Physiological Mitochondrial Fragmentation Is a Normal Cardiac Adaptation to Increased Energy Demand

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Rationale: **Mitochondria play a dual role in the heart, responsible for meeting energetic demands and regulating cell death. Paradigms have held that mitochondrial fission and fragmentation are the result of pathological stresses, such as ischemia, are an indicator of poor mitochondrial health, and lead to mitophagy and cell death. However, recent studies demonstrate that inhibiting fission also results in decreased mitochondrial function and cardiac impairment, suggesting that fission is important for maintaining cardiac and mitochondrial bioenergetic homeostasis.**

Objective: **The purpose of this study is to determine whether mitochondrial fission and fragmentation can be an adaptive mechanism used by the heart to augment mitochondrial and cardiac function during a normal physiological stress, such as exercise.**

Methods and Results: **We demonstrate a novel role for cardiac mitochondrial fission as a normal adaptation to increased energetic demand. During submaximal exercise, physiological mitochondrial fragmentation results in enhanced, rather than impaired, mitochondrial function and is mediated, in part, by β1-adrenergic receptor signaling. Similar to pathological fragmentation, physiological fragmentation is induced by activation of dynamin-related protein 1; however, unlike pathological fragmentation, membrane potential is maintained and regulators of mitophagy are downregulated. Inhibition of fission with P110, Mdivi-1 (mitochondrial division inhibitor), or in mice with cardiac-specific dynamin-related protein 1 ablation significantly decreases exercise capacity.**

Conclusions: **These findings demonstrate the requirement for physiological mitochondrial fragmentation to meet the energetic demands of exercise, as well as providing additional support for the evolving conceptual framework, where mitochondrial fission and fragmentation play a role in the balance between mitochondrial maintenance of normal physiology and response to disease. (*Circ Res*. 2018;122:282-295. DOI: 10.1161/CIRCRESAHA.117.310725.)**

Key Words: cell death ■ dynamins ■ exercise ■ mitochondria ■ receptors, adrenergic

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Cardiac muscle is especially sensitive to alterations in energetic homeostasis because of the high ATP demands of rhythmic contraction.[1] The heart initially responds to increased demands by using local ATP stores and anaerobic metabolism; however, persistent increases are met by sustained aerobic ATP production through increased mitochondrial oxidative phosphorylation and changes in mitochondrial dynamics (fission, fusion, mitophagy, and biogenesis).[2,3] In the heart, mitochondrial fission and subsequent fragmentation are usually the result of pathological stressors, such as ischemia, and are an indicator of dysfunctional mitochondria.[4,5] However, recent studies have shown that inhibiting fission also results in cardiac impairment,[6–9] suggesting that fission is also important for maintaining normal mitochondrial homeostasis. Song et al[10] have shown that conditional Drp1 (dynamin-related protein) knockout (Drp1$^{-/-}$) mice have preserved mitochondrial function but increased mitophagy and eventual heart failure. Others have shown that disruption of fission results in the accumulation of dysfunctional mitochondria.[11] In contrast, the Drp1 inhibitor Mdivi-1 (mitochondrial division inhibitor) decreases mitophagy and rescues pressure overload-induced heart failure.[12] It has also been proposed that, in cardiomyocytes, fission does not substantially alter mitochondrial morphology but is mainly required to facilitate clearance of dysfunctional mitochondria through asymmetrical division and mitophagy.[8,10] Because these studies have all relied on transgenic manipulation of dynamic regulators, such as Drp1, or were done in the context of pathology such as ischemia, the role for mitochondrial fission as a normal cardiac physiological adaption is still unknown.

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Original received January 30, 2017; revision received December 7, 2017; accepted December 11, 2017. In November 2017, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 11.99 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.117.310725/-/DC1.

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Novelty and Significance

What Is Known?

- Mitochondrial fission/fragmentation occurs in response to pathological stimuli.
- Pathological mitochondrial fission/fragmentation is associated with decreased mitochondrial function and the elimination of damaged mitochondria through mitophagy and cell death.

What New Information Does This Article Contribute?

- Mitochondrial fission/fragmentation occurs in the heart in response to normal physiological stimuli, such as routine exercise.
- Physiological mitochondrial fission/fragmentation is associated with enhanced mitochondrial function and does not activate mitophagy.
- Physiological mitochondrial fission/fragmentation is an adaptation to enhance energy delivery to meet increased demands and is required for reaching maximal exercise capacity.

Mitochondrial dynamics (fission, fusion, mitophagy, and biogenesis) are important mediators of tissue homeostasis. In the heart, pathological stress, such as ischemia, induces mitochondrial fission/fragmentation and is associated with decreased mitochondrial function, increased oxidative stress, and activation of mitophagy. In contrast, the role of mitochondrial dynamics in normal cardiac physiology is not well understood. We studied the role of mitochondrial dynamics in normal physiological stress, such as occurs during routine exercise. We found that mitochondrial fission/fragmentation occurs in response to exercise but is associated with enhanced rather than degraded mitochondrial function, absence of oxidative stress, and inhibition of mitophagy. This physiological fragmentation is mediated, in part, by β1-adrenergic stimulation and activation of profission dynamin-related protein 1. Physiological fragmentation is required for achieving maximal exercise capacity: pharmacological inhibition or genetic ablation of dynamin-related protein 1 significantly reduces exercise capacity. Thus, mitochondrial fission/fragmentation is not only a pathological process but also a physiological one that is activated during exercise to maintain cardiac energetic homeostasis. Given the known benefits of exercise on cardiovascular health, these findings suggest that mitochondrial fission may be a promising pathway that can be targeted to simulate the beneficial effects of exercise.

Nonstandard Abbreviations and Acronyms	
AR	adrenergic receptor
Drp1	dynamin-related protein 1

Several studies in skeletal muscle suggest, but do not confirm, a role for mitochondrial dynamics in maintaining energetic homeostasis. Kitaoka et al[13] investigated simulated resistance exercise in gastrocnemius muscle and showed increased Drp1 phosphorylation at Serine 616, suggesting activation of mitochondrial fission. Picard et al[14] subjected mice to voluntary treadmill exercise for 3 hours, and although they found no change in regulators of mitochondrial dynamics, they did find increased mitochondrial interactions, suggesting active remodeling of skeletal muscle mitochondria. Finally, Feng et al[15] showed that during early exercise, expression of fission mediator Fis1 (mitochondrial fission 1 protein) is increased in skeletal muscle, suggesting a role for physiological mitochondrial fragmentation in maintaining bioenergetics during normal physiological stress. Although these studies support the role of mitochondrial dynamics in skeletal muscle energetic adaptation to exercise, they do not confirm whether mitochondrial dynamic processes, such as fission and fusion, are necessary components for exercise performance. Skeletal muscle is also different from cardiac muscle both in sarcomere morphology and function and in mitochondrial organization and density.[16,17] Only 1 study has been performed in cardiac muscle, by Dworatzek et al,[18] who showed that 8 weeks of exercise conditioning increased cardiac mitochondrial number and decreased their size. In the current study, we sought to determine whether mitochondrial fission and fragmentation occur in the heart as a physiological adaptive response to acute submaximal exercise, whether it is required to meet the increased bioenergetic demands of normal levels of exercise, and the similarities and differences between exercise-induced and pathological-induced fragmentation.

