediated by Drp1 can limit infarct size in I/R injury [18].

A critical yet unaddressed issue is whether mitochondrial protection limits ischemia related diastolic dysfunction. Dynasore is a cell-permeable small molecule that non-competitively inhibits the GTPase activity of dynamin1, dynamin2 and Drp1 [19]. We found that low dose Dynasore significantly preserves lusitropy of ex vivo perfused hearts subject to I/R injury. Dynasore also increases cardiomyocyte survival, and decreases cellular ATP consumption in stressed cardiomyocytes. In support of mitochondrial protection, we found that low dose Dynasore is sufficient to prevent oxidative stress induced mitochondrial fission in cultured cells. Thus small molecule based Drp1 inhibition is a potential therapeutic approach to ischemia related DHF.

Materials and Methods

Guidelines for Animal Research

This study was approved by the University of California San Francisco Committee on the Use and Care of Animals (IACUC). All procedures used in this study are in agreement with the guidelines of the University of California San Francisco IACUC. Animals were housed in UCSF facility of Laboratory Animal Resource Center. All investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, received 1996).

Materials

Unless otherwise stated, Dynasore (Figure 1) and all other materials were purchased from Sigma Chemical (St. Louis, MO). Langendorff-Perfused Heart

Male C6/Black mice (8–12 weeks; Charles River) were anesthetized with isoflurane (flow 3%) and 100% O2 in an anesthesia chamber and anti-coagulated with heparin (50 IU, i.p.). After cervical dislocation, hearts were rapidly excised, mounted on a Langendorff apparatus (ADIstrument, Colorado Springs, CO) and perfused retrogradely [20,21] at a constant rate of 2.6 ml/min with oxygenated Krebs-Henseleit buffer containing (mmol/L): NaCl 118, NaHCO3 24, CaCl2·2H2O 2.5, KCl 4.7, KH2PO4 1.2, MgSO4·7H2O 1.2, Glucose 11, EDTA 0.5, adjusted to a pH of 7.4 [22,23]. The apparatus was water-jacketed for temperature control to maintain a core temperature of the heart at 37°C. The buffer passed through a membranous “lung” made of SilasticTM Medical Grade Tubing, which was gassed continuously with 95% O2-5% CO2. Fine platinum electrodes were placed on the right atrium and apex of the left ventricle to record the electrocardiogram and heart rate throughout the experiment. A Millar MIKRO-TIP catheter transducer (Millar Instruments, Houston, TX) was inserted into the left ventricle from the left atrium to measure left ventricular pressure. Left ventricular end diastolic pressure (LVEDP), left ventricular end systolic pressure (LVESP) and heart rate were monitored and recorded continuously using PowerLab system (ADInstruments). Left ventricular developed pressure (LVDP) was calculated by subtracting LVEDP from LVESP. Hearts were paced at 360 bpm with bipolar electrodes attached to the right atrium, using stimuli delivered from a stimulator (ADIstruments Colorado Springs, CO).

After the initial 15 min stabilization, hearts were excluded from further study if they exhibited one or more of the following exclusion criteria: LVEDP higher than 20 mmHg; LVDP less than 50 mmHg; intrinsic heart rate less than 280 bpm or irregular; or aortic regurgitation. The volume of the perfusate was reduced to 200 ml and allowed to recirculate. The hearts were then randomized to one of the following two treatment groups: Dynasore group (n = 8, added into the recirculating perfusate in stepwise fashion to reach a final concentration of 1 µM within 120 min of recirculation) or DMSO control group (n = 8, added in
a similar manner of Dynasore). Hearts were then subjected to 30 min of global ischemia followed by 1 hour of reperfusion. Pacing was initiated after stabilization except during ischemia and was reinitiated 2 min after reperfusion.

Myocardial cTnI Release
Cardiac effluent was collected from apex at baseline, before ischemia and during reperfusion. The samples were snap frozen immediately in liquid nitrogen and stored at −80°C for later analysis. Cardiac effluent samples from 5 hearts in each group were then used to determine cardiac specific troponin I (cTnI) concentration using a commercial cTnI ELISA kit (Life Diagnostics, Inc., West Chester, PA).

