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RESEARCH ARTICLE

Dynamin-related protein 1 is a critical regulator of mitochondrial calcium homeostasis during myocardial ischemia/reperfusion injury

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Abstract

Dynamin-related protein 1 (Drp1) is a cytosolic GTPase protein that when activated translocates to the mitochondria, meditating mitochondrial fission and increasing reactive oxygen species (ROS) in cardiomyocytes. Drp1 has shown promise as a therapeutic target for reducing cardiac ischemia/reperfusion (IR) injury; however, the lack of specificity of some small molecule Drp1 inhibitors and the reliance on the use of Drp1 haploinsufficient hearts from older mice have left the role of Drp1 in IR in question. Here, we address these concerns using two approaches, using: (a) short-term (3 weeks), conditional, cardiomyocyte-specific, Drp1 knockout (KO) and (b) a novel, highly specific Drp1 GTPase inhibitor, Drpitor1a. Short-term Drp1 KO mice exhibited preserved exercise capacity and cardiac contractility, and their isolated cardiac mitochondria demonstrated increased mitochondrial complex 1 activity, respiratory coupling, and calcium retention capacity compared to controls. When exposed to IR injury in a Langendorff perfusion system, Drp1 KO hearts had preserved contractility, decreased reactive

Abbreviations: AMI, acute myocardial infarction; CA, cardiac arrest; CRC, calcium retention capacity; Drp1, dynamin-related protein 1; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Fis1, fission 1 protein; IR, ischemia/reperfusion; KO, knock out; MCUB, mitochondrial calcium uniporter dominant negative beta subunit; MCUR1, mitochondrial calcium uniporter regulator 1; Mfn1, mitofusin 1; Mfn2, mitofusin 2; MICU1, mitochondrial calcium uptake 1; MICU2, mitochondrial calcium uptake 2; MICU3, mitochondrial calcium uptake 3; MPTP, mitochondrial permeability transition pore; MUC, mitochondrial calcium uniporter; NBT, nitro blue tetrazolium chloride; OCR, oxygen consumption rate; OMM, outer mitochondrial membrane; OPA, optic atrophy 1; ROS, reactive oxygen species; RyR2, ryanodine receptor 2; WT, wild type.

oxygen species (ROS), enhanced mitochondrial calcium capacity, and increased resistance to mitochondrial permeability transition pore (MPTP) opening. Pharmacological inhibition of Drp1 with Drpitor1a following ischemia, but before reperfusion, was as protective as Drp1 KO for cardiac function and mitochondrial calcium homeostasis. In contrast to the benefits of short-term Drp1 inhibition, prolonged Drp1 ablation (6 weeks) resulted in cardiomyopathy. Drp1 KO hearts were also associated with decreased ryanodine receptor 2 (RyR2) protein expression and pharmacological inhibition of the RyR2 receptor decreased ROS in post-IR hearts suggesting that changes in RyR2 may have a role in Drp1 KO mediated cardioprotection. We conclude that Drp1-mediated increases in myocardial ROS production and impairment of mitochondrial calcium handling are key mechanisms of IR injury. Short-term inhibition of Drp1 is a promising strategy to limit early myocardial IR injury which is relevant for the therapy of acute myocardial infarction, cardiac arrest, and heart transplantation.

1 | INTRODUCTION

Ischemic heart disease resulting in sudden cardiac arrest (CA) and acute myocardial infarction (AMI) is the leading cause of death worldwide.[1,2] Although early and immediate restoration of circulation with cardiopulmonary resuscitation for CA and the use of percutaneous coronary intervention for AMI are essential for limiting ischemic injury, restoration of blood flow paradoxically results in poor myocardial contractility and accelerates cardiomyocyte death. This phenomenon known as ischemia/reperfusion (IR) injury contributes to transient myocardial dysfunction following CA and the development of heart failure following AMI.[3–5] Pharmacological strategies for minimizing IR injury following CA and AMI have been elusive due to a limited understanding of the underlying molecular mechanisms.[6] However, a growing body of evidence indicates that mitochondria are key upstream regulators of early IR injury and that understanding mitochondrial biology is fundamental to solving the problem of IR injury.[7,8]

Mitochondria comprise nearly 30% of the heart's volume and are essential organelles for the production of energy and cellular signaling in the heart, notably including the regulation of intracellular calcium homeostasis.[9] The quantity, quality, and function of mitochondria are regulated in part by their dynamic cycles of fission and fusion events. Mitochondrial fission (division) is primarily regulated by the large GTPase, dynamin-related protein 1 (Drp1).[10] Fission requires the interaction of Drp1 with its binding partner proteins on the outer mitochondrial membrane (OMM), including mitochondrial fission 1 protein (Fis1), mitochondrial fission factor, MiD49, and MiD51.[10] In contrast, mitochondrial fusion is regulated by mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (OPA-1). Mitochondrial fission occurs when activated Drp1 is recruited from the cytosol to the OMM at sites demarcated by the endoplasmic reticulum and interacts with its binding partners to form a macromolecular fission apparatus.[11] Although it is unclear if interconnected mitochondrial networks exist in the heart as they do in most other cell types, mitochondrial fission, and fusion play important homeostatic and protective roles in the heart.[12]

Mitochondrial function is disrupted following IR injury resulting in the formation of reactive oxygen species (ROS), calcium overload, mitochondrial permeability transition pore (MPTP) opening, and the release of cytochrome C which in aggregate result in contractile dysfunction and cardiomyocyte cell death.[13,14] Drp1 was found to translocate from the cytoplasm to the mitochondria during IR injury and inhibit its GTPase activity with either the putative Drp1 GTPase inhibitor, Mdivi-1, or the peptide P110 (which interferes with the interaction of Fis1 and Drp1) was found to be protective through the reduction of reactive oxygen species (ROS) generation.[15–17] Subsequently, however, Mdivi-1 specificity as a Drp1 GTPase inhibitor was called into question, clouding the interpretation of studies using this drug to understand the role of Drp1 in IR injury.[9,15] Recently, we developed a highly specific, small molecule, ellipticine inhibitor of Drp1 GTPase activity, Drpitor1a. The efficacy of Drpitor1a in left ventricular IR injury is unknown.[18]

Genetic models of Drp1 ablation have uniformly demonstrated that Drp1 is essential for neonatal and adult cardiac development, mitophagy, and long-term homeostatic cardiac health.[19,20] However, the potential benefit of Drp1 ablation in cardiac IR injury is less clear. Ikeda et al. first reported that 12-week-old Drp1 heterozygous mice had a 40% decrease in Drp1 expression and increased susceptibility to IR injury that was associated with impaired

autophagy.[20] They further reported that Drp1 haploinsufficiency exacerbates the development of mitochondrial dysfunction and heart failure after transverse aortic constriction.[21] These findings were contradicted by Bouche et al. that reported 12-week-old Drp1 heterozygous mice had a 60% decrease in Drp1 and were protected from IR injury, a finding they attributed to increased autophagy.[22] A limitation of studies utilizing nonconditional Drp1 haploinsufficiency is that Drp1 expression occurs during development, which does not allow for differentiation of short-term versus long-term effects of Drp1ablation; an important consideration given the established role of Drp1 in mitochondrial health. To date, there have been no studies investigating the effects of IR injury in adult mice with conditional, cardiac-specific, Drp1 KO prior to 6 and 8 weeks post-knockout, by which time mice begin to develop a cardiomyopathy. The role of Drp1 in IR injury and its mechanism of injury thus remains in question.

Here we sought to overcome the limitations of prior studies on the role of Drp1 in IR injury using two complementary approaches; the first being use of an adult conditional Drp1 cardiomyocyte-specific model with IR studies performed just 3 weeks after Drp1 KO (prior to the development of cardiomyopathy) and the second being the highly specific Drp1 GTPase inhibitor, Drpitor1a. Using a Langendorff perfused heart system we found that inhibiting Drp1 activity, using Drpitor1a or ablating Drp1, prevents acute myocardial injury following IR. Short-term Drp1 inhibition whether achieved genetically or pharmacologically, improves left ventricular function, preserves mitochondrial respiration, reduces ROS production, and decreases both the opening of the mitochondrial permeability transition pore (MPTP) and cytochrome C release. Both molecular and pharmacologic strategies for Drp1 inhibition yielded concordant results, revealing that Drp1 inhibition increased mitochondrial calcium buffering capacity and blocked calcium-induced ROS injury. These findings establish a pathological role for Drp1 in myocardial IR injury. In contrast to the established harmful effects of long-term Drp1 inhibition, short-term targeting of Drp1 following acute IR injury is a promising therapeutic strategy.