Methods

Data and Material Availability

Microarray data have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus database in MIAME format. Request for data can be addressed to corresponding author.

Please refer to the Materials and Methods section in the Online Data Supplement for a detailed description of the experimental method.

Animal Protocols

Male 8- to 10-week-old FVB/NJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were subjected to exercise on a treadmill equipped for simultaneous measurement of Vo_2 and Vco_2 (Columbus Instruments, Columbus, OH), and distance ran and respiratory exchange ratio were determined using our established protocols.[19] Mice were exercised using 2 different protocols. First, submaximal exercise was performed for 1 hour at a speed of 20 m/min with a constant grade of 5°. Exercise capacity using this protocol was assessed by running mice to exhaustion at a speed of 20 m/min at a constant grade of 5° and recording total distance ran. Second, maximal exercise capacity was assessed using a protocol that incrementally increased the degree of elevation by 2° and the speed by 2 m/min every 3 minutes. Exercise time was determined as the time until exhaustion or 10 seconds while resting on the shock grid. Exercise distance was calculated as the total vertical distance traveled (meters)=treadmill speed (meters/min)×percent grade (Sin θ)×exercise time (min). Work (Joules) was calculated as the body weight (kg)×total vertical distance traveled (meters)×9.8.

β-Adrenergic Signaling and Mitochondrial Dynamics Modulation

To assess subtype-specific β-adrenergic receptor (AR) signaling, we used β2-AR knockout ($^{-/-}$) mice generated on an FVB background in our laboratory.[20] To assess mitochondrial fission, mice were treated with the Drp1-Fis1 inhibitor P110, provided by Dr Daria Mochly-Rosen (0.5 mg/kg) or Mdivi-1 (25 mg/kg) by intraperitoneal

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injection, followed immediately by exercise testing. Either saline, TAT47-57 peptide (0.35 mg/kg) or 2% dimethyl sulfoxide in saline was used as a control treatment. To assess the role of β1-AR signaling, mice were treated with the β1-AR antagonist metoprolol (5 mg/kg) by intraperitoneal injection 90 minutes before maximal exercise challenge. To assess cardiac-specific mitochondrial fission, $Drp1^{loxp/loxp}$[21] C57B/6NJ mice were provided by Dr Katsuyoshi Mihara and Dr Masatoshi Nomura and were crossed onto the *myh6*-MER-Cre-Mer C57B/6NJ mouse line (Jackson Laboratory, Bar Harbor, ME), and gene recombination was induced by administration of tamoxifen (20 mg/kg) intraperitoneally for 5 days. On day 7, mice were used for experimentation. Male aged-matched $Drp1^{+/+}Cre^{+/+}$ and $Drp^{-/-}Cre^{-/-}$ were treated with tamoxifen and used as controls.

Isoproterenol, Doxorubicin, and Inhibitor Dosing

An HL-1 cardiac myocyte cell line cells and isolated cardiac myocytes were treated with 10 μmol/L isoproterenol for 1 hour. HL-1 cells were pre-treated with 300 nmol/L of the subtype-specific inhibitors CGP 20712A (β1 antagonist) and ICI 118 551 (β2 antagonist) and 1 μmol/L of L748 337 (β3 antagonist) and propranolol (β1 and β2 antagonist) for 30 minutes before isoproterenol exposure. Cells were also pre-treated with 30 μmol/L of the mitochondrial division inhibitor Mdivi-1 for 30 minutes before isoproterenol exposure.

C2C12 or isolated adult cardiac myocytes were exposed to 100 μmol/L doxorubicin. After exposures, cells were either washed with PBS or used directly for morphology or respiration measurement. Mice were exposed to 10 mg/kg of doxorubicin or saline control by tail vain injection. After 30-minute exposure, cardiac tissue was harvested for protein analysis.

Statistical Analysis

Data are expressed as the mean±SEM. Normally distributed data comparing 2 groups were analyzed by Student *t* test. Normally distributed data comparing multiple groups were analyzed by ANOVA with multiple comparisons analysis (Tukeys). Nonparametric data comparing 2 groups were analyzed using the Mann–Whitney *U* test. Statistical analysis of microarray data was performed using Stanford microarray database software and the Significance Analysis of Microarrays, Database for Annotation, Visualization, and Integrated Discovery, Hi-Throughput GOMiner, and TIGR TM4 software. Multiple comparisons were accounted for by false discovery rate analysis, and genes were considered significantly altered with a $P<0.01$ by Fisher exact test. To control for multiple comparisons, we required a false discovery rate of 5%. Microarray data have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus database in MIAME format.

Results

Exercise Induces Physiological Mitochondrial Fragmentation

In mice subjected to submaximal treadmill exercise for 1 hour,[19] cardiac mitochondria demonstrated significant fragmentation: increase in number and decrease in area and perimeter by quantitative electron microscopy (Figure 1A). We confirmed exercise-induced mitochondrial fragmentation using flow cytometry side scatter area, showing that mitochondria from exercised mice were smaller in size versus sedentary controls (Figure 1B). Side scatter area results were validated by calibrating with beads of known size (2, 1, 0.5, 0.2 μm; Online Figure I). Evidence of mitochondrial fission can be confirmed by translocation of the mitochondrial fission mediator Drp1 from the cytosol to the mitochondrial fraction.[22] Purity of our mitochondrial fraction was assessed by low abundance of the cytosolic protein α actinin and robust abundance of mitochondrial Vdac1 (voltage-dependent anion-selective channel 1). Exercise induced mitochondrial translocation of the profission mediator Drp1 (Figure 1C) and phosphorylation of the fission activating site Drp1 Serine 616 (Figure 1D). The profission AMP-activated protein kinase was activated, as evidenced by phosphorylation at the activating site Thr172 (Figure 1D); total AMP-activated protein kinase expression was unchanged (increased but did not reach statistical significance). In contrast, the profusion mediator mitofusin 2 was unchanged (Figure 1C). After 1 hour of recovery from exercise, mitochondrial architecture begins to revert to sedentary morphology (Figure 1A).

To highlight differences between physiological and pathological mitochondrial fragmentation, we compared exercise to a model of pathological fragmentation, doxorubicin cardiotoxicity. Doxorubicin has been shown to induce mitochondrial fission and fragmentation associated with mitochondrial dysfunction.[23,24] During pathological fragmentation (Online Figure IIA), mitochondrial oxidative stress is increased (Online Figure IIB).[25] In contrast, in exercise-induced physiological fragmentation, levels of oxidative stress (4-HNE [4-hydroxynonenal] lipid peroxidation and MitoSox [mitochondrial superoxide indicator]) were unchanged (Figure 2A). In pathological fragmentation, membrane potential (Δψm) is decreased, increasing PINK1 (PTEN-induced putative kinase 1) accumulation on the mitochondrial membrane, resulting in translocation of parkin, accumulation of LC3 (Online Figure IIC), and activation of mitophagy.[26–31] In contrast, during physiological fragmentation, Δψm was preserved (Figure 2B). Furthermore, Δψm remained normal even when isoproterenol was added to simulate the hypersympathetic state during exercise. Preservation of Δψm prevented mitochondrial PINK1 accumulation and recruitment of parkin (Figure 2C), typical of pathological fragmentation and markers of mitophagy.[7,32] Finally, pathological fragmentation is associated with decreased mitochondrial function (Online Figure IIB), whereas physiological fragmentation is associated with enhanced mitochondrial function (Figure 2D). We have previously shown similar deleterious effects of pathological fragmentation in models of diabetes mellitus[33] and ischemia reperfusion.[34]