Determination of Infarct Size
Propidium iodide (excitation, 535 nm; emission, 617 nm) was used to determine infarct size according to a previously established method [24,25] with modest modification. Briefly, at final 15 min reperfusion period, 300 μg of propidium iodide (Sigma Chemical, St. Louis, MO) was injected into the right atrium and allowed to perfuse through the coronary vasculature. At the end of reperfusion, hearts were then removed from the apparatus, sliced perpendicularly to the long axis of the heart into 4~5 equal-thickness transverse slices. The top and bottom surfaces of each slice were imaged by widefield epifluorescence microscopy with a Cy3 filter at an exposure of 500 ms per frame, and grayscale images were captured using a sensitive CCD camera with white pixels corresponding to PI positive signals. Total left ventricular area and infarct area for each image were analyzed using ImageJ software. Infarct size was calculated by total infarct area summed from all the slices and expressed as percentage of the total left ventricular area.

Isolation and Culture of Adult Mouse Cardiomyocytes
Mouse ventricular myocytes were isolated from male adult C57/Black mouse (8~12 weeks; Charles River) after dissociation with

Figure 2. The cardiac lusitropic effect of Dynasore. Effect of 1 μM Dynasore pretreatment on ventricular function was studied in Langendorff perfused mouse hearts subjected to 30 min no-flow global ischemia followed by 60 min reperfusion. Hearts were paced at 360 bpm during the whole experimental protocol except the ischemia period and pacing was reinitiated at 2 min into the reperfusion period. A. Representative Left ventricular pressure tracing in a control heart and a Dynasore treated heart. B, C. Left ventricular end diastolic pressure (LVEDP, B) and left ventricular developed pressure (LVDP, C) are summarized and compared between control group and Dynasore group. (* P<0.05). doi:10.1371/journal.pone.0060967.g002
collagenase II (2 mg/ml, Worthington, Lakewood, NJ) using a previously described method \[26,27\]. After dissociation, cardiomyocytes were plated on laminin-precoated 35 mm\(^2\) culture dishes at a density of \(1,500/mm^2\) and maintained in a humidified atmosphere of 5% CO\(_2\) at 37\(\,^\circ\)C. After 1 hour of plating, cardiomyocytes were replenished with fresh medium (serum supplemented or depleted) and subjected to 2 hours of drug treatment (Dynasore or vehicle) followed by oxidative stress (30 \(\mu M\) H\(_2\)O\(_2\) for 35 min). For ATP supplement experiments, the cells were treated with 3 mM ATP for 30 min before exposure to H\(_2\)O\(_2\).

### In vitro Cardiomyocyte Survival and Viability Assay

After exposure to 30 \(\mu M\) H\(_2\)O\(_2\) for 35 min \[27\], cardiomyocyte survival and viability were analyzed by trypan blue exclusion (TBE) assay, which is a method to determine cell survival and changes in cell morphology in experimental models \[28,29\]. In brief, cardiomyocytes were stained with 0.04% (w/v) trypan blue solution (Gibco, Invitrogen, Carlsbad, CA) at room temperature for 7 min. When cell membranes are irreversibly damaged, the anionic dye trypan blue is taken up by dead cells. Cardiomyocytes were then visualized at 40\(\times\) magnification by microscopy. For each experiment, a total of 200 cardiomyocytes were analyzed from 10 different fields/dish. Cells that excluded trypan blue (TBEs) were considered to have survived. Healthy rod-shaped myocytes (rods) were identified when the length/width ratio was >3:1 \[30\]. Contracted cells were defined when the length/width ratio was <3:1. Trypan blue-positive cells were identified when the trypan blue was present irrespective of whether the cells were rod-shaped or contracted \[28,31\]. Morphologic changes (viability) were measured by determining the number of rods relative to all TBEs (rods and contracted cells) of the 200 cardiomyocytes analyzed. The percent survival and viability were calculated as follow:

\[
\text{Percent survival} = \frac{\text{Total number of TBEs} \times 100\%}{\text{Total number of myocytes (TBEs + nonTBEs)}}
\]

### Table 1. Effect of 1 \(\mu M\) Dynasore pretreatment on ventricular function in Langendorff perfused mouse hearts during ischemia/reperfusion injury.