2 | METHODS AND MATERIALS

2.1 | Langendorff ischemia/reperfusion model

The Langendorff preparation was performed as described previously.[23] Briefly, the mouse (3–4 months, female, and male) was deeply anesthetized, a sternotomy was performed, and the heart was rapidly removed. The aorta was mounted on a Langendorff perfusion apparatus and perfused with oxygenated Krebs–Henseleit (KH) buffer (95% O_2 + 5% CO_2) at a constant pressure of 85 cmH_2O at room temperature. The atrioventricular node was ablated, and the heart was paced at a cycle length of 200 ms (300 bpm). LV pressure was measured by using a water-filled, homemade balloon connected to a pressure transducer (AD Instruments, Sydney, NSW, Australia). Blood pressure and heart rate were recorded using PowerLab and analyzed using Chart V 7.3.3. (AD Instruments). Ischemia-reperfusion (IR) hearts were subjected to 30 min of global ischemia followed by 30 min of reperfusion after 10 min of stabilization. Drpitor1a (0.1 μM) or ruthenium red (5 μM) was added to the perfusate after the heart was reperfused following 30 min of ischemia. The doses of both drugs used in the heart in this experiment were determined from previously published studies.[18,24]

2.2 | Mouse Drp1 knock-out model

The generation of Drp1 flox homozygous (fl/fl) mice has been described previously and was a kind gift of Dr. Gerald Dorn.[19] Floxed mouse lines were combined with the myh6-Mer-Cre-Mer mice for conditional cardiac ablation. Gene recombination was induced by intraperitoneal (i.p.) administration of tamoxifen (20 mg/kg) daily for 6 days. Controls are randomly selected, sex- and age-matched Drp1 wild type (WT, Drp1 +/+) mice (3–4 months, female, and male) with tamoxifen administration. Control and Drp1KO mice were all age- and sex-matched. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Chicago.

2.3 | Mouse echocardiography

Free-breathing mice were anesthetized with 3% inhaled isoflurane. They were placed supine on a Vevo 2100 (VisualSonics, Toronto, ON, Canada) heated imaging table (37°C). Body temperature and electrocardiogram were recorded, as previously described.[23] M-mode transthoracic echocardiography was performed using a parasternal long-axis approach to obtain 2D left ventricular images with a 37.5 MHz transducer. M-mode images were used to measure left ventricular end-diastolic and end-systolic size and to calculate the percent fractional shortening (FS%).

2.4 | Immunoblot

Protein samples from heart tissues were isolated following the manufacturer's instructions (Thermo Scientific,

Rockford, IL). Western blots were performed using standard procedures, as we described previously.[25,26] Anti-Drp1 (1:1000), anti-Mfn1 (1:500), anti-Mfn2 (1:500), anti-Parkin (1:500), anti-MCU (1:500), anti-MICU1 (1:500), and the secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Fis (1:500), anti-OPA1 (1:500), anti-GAPDH (1:1000), and anti-α-tubulin (1:2000) were from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-RyR2 (1:500) was from Proteintech (Rosemont, IL, USA). OXPHOS (a premixed cocktail of antibodies against CI-NDUFB8, CII-SDHB, CIII-UQCRC2, CIV-MTCO1, and CV-ATP5A) was purchased from Abcam (Cambridge, MA, USA).

2.5 | Mitochondria isolation

As previously described, the mouse heart was collected post-euthanasia, minced, and incubated with trypsin before homogenization with a motor-driven glass tissue grinder (Corning Incorporated, Corning, NY).[19] Heart homogenates were centrifuged at 800*g*×10 min at 4°C and the supernatant was collected and centrifuged at 8000*g*×10 min at 4°C. The pellet was collected and washed again then centrifuged at 8000*g*×10 min at 4°C to obtain normal-size mitochondria. The quality of the mitochondria was tested using Seahorse oxygen consumption measurement.

2.6 | Seahorse oxygen consumption measurement

After the mitochondria were isolated, the oxygen consumption rate (OCR) was measured using the Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA), as previously described.[19,27–29] Complex I substrates (pyruvate 5 mM + malate 2 mM) were used to measure OCR in 0.5 μg/mL cardiac mitochondria. Baseline OCR and stimulated (with 4 mM ADP) OCR were measured, constituting state 2 and state 3 respiration, respectively. Following the addition of ADP, oligomycin (2 μM) was added to check ATP-coupled respiration (state 4). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (4 μM) was added to measure maximal respiration and antimycin A (4 μM) was used to measure the proton leak.

2.7 | Complex I enzyme activity, complex I, and complex III activity

Complex I enzyme activity was measured using an enzyme activity dipstick assay (Abcam, Cambridge, MA) following the manufacturer protocol. Briefly, immunocaptured complex I oxidizes NADH and the resulting H^+ reduces nitro blue tetrazolium chloride (NBT) to form a blue precipitate at the complex I antibody line on the dipstick immersed in complex I activity buffer containing NADH (substrate) and NBT (electron acceptor). The signal intensity of this precipitate corresponds to the level of complex I enzyme activity (blue band) in the heart tissue samples. The intensity was analyzed by using Fiji 6 (NIH, public domain).

Complex I and III activities were measured in isolated cardiac mitochondria according to the instructions provided by the manufacturer's instructions (Biovision, Milpitas, CA). For complex I activity measurement, a colorimetric assay was used to monitor the reduction of Complex I dye by tracking the oxidation of NADH, while for complex III activity measurement, the change in the reduction of cytochrome C was monitored. The absorbance at 600 nm (for complex I activity) or 540 nm (for complex III activity) was measured using Spectra MAX M5 (Molecular Devices, Sunnyvale, CA) respectively.

2.8 | Transmission electron microscopy (TEM)

Left ventricular sections from Langendorff perfused hearts were fixed and processed as described previously.[23] Images were collected ($n = 8$) using a scanning transmission electron microscope at 300 kV (Tecnai F30; FEI, Hillsboro, OR USA) with a Gatan charge-coupled device camera. Mitochondrial images (density and cross-sectional areas) were analyzed in a blinded manner using ImageJ software.

2.9 | Opening of mitochondria permeability transmission pore

Calcium-induced mitochondrial permeability transition pore (MPTP) opening was determined in freshly isolated cardiac mitochondria, as described previously.[19] Briefly, mitochondria were suspended in 200 μL reaction buffer (120 mM KCl, 10 mM Tris, and 5 mM KH_2PO_4) at 250 μg/mL and stimulated by the addition of 1 mM $CaCl_2$. Absorbance was continuously measured at 540 nm using a Spectra MAX M5 in 96 well plate reader. Mitochondrial swelling, a marker of MPTP opening, was measured as the decrease in absorbance within 30 min.

2.10 | Calcium retention capacity assay

A calcium retention capacity (CRC) assay was used to measure calcium uptake in WT and Drp1 KO mice.[19] The CRC assay was assessed in a CRC buffer containing

120 mM KCl, 10 mM Tris–HCl, 5 mM MOPS, 5 mM Na_2HPO_4, 10 mM glutamate, 5 mM malate, and 0.01 mM EGTA. The fluorescent calcium indicator Calcium Green-5 N (final concentration 1 μM, Molecular Probes, Eugene, OR) was added to the CRC buffer and then aliquoted on a 96-well black plate. One hundred micrograms of mitochondria diluted in 50 μL of CRC Buffer was then added to the plates. Multiple injections of 175 μM $CaCl_2$ were used to increase the mitochondrial matrix calcium load. Fluorescence was measured at 506 nm excitation and 532 nm emission on a Spectra MAX M5 plate reader. $CaCl_2$ (175uM) was injected into the plate every 240 s.

2.11 | Calcium-induced mitochondrial H_2O_2 production by succinate

The measurement of the rate of H_2O_2 production by mitochondria is a reliable method to evaluate ROS production.[30] H_2O_2 production in isolated mitochondria (0.1 μg/μl) was measured using a Spectra MAX M5 following the instruction of Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit from Molecular Probes (Eugene, USA). Succinate at a concentration of 5 mM was added to plate to induce H_2O_2 production. $CaCl_2$ at the concentrations of 3, 30, 300, and 3000 nM was added to different wells to test the effect of $CaCl_2$ on succinate-induced mitochondrial H_2O_2 production.

2.12 | Tissue staining and MitoSox staining

Trichrome staining and H&E staining on heart sections (10 μm) were done using the standard procedure described previously.[19,31] Mitochondrial-derived ROS production was measured using Mitosox. Briefly, the heart sections (thickness: 10 μm) were cut on a cryostat, mounted on glass slides, and stored at −80°C. At the time of mitochondrial ROS measurement, slides were thawed, washed in PBS, and stained with 5 μM MitoSox for 20 min in the dark. After staining, were washed in PBS and imaged immediately on a Zeiss fluorescent microscope. The mitochondrial ROS production was quantified by measuring red fluorescence (excitation wavelength: 485, emission wavelength: 530 nm).

2.13 | Data analysis

Data was presented as mean ± SEM. Statistical analyses were performed using Prism software (Graph Pad, La Jolla, CA, USA). Inter-group differences were assessed by t-test or one-way ANOVA with post hoc analysis using Tukey's test, as appropriate. Values of $p < .05$ were considered statistically significant.