We next sought to determine whether the absence of autophagy in our studies was reflective of a true failure to activate autophagy or was related to an increase in autophagic flux by treating with chloroquine. Although LC3-I, LC3-II, and P62 levels were similar between chloroquine-treated exercised and sedentary mice, we could not see enhanced LC3/P62 accumulation with chloroquine alone, therefore we cannot exclude a change in autophagic flux in our model. To evaluate an additional end point for autophagy activity, we assessed the activation state of the proautophagy protein ULK1 (Unc-51 like autophagy activating kinase) [35] and found dephosphorylation at the activating site Ser555, another confirmation of the inactivation of autophagy (Figure 2E). A previously published exercise study showed evidence for activation of autophagy, but this study used a more extreme level of exercise, beyond Vo_2 max and anaerobic threshold.[36] This suggests that there may be tiered levels of mitochondrial fission during exercise, where a low grade of fission (physiological fragmentation) may be beneficial during submaximal exercise (as normally experienced by exercising humans). In contrast, a higher grade of fission during extreme exercise (as experienced during laboratory exercise stress testing or marathon running) might be at least partly detrimental. To address this

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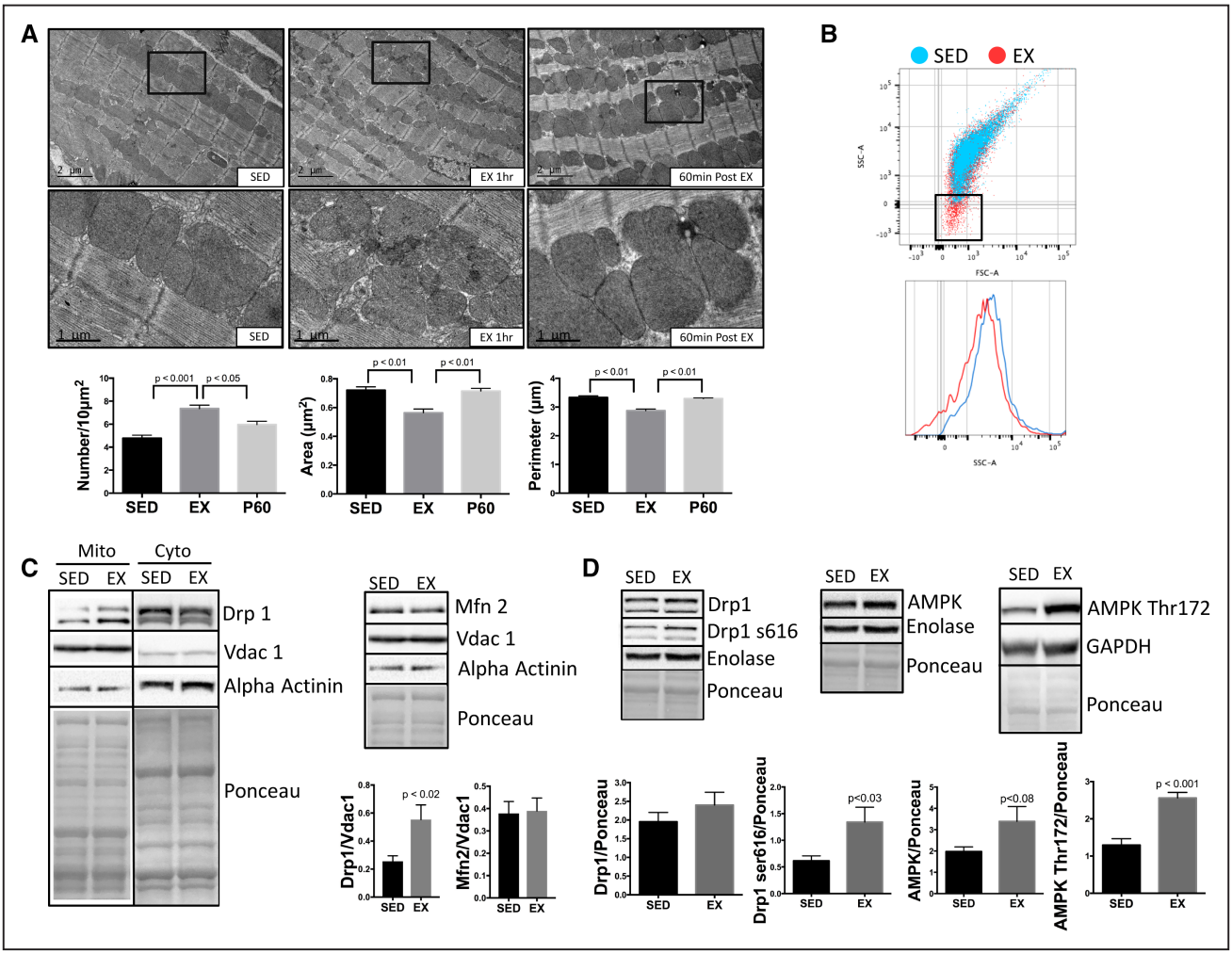

Figure 1. Exercise (EX) induces physiological mitochondrial fragmentation. **A**, Electron micrographs from left ventricular myocardium show mitochondrial fragmentation after 1 h of EX. Mitochondrial number was increased, and area and perimeter decreased vs controls. Mitochondrial morphology returned to the sedentary state after 60 min of post-EX rest (n=4 biological replicates/group, at least 10 fields/subject were averaged for each biological replicate). **B**, Mitochondrial size was assessed by flow cytometry as side scatter area (SSA). Isolated mitochondria from EX mice were smaller than sedentary (SED; n=4/group). **C**, Profission Drp1 (dynamin-related protein 1) is increased in mitochondrial fractions while profusion Mfn 2 (mitofusin 2) is unchanged (n=8/group). **D**, Activating Drp1 phosphorylation site serine 616 and AMPK (AMP-activated protein kinase) phosphorylation site Thr172 is also increased with EX in whole-heart fractions, along with a marginal but nonsignificant increase in total AMP-kinase (n=5/group).

question, we compared tissues from mice subjected to submaximal (1-hour exercise) and those from mice subjected to extreme/maximal (run to exhaustion, beyond anaerobic threshold) exercise. We found a similar increase in total mitochondrial number in both exercise groups compared with sedentary mice (Online Figure IIIA). Similar to submaximal exercise, this was associated with Drp1 translocation to the mitochondrial fraction (Online Figure IIIB). These data confirm that mitochondrial fission also occurs with extreme/maximal exercise. Again, we found no change in LC3 or P62 with submaximal or maximal exercise (Online Figure IIIC). Similarly, parkin was not increased; however, the decrease in parkin translocation to the mitochondria that was seen with submaximal exercise (Figure 2C) was not seen with extreme/maximal exercise (Online Figure IIIB). Together, these data suggest that mitophagy and autophagy do not occur during either submaximal or extreme/maximal exercise. It still remains possible that a tiered level of exercise-induced fission may occur, where normal physiological levels of exercise induce physiological fragmentation and extreme/maximal exercise induces features of both physiological and pathological fragmentations; however, we have not been able to induce pathological fission with either form of exercise.