|                  | LVEDP (mmHg) | LVESP (mmHg) | LVDP (% of BL) |
|------------------|--------------|--------------|----------------|
|                  | Control (N=8) | Dynasore (N=8) | Control (N=8) | Dynasore (N=8) | Control (N=8) | Dynasore (N=8) |
| Baseline         | 4.9±5.3      | 2.4±3.5      | 62.0±16.6      | 62.8±14.1      | 100.0±0.0      | 100.0±0.0      |
| Post-drug        | 5.5±5.5      | 0.8±3.8      | 42.8±6.3       | 48.2±6.8       | 51.1±3.8       | 62.8±6.0       |
| Ischemia         |              |              |                |                |                |                |
| 15 min           | 31.4±6.1     | 7.5±8.5*     | 48.0±17.1      | 15.2±6.9       | 11.7±1.0       | 9.3±1.3        |
| 30 min           | 41.9±8.1     | 9.1±6.9***   | 57.8±15.4      | 24.2±9.8       | 12.4±1.8       | 9.8±1.0        |
| Reperfusion      |              |              |                |                |                |                |
| 15 min           | 21.6±3.7     | 4.6±3.7*     | 45.1±12.3      | 32.0±10.0      | 23.9±5.3       | 20.9±5.4       |
| 30 min           | 25.2±6.6     | 3.3±4.3      | 56.5±18.3      | 40.7±15.2      | 33.2±7.6       | 32.5±7.4       |
| 60 min           | 24.9±4.8     | 0.0±4.8*     | 64.7±20.0      | 46.1±6.2       | 52.6±6.7       | 51.4±9.9       |

*, ***indicate \(P<0.05, P<0.001\) when compared between the two treatment groups.

LVEDP, left ventricular end diastolic pressure; LVESP, left ventricular end systolic pressure; BL, baseline.

doi:10.1371/journal.pone.0060967.t001

### Figure 3. Dynasore decreases cardiomyocyte death in I/R injured mouse hearts. A. Myocardial infarct size was analyzed by Propidium Iodide (PI) perfusion. Left, representative fluorescence images of PI staining in a control and Dynasore treated hearts subjected to I/R injury. Right, average infarct size is presented as percentage over total left ventricular area and compared between the two treatment groups. B. Dynasore decreases cardiac troponin I (cTnI) efflux. Myocardial damage was evaluated by measurement of the release of cTnI in the coronary effluent during the 60 min reperfusion period. (* \(P<0.05, ***P<0.001\)).

doi:10.1371/journal.pone.0060967.g003
Figure 4. Dynasore increases cardiomyocyte survival and viability. Trypan blue exclusion assay was used to identify cell survival and viability of cardiomyocytes subjected to oxidative stress in the presence (A) and absence of FBS (B). A. Dynasore increases cell survival and viability in oxidative stressed (exposed to 30 μM H₂O₂ for 35 min) cardiomyocytes. Top, representative phase images of cardiomyocytes. Bottom, summarized cell survival and viability results. B. Dynasore further increased cell survival and viability in serum depleted and oxidative cardiomyocytes. Top, representative phase images of cardiomyocytes. Bottom, summarized cell survival and viability results. (**P<0.01, ***P<0.001 when compared to stressed cardiomyocytes without Dynasore treatment; †P<0.05, †††P<0.001 when compared to non-stressed cardiomyocyte controls).

doi:10.1371/journal.pone.0060967.g004
Note total number of myocytes (TBEs+non-TBEs) = 200.

Percent viability = \frac{\text{Total number of rod-shaped TBEs}}{\text{Total number of TBEs (rods and contracted cells)}} \times 100\%

ATP Measurement

A luminescence assay [Promega, Madison, WI] was used to quantify cardiomyocyte and Hela cell ATP content [32]. Briefly, after Dynasore treatment and H$_2$O$_2$ exposure, cardiomyocytes were lysed and ATP content was measured in the cell lysates. Meanwhile, in a separate set of wells following same experimental protocol, surviving cardiomyocytes were counted using a TBE assay. Cellular ATP per single live cardiomyocyte was then calculated for each treatment condition. Similar procedures were applied to cultured non-stressed Hela cells treated with control or Dynasore.

Live-cell Mitochondria Imaging with Spinning Disc Confocal Microscopy

HeLa cells (ATCC, Manassas, VA) were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 100 µg/ml Normocin (Amaxa, Lonza Walkersville Inc, Walkersville, MD). Cells were maintained in a humidified atmosphere of 5% CO$_2$ at 37°C. Cells were seeded at a density of 7 × 10$^4$ cells/cm$^2$ and allowed to adhere overnight. Cells were then transduced with Organelle Lights™ Mito-RFP *BacMam 1.0 (Invitrogen). Twenty-four hours after transduction, cells were pretreated with either control or 1 µM Dynasore for 1 hour before being exposed to normal conditions or 200 µM H$_2$O$_2$ for 15 minutes. Before and after exposure to H$_2$O$_2$, cells were imaged using a Nikon Ti inverted microscope, Yokogowa CSU-X1 spinning disk confocal unit with 568-nm DPSS laser source, and a high resolution Cool SNAP HQ2 camera (Photometrics, Tucson, AZ). Images were acquired at 400 ms exposure per frame and automatically processed using a bas relief filter to highlight edges.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5. The data are expressed as means ± SEM. Difference between control and experimental groups were determined using a one-way analysis of variance (ANOVA) for multiple groups. Difference between every two groups was determined using Bonferroni post hoc test. For comparison between two groups with timed repeated measurements, a two-way ANOVA (treatment and time are considered as the two variances) was used. P<0.05 was considered to be significant.