3 | RESULTS

3.1 | Acute (3 weeks) Drp1 ablation does not impair cardiac function

To study the effects of short-term Drp1 inhibition on post-IR myocardial function, we utilized a previously published tamoxifen-inducible, cardiomyocyte-specific Drp1 knockout mouse.[19] Following tamoxifen injection, Drp1 ablation in the heart was evident in KO mice by western blotting at 3 and 6 weeks (Figure 1A, Figure S1A). Following 3 weeks of Drp1 ablation, Drp1 KO hearts remained normal morphologically, except for evidence of mild myocardial fibrosis, as detected by Trichrome staining (Figure 1B). Drp1 ablation at 3 weeks also did not alter the heart weight to body weight ratio (HW/BW) or LV fractional shortening (LVFS) (HW/BW: 4.89 ± 0.17 vs 4.59 ± 0.30 mg/g; LVFS: 44.5 ± 1.6 vs 45.8 ± 1.0%; $p > .05$ vs WT, $n = 8–11$, respectively, Figure 1C). Treadmill data furthermore demonstrated that 3-week Drp1 ablation in the heart did not change exercise capacity (treadmill distance: 188.5 ± 20.3 vs 182.0 ± 22.3 m, $p > .05$ vs WT, $n = 12$, respectively, Figure 1D). These data demonstrate that acute Drp1 ablation of 3 weeks does not impair myocardial function and is relatively benign. In contrast to the effects of acute Drp1 ablation, we confirmed prior reports that a longer duration of Drp1 ablation (6 weeks) results in heart failure.[19] This was evidenced by extensive myocardial fibrosis (Figure S1B), increased HW/BW (HW/BW: 5.37 ± 0.16; $p < .001$ vs WT, $n = 9$) (Figure S1C), decreased LVFS FS, 28.3 ± 1.1%; $p < .001$ vs WT, $n = 9$), and reduced exercise capacity (104.9 ± 6.8 m, $p < .001$ vs WT, $n = 12$, Figure S1D). These data demonstrate a temporal dimorphism in the effects of Drp1 ablation in the heart.

3.2 | Acute Drp1 ablation does not alter mitochondrial respiration complex proteins or regulators of mitochondrial dynamics

To determine whether acute Drp1 KO at 3 weeks altered the expression of other key mitochondria proteins we measured the expression of mitochondrial subunits from complexes I-V. The expression of mitochondrial subunits in the heart after 3 weeks of Drp1 ablation was not altered compared with the expression in WT hearts ($p > .05$ vs WT, $n = 3$, Figure S2A). In addition, we assessed the possibility there would be compensatory changes in the expression of other key mediators of mitochondrial dynamics. We found no evidence of changes

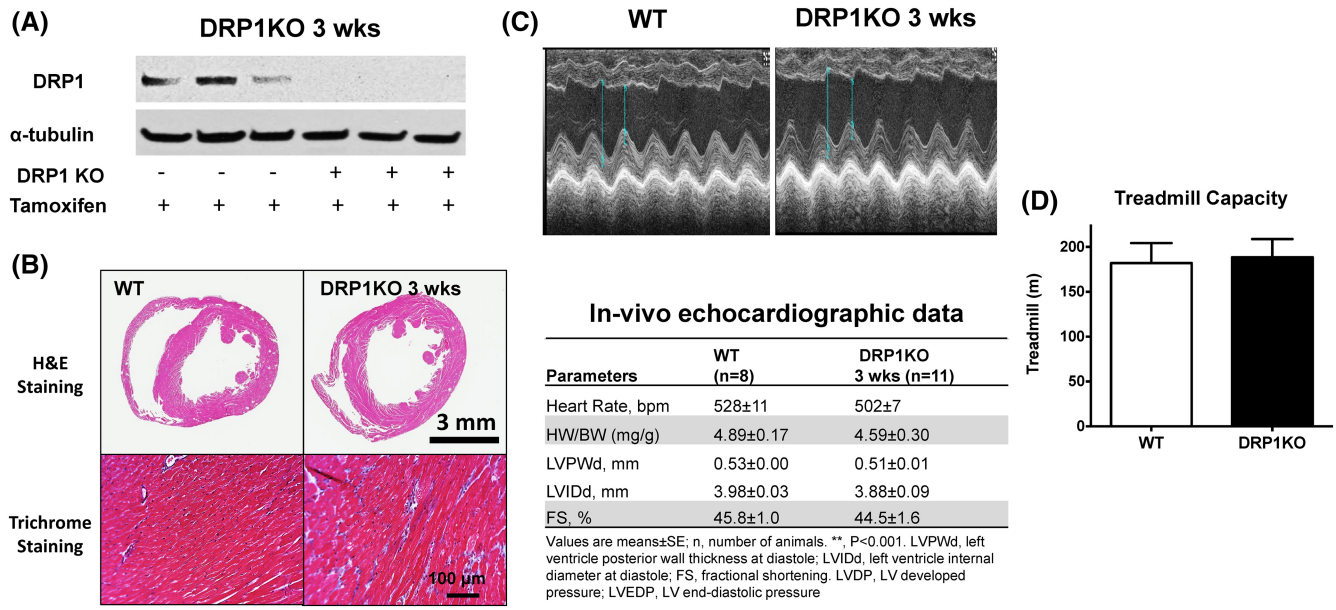

FIGURE 1 Three-week cardiomyocyte-specific Drp1 ablation does not affect cardiac function, exercise capacity, and mitochondrial morphology. (A) Immunoblotting of Drp1 protein demonstrates loss of Drp1 in the heart 3 weeks following tamoxifen injections. No Drp1 bands appear in the heart with Drp1 ablation. (B) The transverse section of the hearts. Trichrome staining on the heart tissue section shows mild signs of fibrosis in the heart with 3 weeks of Drp1 ablation. (C) M-mode echocardiographic analysis shows no changes in fractional shorting (%) and heart weight with 3 weeks of Drp1 ablation. (D) The treadmill distance is not changed with 3 weeks of Drp1 ablation.

in the expression of fission and fusion-related proteins Mfn1, Mfn2, Fis1, and OPA1 in the heart after 3 weeks of Drp1 ablation ($p > .05$ vs WT, $n = 3$, Figure S2B).

3.3 | Acute Drp1 ablation increases mitochondrial complex I activity

To determine oxygen utilization in the heart following acute Drp1 ablation (3 weeks), we measured the oxygen consumption rate (OCR) in cardiac mitochondria isolated from age- and sex-matched WT and KO hearts. Basal OCR, OCR responses to the uncoupler, FCCP, and to the complex III inhibitor, antimycin A showed no differences between WT hearts and the Drp1 KO hearts ($p > .05$, $n = 10$–12, respectively, Figure 2A,B). Interestingly, the OCR responses to ADP were increased in mitochondria from Drp1 KO hearts without evidence of increased electron leak ($p < .05$, $n = 10$–12, Figure 2B). As a result, the respiratory control ratio (State 3/State 4, $p < .05$, $n = 10$–12, Figure 2B) was increased suggesting an increased mitochondrial energetic efficiency. Consistent with this observation, complex I activity was increased in cardiac mitochondria with Drp1 ablation, whether measured using a complex I enzyme activity dipstick assay or a complex I activity assay kit ($p < .05$, $p < .001$ vs WT, respectively, $n = 6$ in each group, Figure 2C,D). However, complex III activity, measured by using complex III activity assay kits, was unchanged in Drp1 ablation mitochondria versus WT cardiac mitochondria ($p > .05$ vs WT, $n = 6$, Figure 2E). These results suggest that Drp1 ablation increases mitochondrial complex I activity as well as increases coupling of complex activity to respiration.