Physiological Mitochondrial Fragmentation Is Required for Maximal Exercise Performance

To determine whether physiological fragmentation is necessary to achieve maximal exercise performance, mice were treated with P110,[19] which inhibits fission by blocking the interaction between Drp1 and Fis1 preventing Drp1s mitochondrial translocation (Figure 3A).[37] Mice were then subjected to both submaximal and maximal exercise capacity tests. P110 treatment resulted in a 45% decrease in submaximal exercise time and 45% decrease in submaximal exercise distance (Figure 3B). To best control for any differences in weight, we determined total work (J) and found that P110-treated mice had a 38% decrease in submaximal exercise

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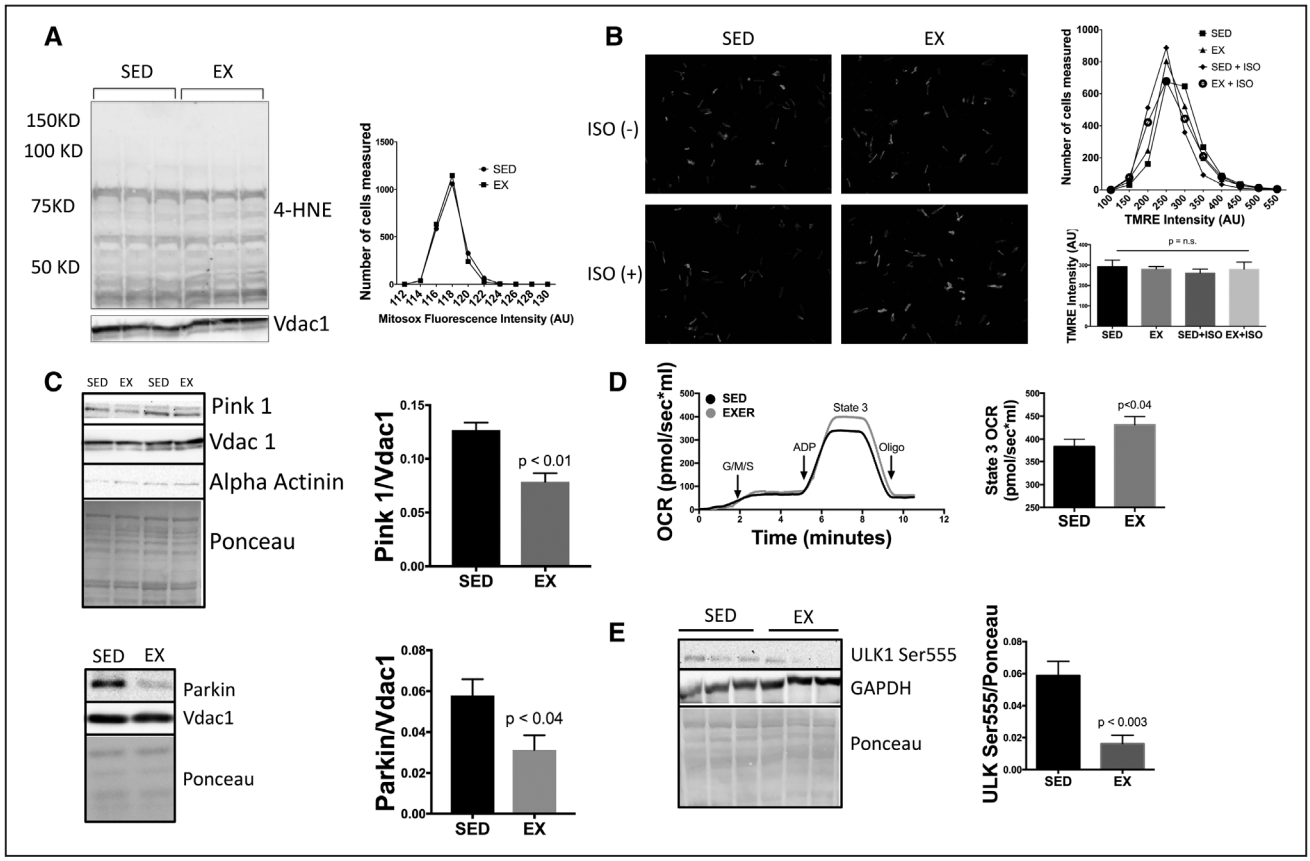

Figure 2. Exercise (EX)-induced physiological mitochondrial fragmentation is associated with inhibition of mitophagy and enhanced mitochondrial function. **A**, Oxidative stress (4-HNE [4-hydroxynonenal] & MitoSOX [mitochondrial superoxide indicator]) was not increased despite fragmentation (n=3/group). **B**, Membrane potential (TMRE [tetramethylrhodamine, ethyl ester]) was maintained in cardiac myocytes isolated from exercised mice, both before and after sympathetic stimulation (isoproterenol [ISO] 10 μmol/L) vs sedentary (SED) controls (n=3/group). **C**, Promitophagy parkin and Pink1 (PTEN-induced putative kinase 1) are both decreased in mitochondrial fractions (n=3–5/group). **D**, Mitochondrial respiration is enhanced with 1 h of exercise as measured by oxygen consumption rate (OCR) by Oroboros oxygraph normalized to 50 μg of mitochondria (n=5/group). **E**, Proautophagy ULK-1 (Unc-51 like autophagy activating kinase) is dephosphorylated (inactive) at Ser555 with 1 h of exercise (n=5/group).

work. Similarly, P110-treated mice subjected to a maximal exercise capacity test had a 16% decrease in maximal exercise time, 37% decrease in distance, and 38% decrease in total work (Figure 3C). P110-treated mice also reached anaerobic threshold (respiratory exchange ratio >1.0) earlier than vehicle-treated mice, demonstrating that blocking mitochondrial fission during exercise results in earlier use of oxidative phosphorylation-independent (anaerobic) ATP production. During early stage exercise (first 5–10 minutes), P110 did not affect respiratory exchange ratio (Online Figure IV), evidence that physiological fragmentation becomes important only after a more prolonged increase in energy demand. Both submaximal and maximal exercise capacity tests induced mitochondrial fragmentation. Submaximal exercise-induced fragmentation was completely blocked by P110 treatment while maximal exercise-induced fragmentation was partially blocked (Figure 3B and 3C), further demonstrating the requirement for physiological mitochondrial fission to reach maximal exercise capacity. Because fission can also occur through Fis-1–independent mechanisms,[38] we confirmed the role of Drp1 in physiological fragmentation using the Drp1 GTPase inhibitor Mdivi-1. Similar to P110, mice treated with Mdivi-1 had decreased maximal exercise capacity (Online Figure V).