Results

In Langendorff Perfused Mouse Hearts, Dynasore Prevents Pathologic LVESP Elevation Following I/R Injury

We investigated the effects of Dynasore on intracardiac pressure during I/R injury. Using Langendorff perfusion, hearts were subjected to no-flow global ischemia followed by restoration of flow, with and without the presence of Dynasore during the entire period. Results are in Figure 2A which contains ventricular pressure tracings at baseline, during ischemia, and post reperfusion from two representative hearts treated with vehicle or 1 µM of Dynasore, which was determined as a safe, complication free, cardiac protective dose based on a pilot study testing the pharmacological dose response curve of Dynasore.
(0.1–10 μM). As indicated, 30 min of global ischemia caused a significant elevation of LVEDP, which only had partial recovery after reperfusion. In addition, global ischemia resulted in a significantly drop of cardiac systolic function indicated by left ventricular developed pressure (LVDP), which returned to its pre-ischemia baseline after 60 min of reperfusion. The concurrent increase of LVEDP with a drop of LVDP indicates that a severe hypo-contractile state is initiated after ischemia and persists into reperfusion. However, pretreatment with 1 μM Dynasore prevented elevation of LVEDP without a change of LVDP. The summarized ventricular pressure data from eight independent experiments of each condition are presented in Figure 2B (LVEDP), Figure 2C (LVDP) and Table 1. Note a significant decrease of LVEDP in Dynasore treated hearts is present during the entire ischemia and reperfusion period. Dynasore’s beneficial effect on LVEDP and not LVDP indicates it benefits lusitropy more than it does on inotropy.

In I/R Injured Mouse Hearts, Dynasore Decreases Myocardial Death

Improved lusitropy implies less stress and ischemia related damage to the myocardium. We investigated myocardial damage with two separate direct assays: staining hearts for cardiomyocyte death and measuring troponin release. Propidium iodide (PI), a nuclear fluorescent dye, permeates through the damaged plasma membrane of cardiomyocytes that are at the early stage of cell death. As seen in the left panel of Figure 3A, the PI signal and hence cell death in heart slices from non-treated hearts is significantly higher. The PI positive area was traced in ImageJ and presented as percentage of total ventricular area. Infarct size calculated using this method indicates that, in control hearts, 30 min of ischemia followed by 1 hour of reperfusion results in 39.6% of PI positive infarct area. Perfusion with Dynasore significantly reduces infarct size to 8.1% (80% reduction, Figure 3A).

Myocardial death was further evaluated by measuring cTnI in the cardiac efflux. As seen in Figure 3B, myocardial damage induced by I/R injury causes cTnI release into cardiac effluent, an event within an hour post reperfusion (clinical cTnI requires as much as 6 hours to be detected [33]. With Dynasore pretreatment, the early cardiac efflux of cTnI was decreased 70% (at 30 minutes of reperfusion), further confirming the protective effects of Dynasore on cardiac muscle.

In Cultured Cardiomyocytes, Dynasore Increases Cell Survival and Viability

The data in Figures 2 and 3 involved protocols of complete no-flow ischemia with whole heart preparations. We were interested if the protective effects of Dynasore extend to a more traditional cellular cardiomyocyte assay with a more controlled stress insult. Isolated adult mouse cardiomyocytes in culture were subjected to oxidative stress by addition of hydrogen peroxide in the presence or absence of serum. Healthy adult cardiomyocytes have a typical elongated rod shape. However oxidative stress significantly damage cell morphology resulting in a well described contracted morphology [27]. We observed significant cardiomyocyte contraction with oxidative stress to 30 μM H2O2 (Figure 4). Note that Dynasore prevents cardiomyocyte morphological changes induced by oxidative stress.

Cell survival and viability can further be assayed by TBE and cell morphological changes, respectively (see Methods). As seen in the bar graphs of Figure 4, as expected, oxidative stress causes a

---

**Figure 7. Dynasore prevents oxidative stress-induced mitochondrial fission.** Cultured human Hela cells were used for mitochondrial morphology study. Top, Hela cells have elongated connected mitochondrial network (left), which was fragmented after oxidative stress (right). Bottom, 1 μM Dynasore pretreatment prevents oxidative stress-induced mitochondrial fragmentation.

doi:10.1371/journal.pone.0060967.g007
significant reduction in both cell survival and viability. However
the presence of Dynasore in the culture medium results in
significantly improved survival and a major improvement in
viability. The beneficial effects of Dynasore on viability and
survival were dose dependent and are also more prominent in
serum free conditions (Figure 4B).