3.4 | Acute Drp1 ablation improves post-IR contractile activity and preserves mitochondrial respiration

We next evaluated the effects of acute Drp1 ablation on the heart's response to IR injury in an *ex vivo* isolated Langendorff perfusion system. Following 30 min of ischemia and 30 min of reperfusion, the systolic pressure, end-diastolic pressure, and the left ventricular developed pressure were all well preserved in the Drp1 KO hearts when compared with control hearts (systolic pressure: 120.5 ± 7.4 vs 97.0 ± 7.9 mmHg; end of diastolic pressure: 8.3 ± 1.8 vs 35.0 ± 10.0 mmHg; developed pressure: 107.4 ± 7.4 vs 62.0 ± 7.8 mmHg. $p < .05$, $p < .01$, $p < .001$ vs WT at 30 min post-reperfusion, $n = 8$, $n = 11$, respectively. Figure 3A,B). Thus, acute Drp1 ablation protected the LV from IR injury. Drp1 KO protection was also associated with preserved ventricular mitochondrial function. The basal OCR, OCR response to ADP, oligomycin, FCCP, and antimycin-A were significantly increased in mitochondria isolated from Drp1 ablation hearts vs mitochondria isolated from WT hearts ($p < .05$, $n = 10$–12, respectively, Figure 3D). Importantly, complex I activity was improved in the Drp1KO group post-IR ($p < .05$, $n = 4$, respectively,

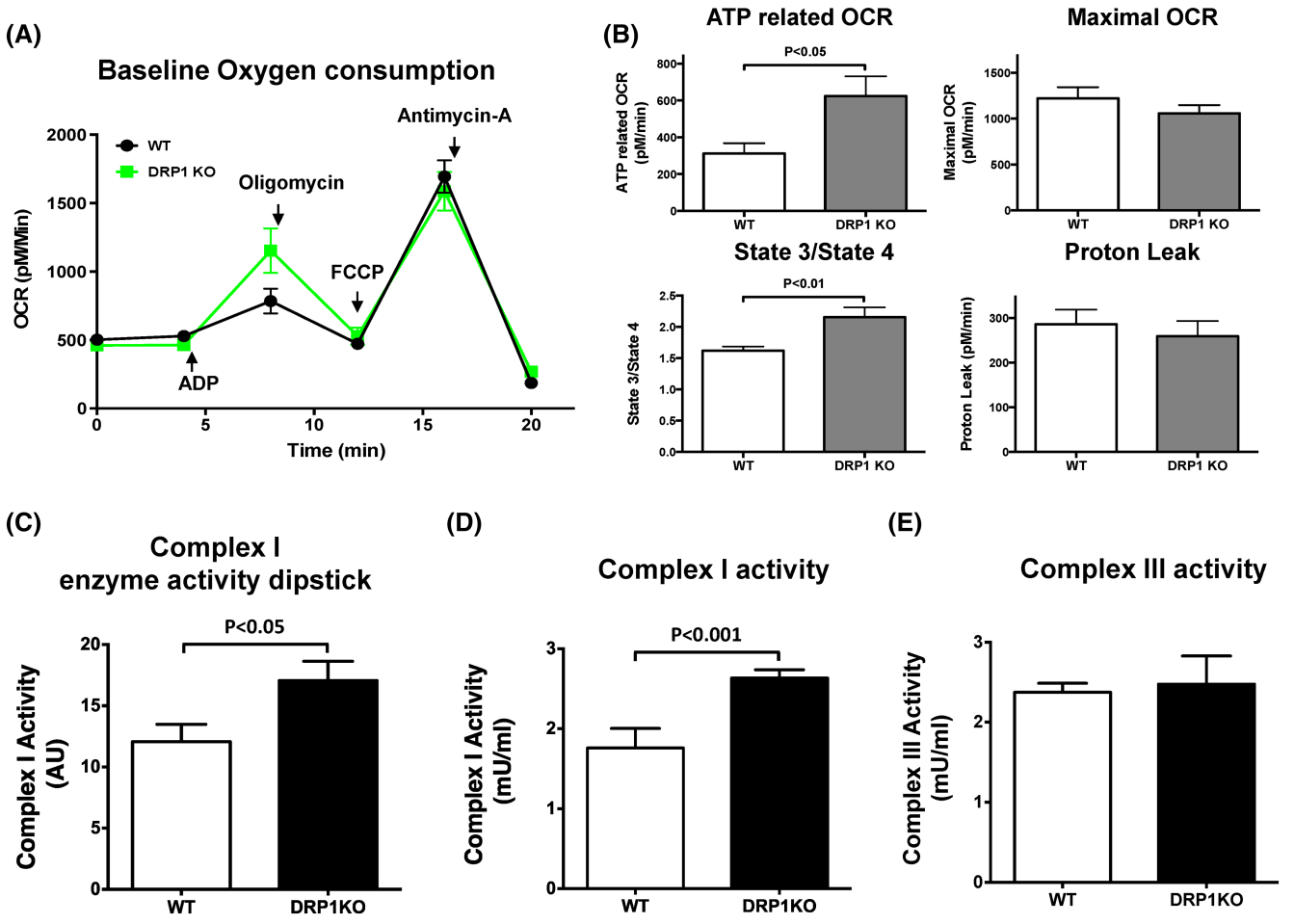

FIGURE 2 Three-week Drp1 ablation improves ATP-related oxygen consumption by increasing complex I activity. (A) Isolated mitochondria from control and Drp1 ablated hearts underwent analysis for oxygen consumption rates (OCR) using a XF24 Analyzer (Seahorse Bioscience). The sequential injection of mitochondrial inhibitors is indicated by arrows. (B) Mean values of baseline OCR, ATP-related OCR, maximal OCR, State 3/State 4, and proton leak are compared between WT mitochondria and the mitochondria with 3-week Drp1 ablation heart. (C) Complex I enzyme activity of isolated mitochondria from control hearts and Drp1 ablated hearts evaluated with commercial dipstick assay. (D) Complex I activity is increased in the Drp1 ablation heart by using a colorimetric assay to track the oxidation of NADH. (E) Complex III remains unchanged by using a colorimetric assay to monitor the reduction of cytochrome C.

Figure 3E). Thus, Drp1 KO's cardioprotective effect post-IR injury is associated with preserved mitochondrial function and improved complex I respiration.

3.5 | Acute Drp1 ablation increases mitochondrial fusion and reduces IR-induced mitochondrial swelling

We next determined the effects of IR on mitochondrial structure and organization in the left ventricle using transmission electron microscopy. Mitochondria size was increased 1.5-fold and mitochondria density decreased to 89% in the acute Drp1 KO hearts ($p < .01$, respectively, $n = 76–78$, Figure 4A), consistent with the expected effects of Drp1 ablation, namely decreased mitochondrial fission. Following 30 min of IR, mitochondria in WT heart appeared swollen and were 3-fold larger than at baseline while mitochondria density was reduced to 87% versus WT hearts without IR injury ($p < .001$ vs WT, respectively, $n = 70–78$, Figure 4A). In contrast, Drp1 KO hearts had preserved mitochondrial size and density post-IR ($p > .05$ vs Drp1 KO without IR injury, respectively, $n = 76–78$, Figure 4A). Acute ablation of Drp1 thus preserves mitochondrial morphology following IR injury.

3.6 | Acute Drp1 ablation prevents IR-induced ROS generation and decreases mitochondrial calcium-dependent ROS generation

As expected, IR injury increased ROS production in WT hearts ($p < .001$ vs WT post-IR, $n = 8–10$, Figure 4B). In

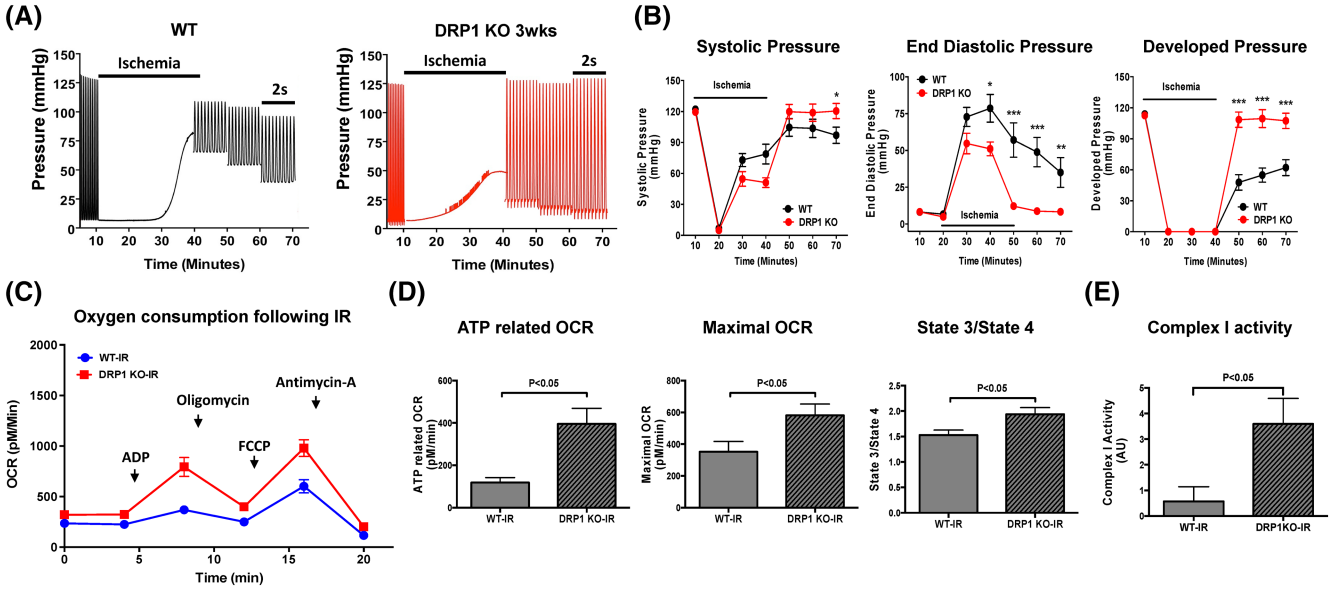

FIGURE 3 Three-week Drp1 ablation improves the hemodynamics and oxygen consumption following ischemia-reperfusion (IR). (A) Representative traces of Langendorff perfused control and Drp1 ablated hearts following 30 min of ischemia followed by reperfusion. (B) Mean values of traces in A. (C) Oxygen consumption rate (OCR) traces determined by seahorse flux analysis on isolated mitochondria from control and Drp1 ablated hearts following IR injury. ADP addition was used to stimulate ATP-coupled respiration. ATP synthase inhibitor oligomycin was used to block ATP-coupled respiration, FCCP was used to uncouple mitochondrial respiration, and antimycin was used to inhibit mitochondrial respiration. The sequential injection of mitochondrial inhibitors is indicated by arrows. (D) Mean values of baseline OCR, ATP-related OCR, maximal OCR, and State 3/State 4 are compared between WT mitochondria and the mitochondria in a 3-week Drp1 knockout heart. (E) Complex I activity dipstick assay of mitochondrial complex I activity in control and Drp1 KO mitochondria following IR.