Finally, to confirm the role of cardiac, as opposed to skeletal muscle, mitochondrial fragmentation in adaptation to exercise, we used conditional cardiac-specific Drp1$^{-/-}$ mice.[6,21] Ablation of Drp1 was induced with tamoxifen (20 mg/kg) for 5 days, and mice were subjected to a maximal exercise capacity test on day 7. Drp1$^{+/+}$Cre$^{+/+}$ and Drp1$^{-/-}$Cre$^{-/-}$ were used as controls and were also exposed to tamoxifen for 5 days and subjected to an exercise capacity test on day 7. Drp1 was absent in cardiac tissue but not in skeletal muscle (Figure 4A; Online Figure VIA) and was the only mitochondrial dynamic regulator decreased as mitofusin 1 and 2, LC3, and parkin remained unchanged (Online Figure VIB). Although long-term knockout of Drp1 can have deleterious effects,[6,10] this brief period of ablation did not affect mitochondrial reactive oxygen species (ROS), membrane potential, or baseline cardiac function (ejection fraction and cardiac output; Online Figure VIC and VID). Similar to P110 treatment, cardiac-specific Drp1$^{-/-}$ mice subjected to a maximal exercise capacity test resulted in a 24% decrease in exercise time, 38% decrease in exercise distance, 58% decrease in total work, and a shorter time to reach anaerobic threshold (Figure 4C). As expected, maximal exercise induced mitochondrial fragmentation in exercised Drp1$^{+/+}$ mice, which was blocked in exercised Drp1$^{-/-}$ mice

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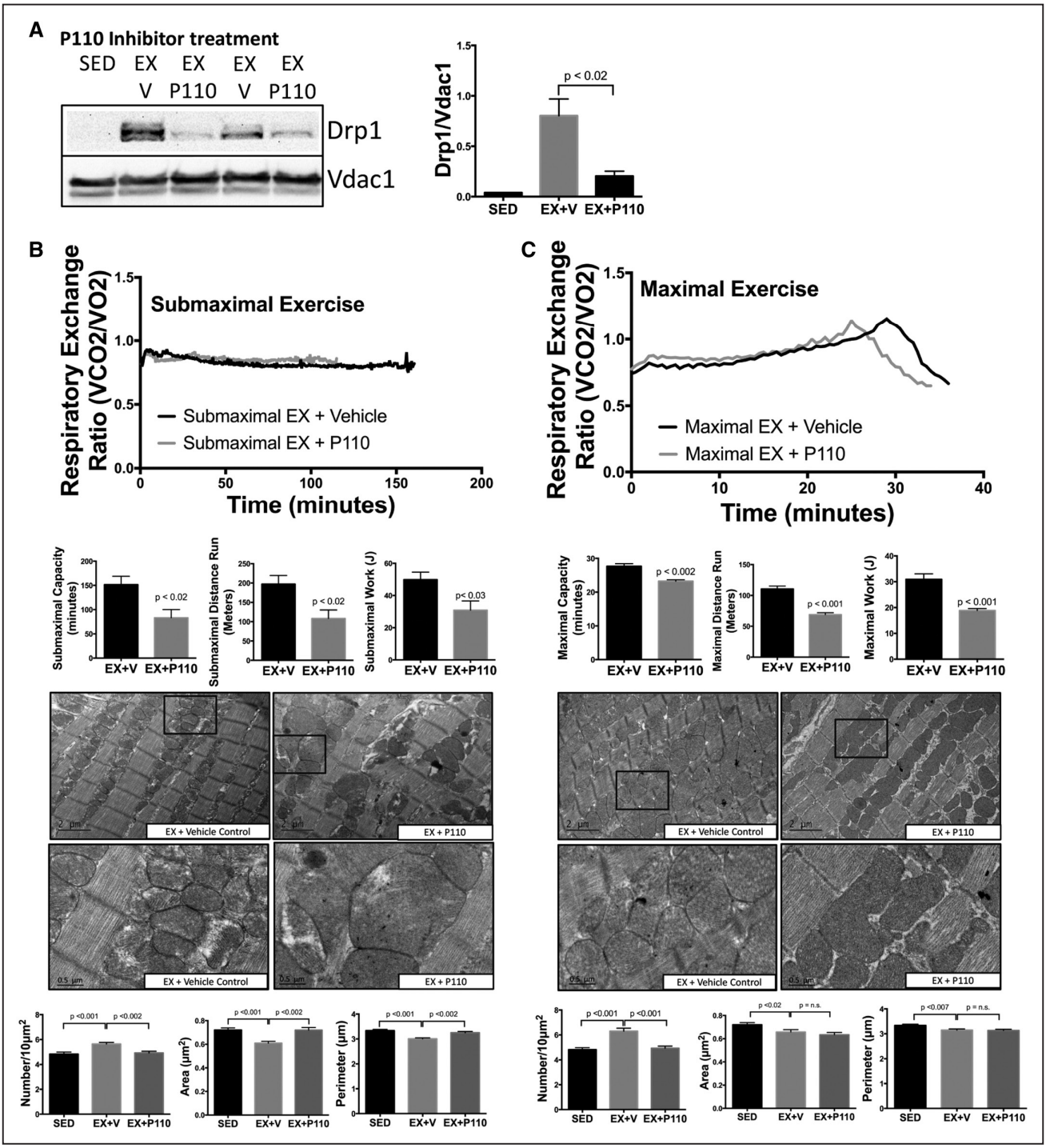

Figure 3. Mitochondrial fragmentation is required for submaximal and maximal exercise (EX) capacity. **A**, The Drp1 (dynamin-related protein 1) inhibitor P110 (0.5 mg/kg IP) decreased Drp1 mitochondrial translocation vs TAT vehicle (V) control (n=3/group). **B**, Blocking Drp1 decreased submaximal and maximal (**C**) exercise time (minutes), distance (meters), and work (J). P110 treatment also prevented mitochondrial fragmentation, as evidenced by failure to increase mitochondrial number during both (**B**) submaximal and (**C**) maximal exercise (n=5/group). SED indicates sedentary.

(Figure 4D). Of interest, exercised Drp1$^{-/-}$ mice exhibited large, highly abnormal mitochondria, some extremely elongated and others with multiple finger-like projections ("puzzle piece mitochondria"), which we speculate are related to an incomplete stimulus for fission (Figure 4C; Online Figure VII). To confirm that these abnormal mitochondria were a result of exercise and not because of the gene knockout itself, we compared sedentary Drp1$^{+/+}$ mice to sedentary Drp1$^{-/-}$ mice and found no change in mitochondrial morphology, confirming that this abnormal mitochondrial phenotype occurs only in response to exercise (Figure 4C). As expected, sedentary Drp1$^{-/-}$ had a 40% reduction in state 3 mitochondrial respiration, similar to that previously described.[6] Interestingly, exercised Drp1$^{-/-}$ mice had a dramatic 65% reduction in state 3