Dynasore Preserves Cellular ATP Content in Stressed
Cardiomyocytes
Dynasore’s beneficial effect on cell fate and the ameliorating
effect of serum (Figure 4) suggest that the mechanism of
Dynasore’s effect is related to energetics. This hypothesis was
tested by recording cellular ATP content in oxidative stressed
cardiomyocytes. To control for different amount of living
cardiomyocytes per culture well, net ATP content was normalized
to the amount of surviving cardiomyocytes counted per dish. As
seen in Figure 5A, as low as 1 μM dose of Dynasore increases
cellular ATP content in live cardiomyocytes. To confirm that
Dynasore induced higher levels of ATP are protective, we
performed an ATP rescue experiment and found, in Figure 5B,
that direct supplementation with exogenous ATP has similar effect
to Dynasore on cardiomyocyte viability subjected to oxidative
stress.

Next, we explored whether Dynasore simply preserves ATP
during stress or can actually generate ATP independent of a
metabolic insult. Since the process of dissociating and culturing
adult mouse cardiomyocytes is already a stress to these primary
cells, a typical cell line of Hela cells in standard cell culture
hemostasis was used in this analysis. As seen in Figure 6, low dose
Dynasore (1–3 μM) has no effect on cellular ATP content,
indicating that Dynasore may preserve ATP in stressed cells
rather than be responsible for ATP production. Note high dose
Dynasore (>10μM) increases cellular ATP content, possibly
indicating direct ATP production or, more likely, the energetic
benefit of limiting Dynamin GTPase dependent endocytosis at
higher dosage.

Dynasore Inhibits Oxidative Stress-induced Mitochondrial
Fission
Given the reported inhibitory effects of Dynasore on Drp1
[19] and the observed effect on increasing cell survival and
viability (Figure 4) as well as preserving cellular ATP content
(Figure 5), we hypothesized that the cardioprotective effect of
Dynasore (Figure 2 and 3) is mediated by inhibition of Drp1
dependent mitochondrial fission. To test this hypothesis,
cultured cells were transduced with baculovirus expressing a
mitochondrial targeted fluorescent protein. Instead of adult
mouse cardiomyocytes, HeLa cells were used for this study
because the relatively flat HeLa cell morphology is permissive to
detailed high resolution mitochondrial imaging. The cardiac
atrial origin HL-1 cells and neonatal cardiomyocytes are not
used for this study due to their resistance to oxidative stress
[34], probably related to differential mitochondrial fusion-fission
dynamics. The morphology of mitochondria was examined by
spinning disc confocal microscopy before and after the exposure
to 200 μM H$_2$O$_2$ for 15 minutes. As seen in Figure 7, the non-
stressed mitochondria have an elongated and well-organized
reticulum network, whereas oxidative stress induces mitochon-
drial fragmentation. Note the cellular morphology is also altered
after oxidative stress, resulting in contracted and smaller cells.
Consistent with the above hypothesis, pretreatment of the cells
with Dynasore prevented oxidative stress induced mitochondrial
fission (Figure 7, bottom row), retaining the original organized
mitochondrial reticulum network and maintaining normal cell
morphology.

Discussion
We have found that the small dynamin-GTPase inhibitor
Dynasore protects mitochondria and significantly benefits cardiac
lusitropy in hearts subjected to I/R injury. The cardioprotective
effect is observed in both ex vivo perfused mouse heart preparations
and isolated cultured cardiomyocytes. Dynasore also, in dose
dependent fashion, increases cell survival in cultured primary adult
mouse cardiomyocytes exposed to oxidative stress. In the surviving
cells, Dynasore preserves cellular ATP content whereas adding
exogenous ATP provides a similar rescue in the absence of
Dynasore. Of note, the cardioprotective dose of Dynasore is
significantly lower than the dose used to effectively block
endocytosis.

A Novel Cardiac Lusitropic Role of Dynasore
The small molecule Dynasore was identified as an endocytosis
inhibitor seven years ago [19]. It was found to be a non-
competitive inhibitor of the GTPase activity of dynamin1,
dynamin2, and the mitochondrial pro-fission dynamin isotype
Drp1 (Cartoon in Figure 1). By blocking plasma membrane
dynamin) and dynamin2, Dynasore acts as a potent blocker of
dynamin-dependent coated vesicle formation, resulting in stabilizi-
ation of intermediates including U-shaped and O-shaped pits
[19]. Since its discovery, Dynasore has been used as an effective
cardiac related endocytosis inhibitor due to its potency and limited
cytotoxicity [34,35].