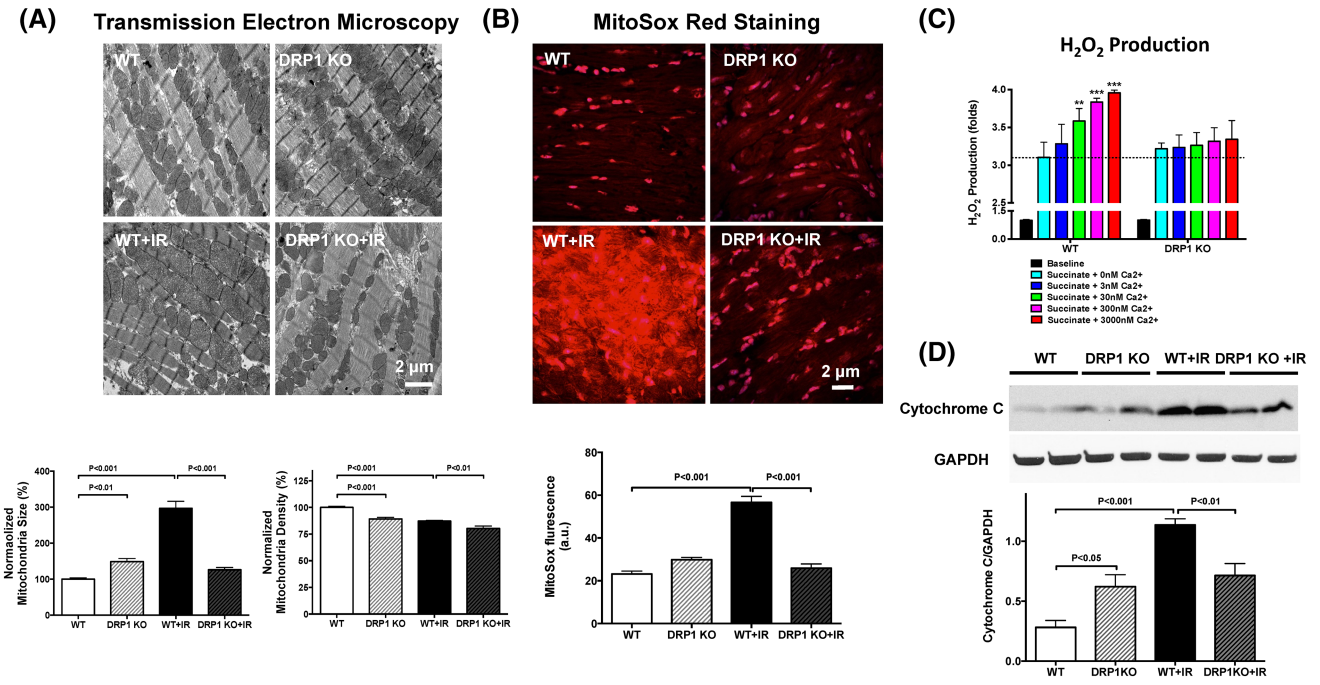

FIGURE 4 Three-week Drp1 ablation rescues mitochondrial morphology and limits ROS generation in the heart post-IR. (A) Representative transmission electron microscopy images of cardiac mitochondria and the mean values of mitochondria size and density in the WT group and Drp1 knockout group following IR. (B) MitoSox Red staining shows that mitochondrial ROS generation is normalized in the Drp1 knockout group post-IR. (C) Succinate-induced H_2O_2 production in WT cardiac mitochondria increases along with the increase of calcium concentration. Succinate-induced H_2O_2 production in the Drp1 knockout group is insensitive to the increased calcium concentration. (D) Western blot bands and the bar graphs show that the increased cytochrome c expression is reduced in the Drp1KO group post-IR.

contrast, Drp1 ablation decreased ROS production induced by IR ($p < .001$ vs WT post-IR, $n = 8–10$, Figure 4B). Acute Drp1 ablation in the heart of KO mice thus decreases IR-induced increases in ROS.

Succinate-induced mitochondrial ROS production is a critical mediator of IR injury. Succinate levels increase during ischemia. During reperfusion, succinate induces backflow of electrons through complex I thereby uncoupling electron flow from respiration and inducing ROS production.[32,33] Consequently, we measured the effect of acute Drp1 ablation on the rate of succinate-induced H_2O_2 generation. Using different concentrations of extracellular $CaCl_2$ to mimic the calcium overload that occurs following IR injury, we measured succinate-dependent H_2O_2 production in mitochondria in both WT and KO hearts. In the WT heart, mitochondrial H_2O_2 production in the presence of succinate increased in proportion to the calcium concentration. In contrast, mitochondria from Drp1 ablated hearts did not demonstrate calcium concentration-dependent increases in succinate-induced mitochondrial H_2O_2 production, even at maximum calcium concentrations ($p < .01$ with 30 nM $CaCl_2$; $p < .001$ with 300 nM $CaCl_2$; $p < .001$ with 3 mM $CaCl_2$ vs WT, $n = 4$, respectively, Figure 4C). These findings indicate that both mitochondrial calcium overload and Drp1 are critical factors in promoting succinate-driven ROS production during IR injury.

Cytochrome C expression is often increased following IR injury in the heart and is a marker of injury. Following IR, we found that Drp1 KO hearts exhibited a 38% reduction in cytochrome C expression compared to wild-type controls ($p < .001$ vs WT, $n = 4$, Figure 4D), despite the slight increase of cytochrome C in Drp1 KO heart at baseline ($p < .05$ vs WT, $n = 4$, Figure 4D). Together, these data suggested that mitochondria from Drp1 ablated hearts are relatively insensitive to increases in calcium, thus producing less ROS and resulting in less cytochrome C induction following IR injury, even with simulated calcium overload. This provides a potential explanation for the observed cardioprotective effect of Drp1 inhibition or deletion.

3.7 | Acute Drp1 ablation reduces calcium uptake and ROS generation by decreasing RyR2 expression following IR injury

We next studied the effect of acute Drp1 ablation on mitochondrial calcium homeostasis and retention at baseline and post-IR to determine whether Drp1's cardioprotective effects were related to changes in MPTP opening. As shown in Figure 5A, calcium-induced MPTP opening did not differ between WT and Drp1 KO hearts under control conditions ($p > .05$ at all time points vs WT, $n = 4$, Figure 5A). However, following IR injury, mitochondria from Drp1 KO hearts exhibited slowed calcium-induced MPTP opening ($n = 4$, Figure 5B) versus control mitochondria. We then similarly evaluated calcium retention capacity in control and Drp1 KO mitochondria under baseline and post-IR conditions. Mitochondria from Drp1 ablated hearts better withstood calcium overload compared to control mitochondria both under baseline conditions ($n = 4$, Figure 5C) and following IR injury ($n = 4$, Figure 5D).

We then investigated the gene expression of the key regulators of mitochondrial calcium uptake in the hearts with Drp1 ablation. As shown in Figure 5E and Figure S3, gene expression (mRNA) was unchanged for the following relevant calcium regulators in 3-week Drp1 KO hearts: ryanodine receptors type 2 (RyR2), mitochondrial calcium uniporter (MUC), mitochondrial calcium uniporter dominant negative beta subunit (MCUB), mitochondrial calcium uniporter regulator 1 (MCUR1), mitochondrial calcium uptake 1 (MICU1), mitochondrial calcium uptake 2 (MICU2) and mitochondrial calcium uptake 3 (MICU3) ($p > .05$, $n = 5$ respectively). The protein expression of MICU1 and MCU was also unchanged by Drp1 ablation ($p > .05$, $n = 3$ respectively, Figure S3B).