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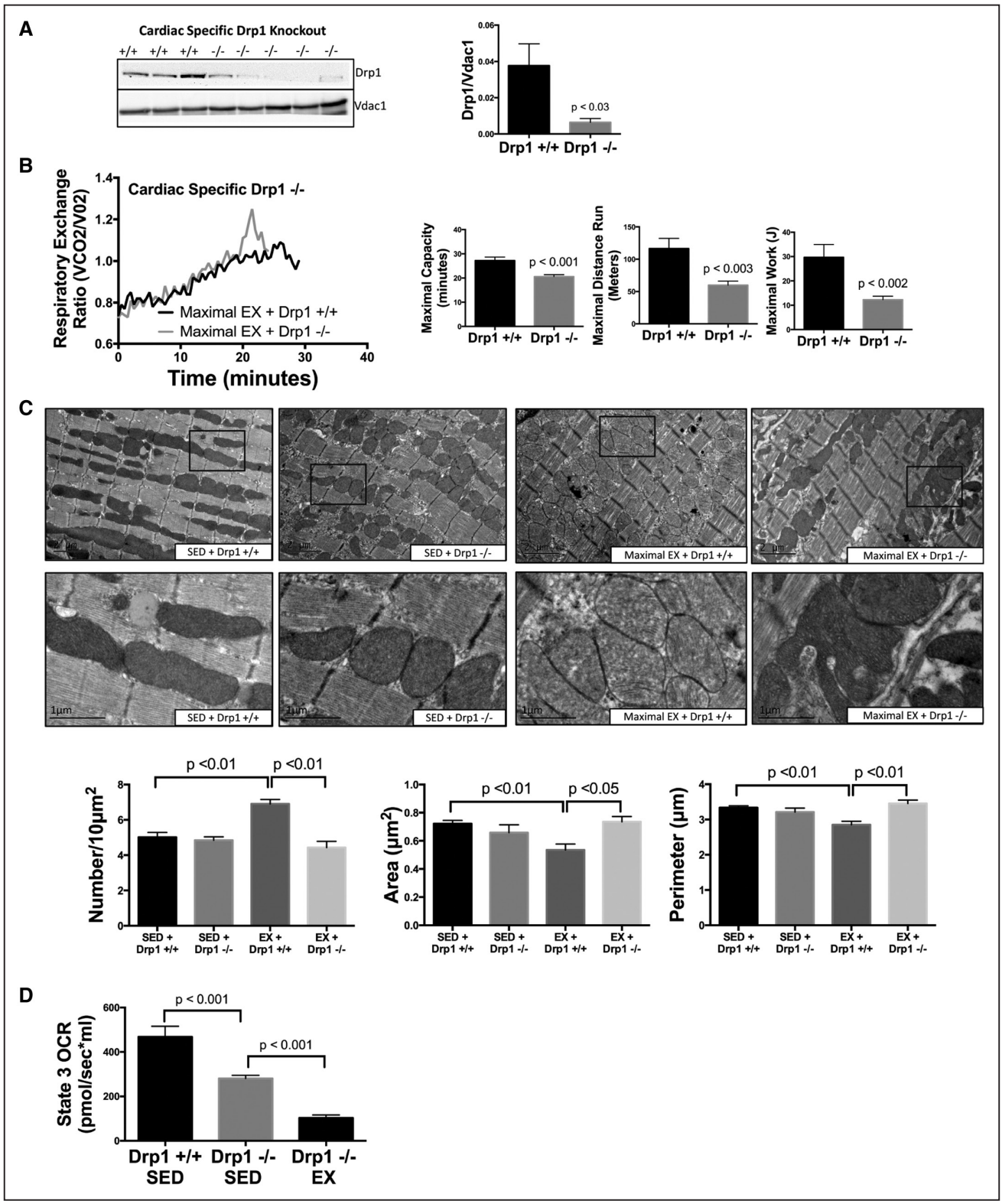

Figure 4. Cardiac mitochondrial fragmentation is required for maximal exercise (EX) capacity. **A**, Cardiac-specific conditional dynamin-related protein 1 knockout (Drp1$^{-/-}$) mice were generated by crossing Drp1$^{loxp/loxp}$ mice with *myh6*-MER-Cre-Mer mice and treating with tamoxifen (20 mg/kg, IP) for 5 d. **B**, Drp1$^{-/-}$ decreased maximal EX time (min), distance (m), and work (J) and (**C**) prevented EX-induced mitochondrial fragmentation but resulted in abnormal appearing mitochondria, both elongated (**arrow**) or hypersegmented (**bottom**). No changes in mitochondrial morphology were observed between sedentary (SED) Drp1$^{+/+}$ and Drp1$^{-/-}$ (n=3–5 biological replicates/group, at least 10 fields/subject were averaged for each biological replicate). **D**, Reduced mitochondrial function was observed in SED and EX Drp1$^{-/-}$ mice by Oroboros oxygraph normalized to 50 µg of mitochondria (n=5/group).

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mitochondrial respiration at the end of 1-hour exercise (20 m/min) versus sedentary Drp1$^{-/-}$, indicating a markedly compromised response to energetic stress (Figure 4D).

β1-AR Signaling Induces Physiological Mitochondrial Fragmentation and Enhances Mitochondrial Function

The sympathetic nervous system is a principal mechanism for meeting the hemodynamic demands of exercise, increasing heart rate and contractility, both of which increase energetic demands. This response is mediated by β-AR signaling,[39] largely through protein kinase A–mediated increases in intracellular calcium, a regulator of both mitochondrial ATP production and mitochondrial dynamics.[40] To examine the role of β-AR signaling in mitochondrial fragmentation, HL-1 cardiomyocytes were labeled with MitoTracker and treated with a physiological dose (10 μmol/L) of the β-AR agonist isoproterenol for 1 hour, which resulted in increased fragmentation (Figure 5). The β1-AR antagonist CGP 20712A (300 nmol/L) blocked isoproterenol-induced fragmentation whereas the β2-AR antagonist ICI 118 551 (300 nmol/L) did not, demonstrating β1-AR subtype-specific regulation of mitochondrial dynamics.

To assess whether this in vitro model of physiological mitochondrial fragmentation is associated with enhanced mitochondrial function, respiration was assessed by Seahorse oximetry. Isoproterenol induced a dose-dependent increase in basal respiration (before oligomycin), maximum respiratory capacity (after FCCP [carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone]), and ATPase-driven respiration (ΔOCR Basal-Oligo; Figure 6A). CGP 20712A blocked the effects of isoproterenol on respiration, whereas ICI 118 551 intriguingly increased basal, maximal, and ATPase-driven respiration above isoproterenol alone (Figure 6B), likely a result of the elimination of β2-AR-G-inhibitory protein (Gi) signaling.[41] Similar to exercise, isoproterenol stimulated Drp1 mitochondrial translocation, and this was blocked by CGP 20712A (Figure 6B). Confirming the role of β1-AR signaling in energy production, isoproterenol significantly increased ATP production (Figure 6B).

We also assessed the role of β3-adrenergic receptor signaling because it is also stimulated by isoproterenol and has been shown to play a role in modulating mitochondrial dynamics in skeletal muscle and brown adipose tissue.[42] We pre-treated cells with 1 μmol/L of the β3-AR antagonist L748 337[43,44] or the β1/β2 antagonist propranolol[45] for 30 minutes before isoproterenol exposure. We found that inhibition of β3-AR signaling (L748 337+isoproterenol) resulted in a synergistic increase in respiration, similar to that seen with the β2-AR antagonist ICI 118 551 while activation of only the β3-AR (propranolol+isoproterenol) resulted in a dramatic decrease in respiration (Figure 6C). These results demonstrate that β3-AR signaling opposes β1-AR–induced physiological fragmentation. Previous reports have shown that β3-ARs, similar to β2-ARs, can couple to the inhibitory protein, Gi[46] and also promote promitofusion pathways.[42]

To confirm the connection between β-AR signaling and mitochondrial fission-enhanced respiration, we pre-treated cells with 30 μmol/L of the mitochondrial division inhibitor Mdivi-1 for 30 minutes before isoproterenol exposure and assessed respiration. Pre-treatment with Mdivi-1 resulted in a significant decrease in respiration (Figure 6D), confirming the connection between βAR signaling, mitochondrial fission, and enhanced mitochondrial respiration.