Since Dynasore inhibits Drp1 in vitro [19], it is interesting that
Dynasore significantly prevents LVEDP elevation during I/R
injury without affecting LVDP (Figure 2) indicating a novel
lusitropic effect. Diastolic dysfunction is usually associated with
prominently altered nucleotide levels [36] or ATP turnover and
catabolism [37]. Therefore the beneficial lusitropic effect may be
mediated by Dynasore’s ability to preserve the ATP reserve in
stressed cardiomyocytes (Figures 5). Dynasore inhibits GTPase
activity at both the plasma membrane (Dynamin 1, 2) and the
mitochondria membrane (Drp1) [19]. However, the current
lusitropic dose of Dynasore (1 μM, Figure 2) is significantly lower
than the previously reported inhibitory dose of Dynasore (IC50
~15 μM) on dynamin dependent endocytosis [19], indicating an
effect separate from dynamin inhibition at the plasma membrane.
The low non-endocytosis related dose of Dynasore that blocks
oxidative stress-induced mitochondrial fission (Figure 7) is most
likely due to Drp1 inhibition [18,38].

Interestingly, Dynasore improves diastolic function with acute
I/R injury, without affecting systolic function (Figure 2). It might
be that no-flow ischemia permits the accumulation of toxic
metabolites, such as low pH, which can limit inotropy independent
of myocardial energetics. However inotropy was unaffected by
Dynasore during the reperfusion period which should wash out
toxic metabolites, even while lusitropy was preserved (Figure 2).
Therefore lusitropy specificity could result from diastolic function
being a more sensitive indicator of cardiac ATP and other
mitochondrial dependent energy production. In healthy individ-
uals, mild hypoxia results in diastolic dysfunction without affecting
systolic function [39]. Diastolic sensitivity to hypoxia could be due
to a decline in high energy phosphate metabolism [40]. Advanced
age is also a well established association with progressive diastolic
dysfunction. Hypoxia and age both result in irreversible damage to
mitochondrial genes involved in oxidative phosphorylation [41].
These mutations limit mitochondrial function and could contrib-
Mitochondrial Morphology and Cardiomyocyte Survival

In non-stressed conditions, mitochondria fusion prevails resulting in elongated, tubular, and interconnected mitochondria networks. During ischemia, the dynamic balance shifts from fusion to fission, resulting in fragmented and discontinuous mitochondria [18,43], as well as mitochondrial outer membrane permeabilization, release of apoptotic factors, and activation of apoptosis. Apoptotic cell death is understood to be a major contributing factor of I/R injury [44]. Recent studies show that alteration of mitochondrial morphology is significant in ischemic hearts [18,43] and modulation of which can protect the heart against I/R injury [18].

The apoptotic cell death that results from mitochondrial fission and fragmentation is mediated by activation of a key mitochondrial pro-fission protein Drp1 [38,45]. Recently, it was reported that a dominant-negative mutant of Drp1 induces mitochondrial elongation and pharmacological inhibition of Drp1 protects against I/R injury in the heart [18]. We found in human cells that Dynasore significantly prevents stress-induced mitochondrial fragmentation and maintains a normal elongated mitochondrial morphology (Figure 7). Drp1 is a known target of Dynasore, and it follows that the mitochondrial protection of Dynasore is mediated by inhibition of Drp1. Future studies could explore the effect of Dynasore on activities of other mitochondrial fusion and fission related regulators, such as MFN1, MFN2, OPA1, FIS1, MFF.

In conclusion, our study provides the first evidence that Dynasore has a potent lusitropic effect during I/R injury. The mechanism is mitochondrial protection and preservation of oxidative phosphorylation. Pharmaceutical therapy that preserves mitochondrial function may not just benefit myocardial survival, but improve diastolic dysfunction as well.

Acknowledgments

We are grateful to Dr. James W Smyth for his critical scientific advice and assistance with the cartoon images.

Author Contributions

Designed the experiments: LZ TTH. Performed data analysis: LZ TTH DCG. Supervised experimental design: JZ RMS. Technical support: RD. Performed the experiments: DCG. Contributed reagents/materials/analysis tools: JZ RMS. Wrote the paper: JZ RMS TTH DCG.