Gene expression of MCU, MCUB, MCUR1, MICU1, MICU2, and MICU3 were also not altered by IR in control or Drp1 hearts ($p > .05$, $n = 5$ respectively, Figure S4A). Interestingly, although ryanodine receptor type 2 (RyR2) gene expression was not altered in Drp1 KO hearts at baseline (Figure 5E), RyR2 protein expression was decreased in Drp1 KO hearts ($p < .05$, $n = 4$, Figure 5F). RyR2 gene and protein expression was also reduced post-IR in Drp1KO hearts compared with controls ($p < .05$, $n = 4$, Figure 5E,F). To explore whether inhibiting RyR2 is beneficial for mitochondrial function, we applied the RyR inhibitor ruthenium red (RR, 5 μM) to the perfusate of Langendorff-suspended hearts following 30 min of ischemia. RR-treated hearts demonstrated decreased ROS generation following IR compared to controls, as evidenced by decreased MitoSox red staining ($p < .001$, $n = 6$, Figure 5G). These results suggest that changes in the RyR2 receptor may have a role in improved calcium homeostasis and mitochondrial function in Drp1 KO hearts.

3.8 | Drpitor1a improves LV systolic function and cardiac mitochondrial function following IR injury

Next, we sought to determine if pharmacological inhibition of Drp1 GTPase activity had similar protective effects

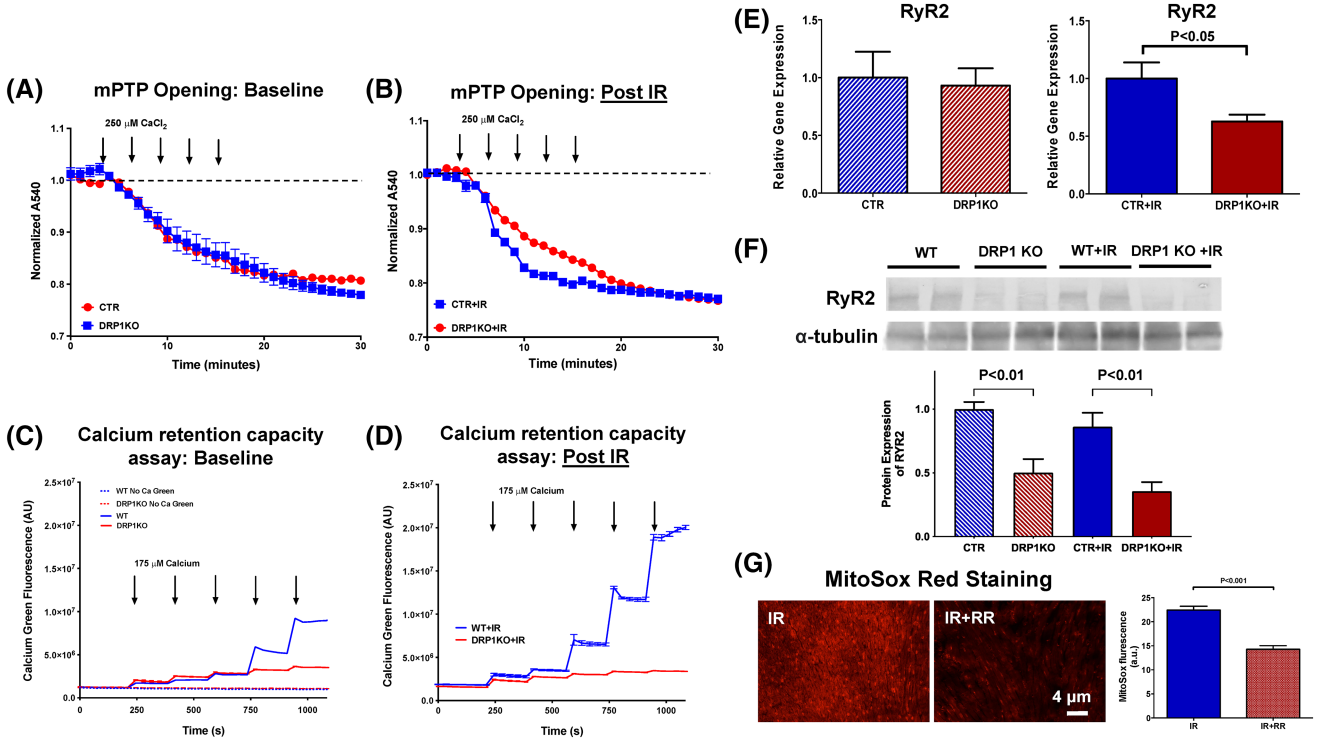

FIGURE 5 Three-week Drp1 ablation limits MPTP opening following IR injury. (A) Mitochondria isolated from control and Drp1 ablated hearts underwent calcium-induced swelling assay to evaluate for MPTP opening. (B) Mitochondria from control and Drp1 ablated hearts following IR injury subjected to calcium-induced swelling. (C) Mitochondria isolated from control and Drp1 ablated hearts subjected to calcium retention assay. (D) Mitochondria isolated from control and Drp1 ablated hearts following IR injury subjected to calcium retention assay. (E) mRNA expression of the RyR2 in control and Drp1 KO hearts at baseline and after IR injury. (F) Protein expression of the RyR2 in control and Drp1 KO hearts at baseline and after IR injury. (G) MitoSox staining of the left ventricle demonstrates decreased ROS generation in Langendorff hearts perfused with ruthenium red (RR).

to short-term Drp1 genetic ablation using the inhibitor Drpitor1a. Drpitor1a has been shown to preserve RV diastolic function in a RV-IR model but its effects on left ventricular (LV) function following IR injury are not known.[18] Using an *ex vivo* Langendorff setup, we tested the effects of Drpitor1a on LV) function following IR injury using doses previously shown to be effective at inhibiting Drp1GTPase activity (0.1 μM). Thirty minutes of ischemia followed by reperfusion in control hearts resulted in significant declines in systolic pressure and increases in diastolic pressure resulting in substantial decreases in developed pressure (defined as systolic pressure—end-diastolic pressure) (Figure 6A,B). In contrast, administration of Drpitor1a following ischemia and continued during reperfusion preserved both systolic pressure and the developed pressure in the heart (systolic pressure: 85.1 ± 3.7 vs 66.3 ± 6.1 mmHg; developed pressure: 66.3 ± 4.6 vs 36.8 ± 2.1 mmHg, measured 30 min after IR injury. $p < .05$, $p < .001$ vs IR group, $n = 4$, respectively. Figure 6A,B). These results demonstrate that pharmacological Drp1 inhibition protects the heart from reduced LV function following IR injury.

We then studied the effects of Drpitor1a on changes in mitochondrial calcium uptake in isolated mitochondria following IR. The goal of these experiments was to determine whether Drpitor1a's cardioprotective effects were related to the preservation of mitochondrial calcium homeostasis. IR induces mitochondrial depolarization, calcium release, and mitochondrial swelling. Mitochondrial size can be determined by forward angle light scattering. Decreases in absorbance indicate swelling of the mitochondrial matrix and MPTP opening. Mitochondria isolated from Dripitor1a-treated hearts subjected to IR had less calcium-induced mitochondrial swelling than those from control IR hearts, suggesting that inhibition of fission decreases mitochondrial calcium uptake and sensitivity and resistance to MPTP opening ($n = 3$, Figure 6C).

Consistent with these findings, mitochondria from Drpitor1a treated IR hearts were resistant to calcium-induced calcium release, indicating that preventing fission increased mitochondrial capacity to uptake and retain calcium (calcium retention capacity) compared to control IR hearts ($n = 3$, Figure 6D). Drpitor1a treated