Because HL-1 cells are an immortalized cell line, we next determined whether similar changes in mitochondrial morphology and function were observed in a primary cardiac myocyte culture. Cardiac myocytes were isolated from adult mice and treated with 10 μmol/L isoproterenol for 1 hour. Electron microscopy again showed significant mitochondrial fragmentation with increased number and decreased area and perimeter (Online Figure VIIIA). Mitochondrial function, assessed by Oroboros oxygraph, showed a significant increase in both basal and maximal oxygen consumption (Online Figure VIIIB). Thus, mitochondrial fragmentation induced by physiological level β1-AR signaling increases mitochondrial function, additional evidence that mitochondrial fragmentation can be associated with enhanced and not degraded mitochondrial function in the heart.

Deletion of the β2-AR In Vivo Results in Drp1-Mediated Mitochondrial Fission and Enhanced Exercise Capacity

To provide additional in vivo evidence for β1-AR–mediated mitochondrial fragmentation, we evaluated β2-AR knockout mice (β2$^{-/-}$). These mice are a model of chronic physiological level β1-AR signaling, without alterations in β1-receptor density or function, and without the counterbalancing effects of β2-ARs and β2-AR-Gi signaling.[20,41] Importantly, we have previously shown that β2$^{-/-}$ mice have normal cardiac structure and function and normal survival.[20] Consistent with our in vitro results, β2-AR$^{-/-}$ mice show marked mitochondrial fragmentation (Figure 7A), Drp1 translocation, and downregulation of mitophagy regulators PINK1 (Figure 7B). Of 35 mitochondrial-related genes identified as upregulated or downregulated using a genome-wide screen (Online Table I), the only regulator of mitochondrial dynamics changed was beclin-1, and this mitophagy mediator was downregulated, suggesting that transcriptional regulation of mitochondrial dynamic regulators is not the principal mechanism underlying chronic β-AR–induced changes in mitochondrial dynamics. Importantly, despite marked fragmentation in the β2$^{-/-}$, mitochondrial function was not impaired at baseline (Figure 7C), and β2$^{-/-}$ mice have enhanced exercise running time, distance, and work (Figure 7D) versus wild type. To confirm the role of enhanced β1-AR signaling, we blocked β1-AR signaling 90 minutes before exercise challenge by treating with the β1-specific antagonist metoprolol (5 mg/kg) intraperitoneally. The enhanced exercise work in the β2$^{-/-}$ was significantly decreased with β1-AR blockade (Figure 7D), additional evidence that mitochondrial fission and fragmentation play a major role in cardiac exercise adaptation.

Discussion

Mitochondrial fission has traditionally been associated with pathological stress and with impaired mitochondrial function; however, more recent studies have suggested a role for mitochondrial fission in normal cardiac homeostatic regulation.[6,8,10] In the current study, we demonstrate that cardiac mitochondrial fission and fragmentation is not limited to

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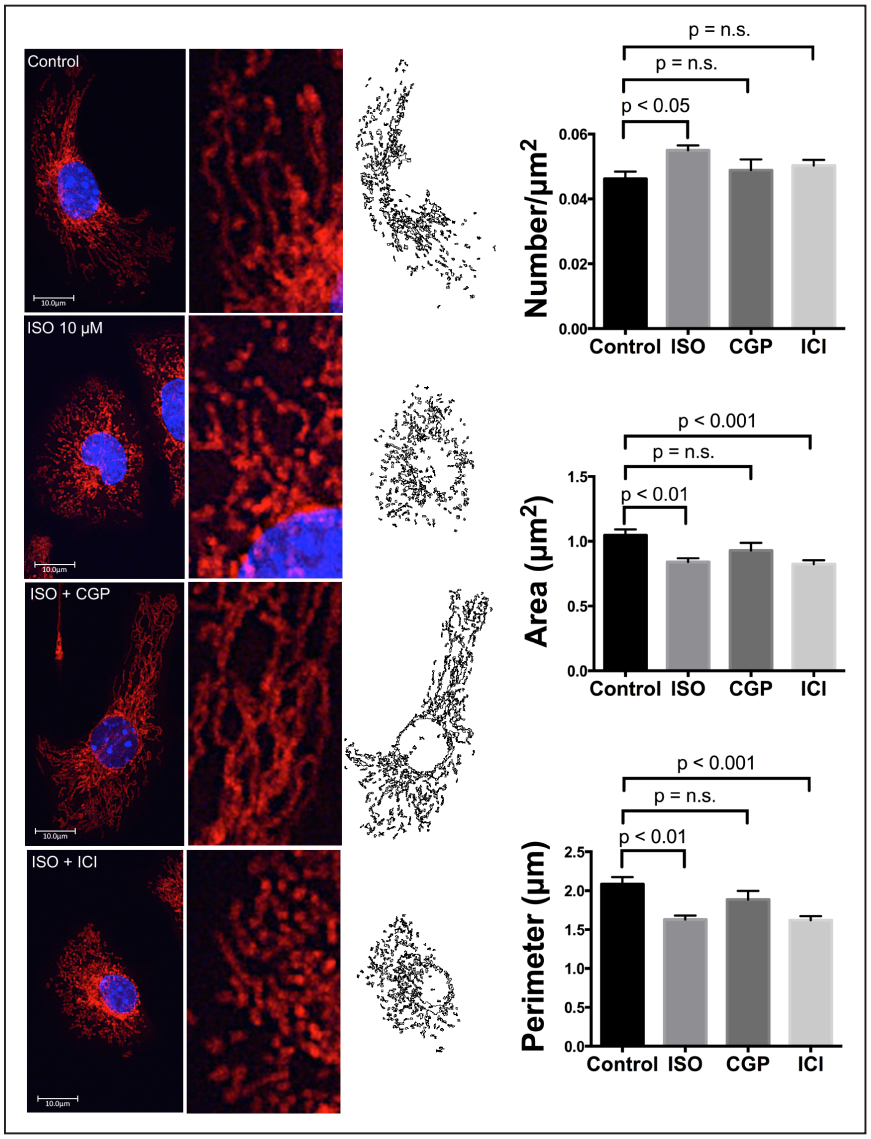

Figure 5. β1-adrenergic receptor signaling induces mitochondrial fragmentation in HL-1 cells. Cells were treated with isoproterenol (ISO; 10 μmol/L) for 1 h and imaged by confocal microscopy. ISO increased mitochondrial fragmentation (decreased perimeter and area and increased number), and this was blocked by CGP 20712A (β1 antagonist) but not by ICI 118551 (β2 antagonist; n=13–25 cells/group). Cells were quantified using a custom macro in Image J.

pathological states and is a component of normal physiological regulation of mitochondrial function during physiological stress, such as submaximal exercise. This is consistent with recent reports that have suggested that fission is necessary for normal mitochondrial quality control[5]; however, because these studies have relied on genetic manipulation, for example, Drp1 ablation, or were done in a pathological context, our study is the first to demonstrate a role for mitochondrial fragmentation in normal cardiac physiology. As a positive mediator of mitochondrial energetics, physiological fragmentation is essential for reaching maximal exercise performance. When mitochondrial fission and fragmentation were blocked with the Drp1 inhibitors P110, Mdivi-1, or by short-term conditional cardiac-specific Drp1 knockout, exercise capacity was significantly decreased, there was an earlier shift to oxygen-independent mechanisms for ATP generation, as evidenced by time to anaerobic threshold, and there was a dramatic reduction in ATP synthase–dependent state 3 respiration. A similar effect of mitochondrial fission on bioenergetics has been reported by Benard et al,[47] who show that Drp1 silencing in HeLa cells decreases state 3 respiration, rate of ATP synthesis, and survival during glucose starvation. The reduction in mitochondrial function seen with Drp1 silencing may be because of its role in mitochondrial remodeling, opening the cristae, and expanding the intermembrane space.[47,48] Prior studies in other cell systems support the concept that fission, fusion, and mitophagy are rapid enough processes to be operative within the time frame of physiological stressors, such as exercise.[49–51] One of the limitations of our study is that there is no method available to directly measure mitochondrial function during exercise in vivo, therefore our measurements were affected by the time delay required to isolate mitochondria from exercised mouse hearts. Thus, our finding of slightly increased mitochondrial function may be an underestimate of the degree to which mitochondrial function is augmented during exercise. Our critical finding here is that we see mitochondrial fission without a decrease in function.