References

1. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, et al. (2010) Heart disease and stroke statistics–2010 update: a report from the American Heart Association. Circulation 121: e6-e215.
2. Zile MR, Brunaert DL (2002) New concepts in diastolic dysfunction and diastolic heart failure: Part I: diagnosis, prognosis, and measurements of diastolic function. Circulation 105: 1567–1593.
3. Bursi F, Weston SA, Redfield MM, Jacobsen SJ, Pahhomov S, et al. (2006) Systolic and diastolic heart failure in the community. Jama 296: 2209-2216.
4. Finzighobos TP, Meyer TE, Aurigemma GP (2009) Mortality in diastolic heart failure: an update. Cardiol Rev 17: 51-55.
5. Wood P, Piran S, Liu PP (2011) Diastolic heart failure: progress, treatment challenges, and prevention. Can J Cardiol 27: 302-310.
6. Zile MR, Brunaert DL (2002) New concepts in diastolic dysfunction and diastolic heart failure: Part II: causal mechanisms and treatment. Circulation 105: 1503-1508.
7. Prasad SB, See V, Brown P, McKay T, Narayan A, et al. (2011) Impact of duration of ischemia on left ventricular diastolic properties following reperfusion for acute myocardial infarction. Am J Cardiol 108: 549-554.
8. Yang EH, Brilakis ES, Reeder GS, Gersh BJ (2006) Modern management of acute myocardial infarction. Curr Probl Cardiol 31: 769-817.
9. Murphy E, Steenbergen C (2008) Mechanisms underlying acute protection from cardiac ischemia/reperfusion injury. Physiol Rev 88: 581-609.
10. Steenbergen C, Murphy E, Levy L, London RE (1987) Lysis in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. Circ Res 60: 700-707.
11. Murphy E, Pedman M, London RE, Steenbergen C (1991) Amiodarole delays the ischemia-induced rise in cytosolic free calcium. Circ Res 68: 1250-1258.
12. Arroyo CM, Kramer JH, Dickens BF, Weglicki WB (1987) Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitroxide DMOPO. FERBS Lett 221: 101-104.
13. Boli R, Patel BS, Joureidi MO, Lai EK, McCay PB (1988) Demonstration of free radical generation in “stunned” myocardium of intact dogs with the use of the spin trap alpha-phenyl-N-tert-butyl nitronate. J Clin Invest 82: 476-485.
14. Griffiths EJ, Ocampo CJ, Savage JS, Rutter GA, Hansford RG, et al. (1990) Mitochondrial calcium transporting pathways during hypoxia and reoxygenation in single rat cardiomyocytes. Cardiovasc Res 39: 423-433.
15. Griffiths EJ, Ocampo CJ, Savage JS, Stern MD, Silverman HS (2000) Protective effects of low and high doses of cyclosporin A against reoxygenation injury in isolated rat cardiomyocytes are associated with differential effects on mitochondrial calcium levels. Cell Calcium 27: 87-95.
16. Hailestrap AP, Clarke SJ, Javadov SA (2004) Mitochondrial permeability transition pore opening during myocardial reperfusion–a target for cardioprotection. Cardiovasc Res 61: 372-383.
17. Jezek P, Plecita-Hlavata L (2009) Mitochondrial reticulum network dynamics in relation to oxidative stress, redox regulation, and hypoxia. Int J Biochem Cell Biol 41: 1780-1788.
18. Ong SB, Subrayan S, Lim SY, Yellon DM, Davidson SM, et al. (2010) Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. Circulation 121: 2012-2022.
19. Macia E, Ehrlich M, Massel K, Boycot E, Brunner C, et al. (2006) Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell 10: 839-850.
20. Zhao T, Xi L, Chelliah J, Levasseur JE, Kukreja RC (2000) Inducible nitric oxide synthase mediates delayed myocardial protection induced by activation of adenosine A(1) receptors: evidence from gene-knockout mice. Circulation 102: 904-909.
21. Gao R, Ma H, Gao F, Zhong L, Ren J (2009) Metallothionein alleviates oxidative stress-induced endoplasmic reticulum stress and myocardial dysfunction. J Mol Cell Cardiol 47: 228-237.
22. Song X, Li G, Vaage J, Valen G (2007) Effects of sex, gonadectomy, and oestrogen substitution on ischaemic preconditioning and ischaemia-reperfusion injury in mice. Acta Physiol Scand 177: 449-466.
23. Sutherland FJ, Shattock MJ, Baker KE, Hearse DJ (2005) Mouse isolated perfused heart: characteristics and cautions. Clin Exp Pharmacol Physiol 30: 867-876.
24. Wolff RA, Chien GL, van Winkle DM (2000) Propidium iodide compares favorably with histology and triphenyl tetrazolium chloride in the assessment of experimentally-induced infarct size. J Mol Cell Cardiol 32: 225-232.
25. Weinbrenner C, Schulz F, Sarvary L, Strasser RH (2004) Remote preconditioning by infrarenal aortic occlusion is operative via delta1-opioid receptors and free radicals in vivo in the rat heart. Cardiovasc Res 61: 591-599.
26. O’Connell TD, Rodriguez MC, Simpson PC (2007) Isolation and culture of adult mouse cardiac myocytes. Methods Mol Biol 357: 271-296.
27. Chatterjee K, Zhang J, Houno N, Simonis U, Shaw R, et al. (2007) Acute vincristine pretreatment protects adult mouse cardiac myocytes from oxidative stress. J Mol Cell Cardiol 43: 327-336.
28. Kallrn J, Houno N, Summers K, Gray MO, Goetzl EJ (2001) The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. J Mol Cell Cardiol 33: 1713–1717.
29. Caemini R, Chentoufi J, Houno N, Long CS, Kallrn J (2000) Hypoxia differentially regulates stress proteins in cultured cardiomycocytes: role of the p38 stress-activated kinase signaling cascade, and relation to cytoprotection. Cardiovasc Res 46: 139-150.
30. Armstrong SC, Ganote CE (1991) Effects of 2,3-butanedione monoxime (BDM) on contracture and injury of isolated rat myocytes following metabolic ischemia and reperfusion. J Mol Cell Cardiol 23: 1001-1014.
31. Zhou YY, Wang SQ, Zhu WZ, Chruscinski A, Kobialka BK, et al. (2000) Culture and adenosinergic inhibition of adult mouse cardiac myocytes: methods for cellular genetic physiology. Am J Physiol Heart Circ Physiol 279: H429-436.
32. Sharikabad MN, Aronson JM, Traugott E, Pedersen J, Muller AS, et al. (2009) Cardiomyocytes from postinfarction failing rat hearts have improved ischemia tolerance. Am J Physiol Heart Circ Physiol 296: H789-795.
33. Babuin L, Jaffe AS (2005) Troponin: the biomarker of choice for the detection of cardiac injury. Cmaaj 173: 1191–1192.
34. Smyth JW, Hong TT, Gao D, Vogan JM, Jensen BC, et al. (2010) Limited forward trafficking of connexin 43 reduces cell-cell coupling in stressed human and mouse myocardium. J Clin Invest 120: 266-279.
35. Hong TT, Smyth JW, Gao D, Chu KY, Vogan JM, et al. (2010) BIN1 Localizes the L-Type Calcium Channel to Cardiac T-Tubules. PLoS Biol 8: e1000312.