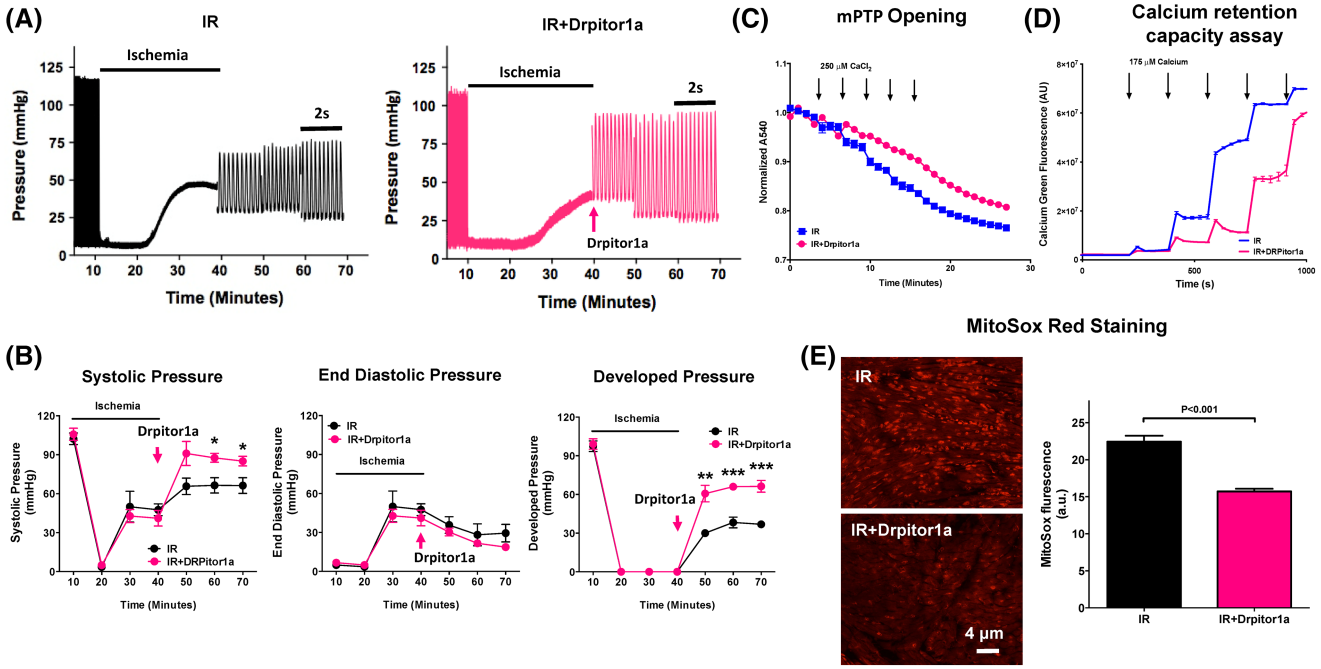

FIGURE 6 Drp1 GTPase inhibitor Drpitor1a improves cardiac function by improving mitochondrial calcium homeostasis and reducing ROS generation. Representative pressure traces (A) and mean values (B) of Langendorff perfused hearts following IR with and without Drpitor1a. Following IR injury, diastolic pressure is elevated, and the systolic pressure is reduced resulting in a significantly decreased developed pressure (systolic pressure—end-diastolic pressure). Administration of Drpitor1a following ischemia and at the beginning of reperfusion results in improved developed pressure. (C) Cardiac mitochondrial swelling (decrease in absorbance at 540 nm) induced by multiple injections of $CaCl_2$ shows MPTP opening was reduced by the treatment of Drpitor1a compared with the control group post-IR. (D) Calcium retention capacity assay measured with the florescence density of calcium green-5 N shows calcium uptake from the cytosol to mitochondria is increased with the treatment of Drpitor1a treatment compared with the control mitochondria post-IR. (E) ROS indicator (MitoSox Red) staining of the left ventricle of IR hearts treated with and without Drpitor1a.

hearts also displayed reduced generation of ROS, as indicated by decreased MitoSox red (a superoxide indicator) staining compared with the untreated controls ($p < .01$, $n = 6$, Figure 6E) consistent with lower mitochondrial ROS production following Drpitor1a treatment. These studies demonstrate that Drpitor1a's beneficial effects on cardiac systolic function were associated with the improvement of mitochondrial calcium homeostasis and a reduction of mitochondrial ROS generation.

4 | DISCUSSION

In this study, we have utilized both genetic (using inducible cardiomyocyte-specific Drp1 KO mice) and pharmacological (using Drpitor1a, a specific inhibitor of Drp1 GTPase) approaches to clarify the role of Drp1 in post-ischemic mitochondrial injury, ROS generation, and myocardial function. This study has four important findings. *First*, we determined that short-term Drp1 ablation (3 weeks) does not impair cardiac contractility or reduce exercise capacity. This contrasts with the deleterious effects observed with more prolonged Drp1 ablation (e.g., 6 weeks duration). Thus, while Drp1 is essential for normal cardiac function in the long-term, in the short-term Drp1 ablation is not harmful and indeed is cardioprotective against the effects of IR. *Second*, acute Drp1 ablation increased mitochondrial complex I activity and ADP-stimulated oxygen consumption. These benefits were preserved following IR injury and were associated with improved post-IR myocardial function. Acute molecular depletion of Drp1 is protective of mitochondrial and myocardial function in the setting of IR, confirming prior pharmacological studies that our group and others have published. However, our findings of cardiomyopathy following 6-week Drp1 KO indicate that therapeutic interventions targeting Drp1 need to be time-limited to avoid potential cardiotoxicity. *Third*, acute Drp1 ablation decreased mitochondrial ROS generation following IR injury and reduced mitochondrial sensitivity to the effects of calcium concentration-dependent increases in ROS production. Drp1 ablation thus therapeutically attenuated mitochondrial complex I's response to ROS generation, which occurs during IR injury. This mechanism is associated with better calcium homeostasis post-IR in hearts in which there has been acute short-term (3 weeks) conditional, cardiac Drp1

ablation. These findings were associated with Drp 1 KO alterations in cardiac RyR2 protein expression, suggesting potential interactions between Drp1 and RyR2 regulation that regulate calcium homeostasis in the heart. *Fourth*, we confirm the beneficial effects of pharmacological inhibition of Drp1 by showing for the first time that the Drp1 GTPase inhibitor Drpitor1a improves mitochondrial and cardiac function in the left ventricle following IR injury, findings concordant with our observations in mice with short-term Drp1 KO. *Collectively*, these four findings affirm Drp1 as a mediator of mitochondrial ROS generation following IR injury and support the benefit of short-term Drp1 inhibition as a therapeutic strategy for IR injury.

Drp1 is an important regulator of mitochondrial dynamics in the heart as a mediator of mitochondrial fission. Early studies implicated it in the pathological injury of the heart following IR injury.[15–17] Drp1 inhibition was also found to reverse Drp1 mitochondrial translocation and interrupt apoptosis in the heart under conditions of mitochondria stress, such as starvation and IR injury.[34] Over-expression of dominant negative forms of Drp1 to reduce the endogenous activity of Drp1 through the use of adenoviruses (Drp1K38A, a dominant-negative form of Drp1), delayed MPTP opening and elongated mitochondria, leading to cardioprotection post-IR injury.[35] P110 (a peptide that inhibits fission by blocking the interaction between Fis1 and Drp1) and Mdivi-1 (a Drp1 GTPase inhibitor) were also found to be cardioprotective.[15,17] Our findings are supportive of these earlier reports. Enhancing mitochondrial fusion through the inhibition of Drp1-mediated mitochondrial fission was accepted as a cardioprotective strategy for a multitude of cardiac conditions.[11] This approach however was called into question by two findings; 1) Mdivi-1, the gold standard for pharmacological inhibition of Drp1 was found to also inhibit mitochondrial complex 1, calling into question the specificity of Drp1 inhibition in studies that had employed its use[36] and 2) the discovery of Drp1's importance in regulating long-term mitochondrial and cardiac health as a key regulator of autophagy and mitophagy. Ikeda et al. determined that long-term Drp1 ablation impaired mitochondrial health, induced cellular apoptosis, and enhanced MPTP opening, despite increasing mitochondrial fusion. Following 8–12 weeks of Drp1 ablation, all mice died and showed evidence of increased autophagy.[20] Kageyama et al. also found that Drp1 deficiency interrupted mitophagy despite enhancing mitochondrial fusion.[37] Similarly, Song et al. found that 6 weeks of Drp1 deletion induced mitophagy in the heart associated with dilated cardiomyopathy resulting in death.[19] Collectively, these studies called into question the benefits of therapeutically inhibiting Drp1 given its essential role as a homeostatic regulator of myocardial function, autophagy, and mitophagy and the severe adverse consequences for the heart resulting from *long-term* Drp1 inhibition.

This study addresses these two concerns and the potential benefits of *short-term* Drp1 inhibition with short-term cardiomyocyte-specific Drp1 ablation in mice and the use of a novel Drp1 inhibitor developed by us, Drpitor1a. Using inducible cardiomyocyte-specific Drp1 knock-out mice, created by Song et al.,[19] we showed that short-term Drp1 ablation (3 weeks) had no harmful effects on mouse exercise capacity, myocardial function, and mitochondrial respiration and confirmed that the deleterious effects of Drp1 ablation only occurred after 6 weeks (Figures 1 and 2, Figure S1). Our studies further suggested short-term KO increased coupling of ADP-dependent mitochondrial respiration to electron transport through enhanced complex1 activity (Figure 2) associated with increased mitochondrial size (Figure 3). These findings contrast with those of Zhang et al. who found that mitochondria respiration is inhibited by in vitro Drp1 inhibition with Mdivi-1, Drp1 K38A, and Drp1 shRNA without altering complex I-IV activities.[38] The inhibition of mitochondrial membrane potential and OCR without affecting intracellular ATP was also found in Drp1 K38A-treated cardiomyocytes.[35] These differences in findings regarding the effects of Drp1 ablation may be due to differences in the underlying mechanisms between in vitro Drp1 ablation versus *in vivo* Drp1 ablation. Thus, we conclude that, while Drp1 is necessary for maintaining cardiac and mitochondrial homeostasis in the long-term, short-term inhibition of Drp1 does not result in overt pathology. This is highly relevant for considering the therapeutic application of our discovery in clinical conditions of cardiac IR, as occurs post-AMI or cardiac arrest, in which only short-term therapy would be needed.

Consistent with this, when we subjected short-term Drp1 KO hearts to IR injury we found that Drp1 KO preserved systolic, diastolic, and developed pressure (Figure 3A,B) and decreased ROS and cytosolic cytochrome C release (Figure 4B,D). Mitochondria from Drp1 KO cardiomyocytes following IR injury also showed improved ATP-related oxygen consumption as well as total capacity of OCR (Figure 3B). This increased OCR at complex 1 suggested that oxygen was still being used for ATP production rather than ROS production.[39] Concordant with these results, we found an increase in complex I activity, but not complex III activity, in the cardiac mitochondria from Drp1 KO hearts (Figure 2D,E). Additionally, complex I activity was increased in the heart with Drp1 ablation post-IR (Figure 3E). These findings are again in contrast to those of Ikeda et al. who reported *decreased* complex I activity following *long-term* Drp1 ablation after 12 weeks.[20] The opposing findings of these studies likely reflect the temporal effects of Drp1 KO at 3 weeks

vs 12 weeks on mitochondrial biology. We conclude that short-term Drp1 ablation is beneficial to mitochondrial biology and function (in the presence of IR) whereas long-term Drp1 ablation is harmful (even at baseline).

Mitochondrial injury following IR injury is complex in nature. MPTP opening following IR in the heart is a common final pathway for IR injury and genetically manipulating mitochondrial factors such as the mitochondrial calcium uniporter (MCU) have been shown to be cardioprotective by inhibiting MPTP response to calcium overload.[40] In contrast to *long-term* Drp1 ablation, which increases the sensitivity of cells to calcium-induced MPTP opening, we found that *short-term* Drp1 ablation had no effects on calcium overload-induced MPTP opening (Figure 5A).[20] Given that Drp1 is predominantly a cytosolic protein under basal conditions with relatively little association with the outer mitochondrial membrane our findings of little differences between Drp1 KO and control mitochondria under basal conditions is not surprising. However, following IR injury, Drp1 translocates from the cytosol to the mitochondria, and we hypothesized that differences in Drp1 KO versus control mice would primarily be observed following IR injury, as was indeed the case. Mitochondria from Drp1 KO hearts following IR injury had increased resistance to calcium-induced swelling and increased mitochondrial calcium retention capacity compared to control mitochondria following IR injury (Figure 5B,D). These findings indicate that Drp1 increases the sensitivity of the MPTP opening following IR injury. Since MPTP opening prompts the loss of mitochondrial membrane potential further accelerating cellular energy depletion,[41] the slowed MPTP opening caused by Drp1 ablation is beneficial, increasing mitochondrial respiration and reducing ROS generation (Figure 5F). Although the precise mechanism of how Drp1 regulates mitochondrial calcium homeostasis following IR injury is unknown, we found that Drp1 ablation was associated with reduced expression of RyR2. This is relevant to this study because alterations in RyR2 function have been implicated in calcium mishandling and arrhythmia, particularly in the setting of heart failure.[42] Constitutive knockout of the ryanodine receptor is embryonically lethal and conditional knockout results in sudden cardiac death although recently, it was reported that haploinsufficiency has no overt physiological phenotype.[43,44] To our knowledge, this is the first time that an interaction between Drp1 and RyR2 expression has been observed. We further discovered that the RyR2 inhibitor ruthenium red reduced IR-induced ROS generation suggesting a relationship a role for RyR2 in post-IR injury. Given the known potential of RyR2-mediated calcium

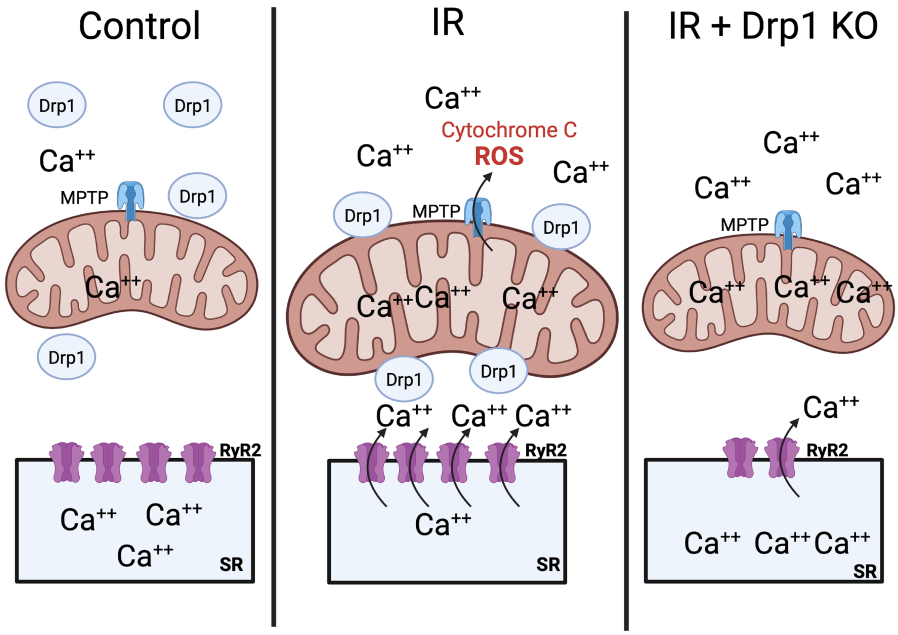

FIGURE 7 Proposed mechanism of acute Drp1 ablation on improving mitochondria and cardiac function post-ischemia-reperfusion (IR). In control of normal hearts Drp1 cycles on and off the outer mitochondrial membrane (OMM) maintaining mitochondrial homeostasis. IR injury results in increased cytosolic and mitochondrial matrix calcium. Drp1 translocated to the OMM resulting in increased mitochondrial permeability transition pore (MPTP) sensitivity resulting in membrane depolarization, mitochondrial swelling, and release of ROS and cytochrome C. In contrast, lack of Drp1 results in MPTP resistance to calcium overload and lack of pore opening resulting in maintained mitochondrial function. Lack of Drp1 also results in decreased expression of the cardiac calcium-regulating ryanodine receptor (RyR2) protein which may also affect post-IR calcium regulation.

leak from the sarcoplasmic reticulum in the progression of cardiac dysfunction for heart failure, future work will be required to determine whether this receptor plays a causal role in the Drp1-mediated effects in IR injury.[42,45]

Finally, to further confirm our genetic findings of Drp1 in IR injury in the heart, we investigated the effects of the potent Drp1 GTPase inhibitor Drpitor1a developed by Wu et al.[18] Our findings showed that Drpitor1a was able to improve LV systolic function following IR and that this beneficial effect was due to the improvement of mitochondrial calcium uptake and the reduction of ROS generation (Figure 6). These results were consistent with our findings that short-term Drp1 ablation improved LV function and reduced ROS generation following LV-IR injury.

5 | CONCLUSIONS

Short-term Drp1 inhibition either genetically or pharmacologically inhibits MPTP opening, improves mitochondrial complex 1 function, and lowers the risk of calcium overload and ROS generation following IR injury in the heart resulting in improved contractile function and reduced cellular injury (Summarized in Figure 7). These effects are associated with reduced RyR2 expression, an observation requiring further exploration. In contrast, long-term Drp1 ablation (>6 weeks) is harmful resulting in lethal cardiomyopathy. Our study indicates that short-term application of Drp1 inhibition may be both safe and effective in counteracting IR injury. Clinical evaluation of Drp1 inhibitors, such as Drpitor1a, should be considered in the future.

6 | LIMITATIONS

Our study has certain limitations. First, although we did not observe overt deleterious effects of short-term Drp1 ablation, it is possible that acute Drp1 ablation had subtle deleterious effects on the heart that we did not observe. The use of Drpitor1a in *in vivo* ischemia–reperfusion studies also needs to be further investigated. Finally, experiments dissecting the relationship between Drp1 and RyR2 interaction are needed to further clarify this potential relationship in myocardial function and calcium homeostasis.

AUTHOR CONTRIBUTIONS

Lin Piao drafted main manuscript, performed data analysis, and performed experiments. Yong-Hu Fang performed experiments and edited manuscript. Michael Fisher worked on the mitochondrial calcium homeostasis experiments. Robert B. Hamanaka assisted with Seahorse experiments, data interpretation, and editing. Alaa Ousta data interpretation and manuscript editing. Rongxu Wu, Gökhan M. Mutlu, and Alfredo J. Garcia III performed data interpretation and manuscript editing. Stephen L Archer developed and provided Drpitor1a for this study, data interpretation, and manuscript editing. Willard W. Sharp conceived of project, obtained funding for the project, writing, and editing of manuscript, interpretation, and review of the data.

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DISCLOSURES

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be preserved and shared with the public via https://knowledge.uchicago.edu, the University of Chicago institutional repository. Knowledge@UChicago is managed by the University Library and uses a cloud-based hosted repository service called TIND as its platform. To support discoverability and citation, DOIs are minted for deposits made to Knowledge@UChicago through the DataCite Fabrica service.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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