36. Ingwall JS (2009) Energy metabolism in heart failure and remodelling. Cardiovasc Res 81: 412–419.

37. Paolocci N, Tavazzi B, Biondi R, Gharband YA, Amorini AM, et al. (2006) Metalloproteinase inhibitor counters high-energy phosphate depletion and AMP deaminase activity enhancing ventricular diastolic compliance in subacute heart failure. J Pharmacol Exp Ther 317: 506–513.

38. Cho DH, Nakamura T, Fang J, Cieplak P, Godzik A, et al. (2009) S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. Science 324: 102–105.

39. Kjaergaard J, Snyder EM, Hassager C, Olson TP, Oh JK, et al. (2006) The effect of 18 h of simulated high altitude on left ventricular function. Eur J Appl Physiol 98: 411–418.

40. Holloway C, Cochlin L, Codreanu I, Bloch E, Fatemian M, et al. (2011) Normobaric hypoxia impairs human cardiac energetics. FASEB J 25: 3130–3135.

41. Corral-Debrinski M, Stepien G, Shoffner JM, Lott MT, Kanter K, et al. (1991) Hypoxemia is associated with mitochondrial DNA damage and gene induction. Implications for cardiac disease. Jama 266: 1812–1816.

42. Ozounian M, Lee DS, Liu PP (2008) Diastolic heart failure: mechanisms and controversies. Nat Clin Pract Cardiovasc Med 5: 375–386.

43. Brady NR, Hamacher-Brady A, Gottlieb RA (2006) Proapoptotic BCL-2 family members and mitochondrial dysfunction during ischemia/reperfusion injury, a study employing cardiac HL-1 cells and GFP biosensors. Biochim Biophys Acta 1757: 667–678.

44. Gottlieb RA, Burleson KO, Kloner RA, Bahior BM, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 94: 1621–1628.

45. Brooks C, Wei Q, Cho SG, Dong Z (2009) Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. J Clin Invest 119: 1275–1285.