The mitochondrial dynamic response to pathological stressors, such as ischemia/reperfusion injury (limited oxygen availability), doxorubicin toxicity (oxidative mitochondrial stress), or diabetes mellitus, (metabolic stress) have been published by

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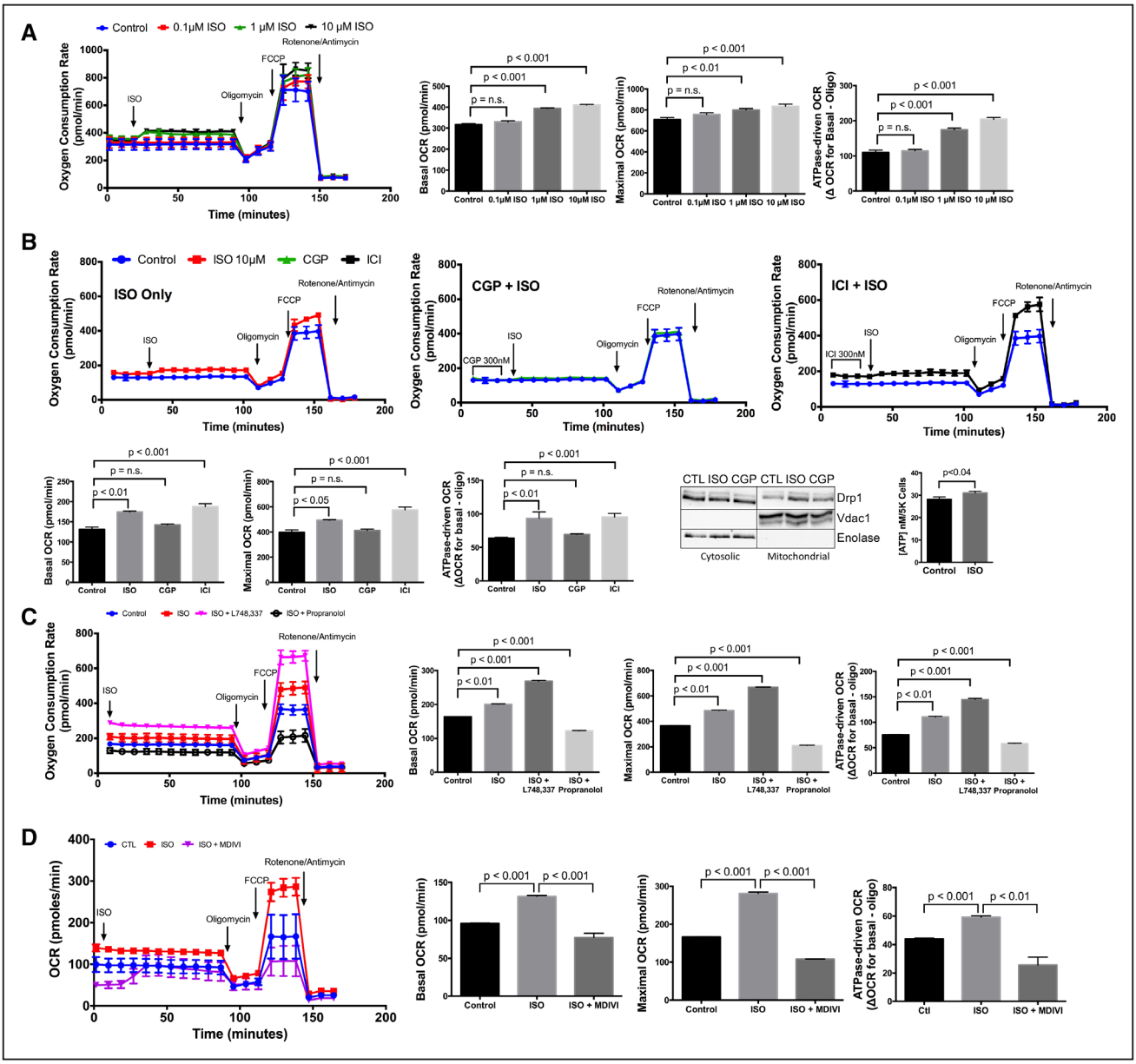

Figure 6. β1-adrenergic receptor (AR) signaling enhances mitochondrial respiration in HL-1 cells, which is opposed by both β2-AR and β3-AR signaling. **A**, Isoproterenol (ISO) increased basal, maximal, and ATPase-driven respiration in a dose-dependent manner starting at 1 μmol/L (n=5/group). **B**, This increase was blocked by CGP and further increased by ICI (n=4/group). ISO increased Drp1 (dynamin-related protein 1) mitochondrial translocation, which was blocked by CGP. ISO also significantly increased ATP production (n=7/group). **C**, ISO-enhanced respiration was synergistically increased with the β3-AR antagonist L748337 and blocked with propranolol (β1/β2 antagonist; n=5/group). **D**, Inhibition of mitochondrial fission with Mdivi-1 (mitochondrial division inhibitor) blocked ISO-induced increase in mitochondrial respiration (n=5/group).

our group and others, all showing activation of mitochondrial fission and fragmentation, increased ROS production, and the initiation of clearance of dysfunctional mitochondria through mitophagy/autophagy.[4,23,24,33,34] This is in contrast to physiological stressors such as submaximal exercise (increased metabolic demand) where ROS is not increased and mitochondrial membrane potential is maintained, preventing the activation of PINK1/parkin, and suppression of mitophagy. Interestingly, proautophagy ULK-1 is inactivated with submaximal exercise despite AMP-activated protein kinase activation.[52] ULK-1 inactivation may be mediated by phosphatases that are activated during exercise, for example, dual specificity protein phosphatase 1, which targets ULK1,[53] is expressed in the heart[54] and has been shown to become activated with exercise.[55]

One prior study by He et al[36] has shown that autophagy is activated in the heart during exercise. In contrast, our model of submaximal exercise did not induce mitophagy or autophagy, supporting the concept that these processes are inactivated during submaximal exercise in the absence of mitochondrial dysfunction. One difference between our model and that of He et al[36] is that we exercised mice below their anaerobic threshold at 80% of their maximum capacity for 1 hour at a consistent speed of 20 m/min, without pre-training; whereas their study subjected mice first to 2 days of exercise training

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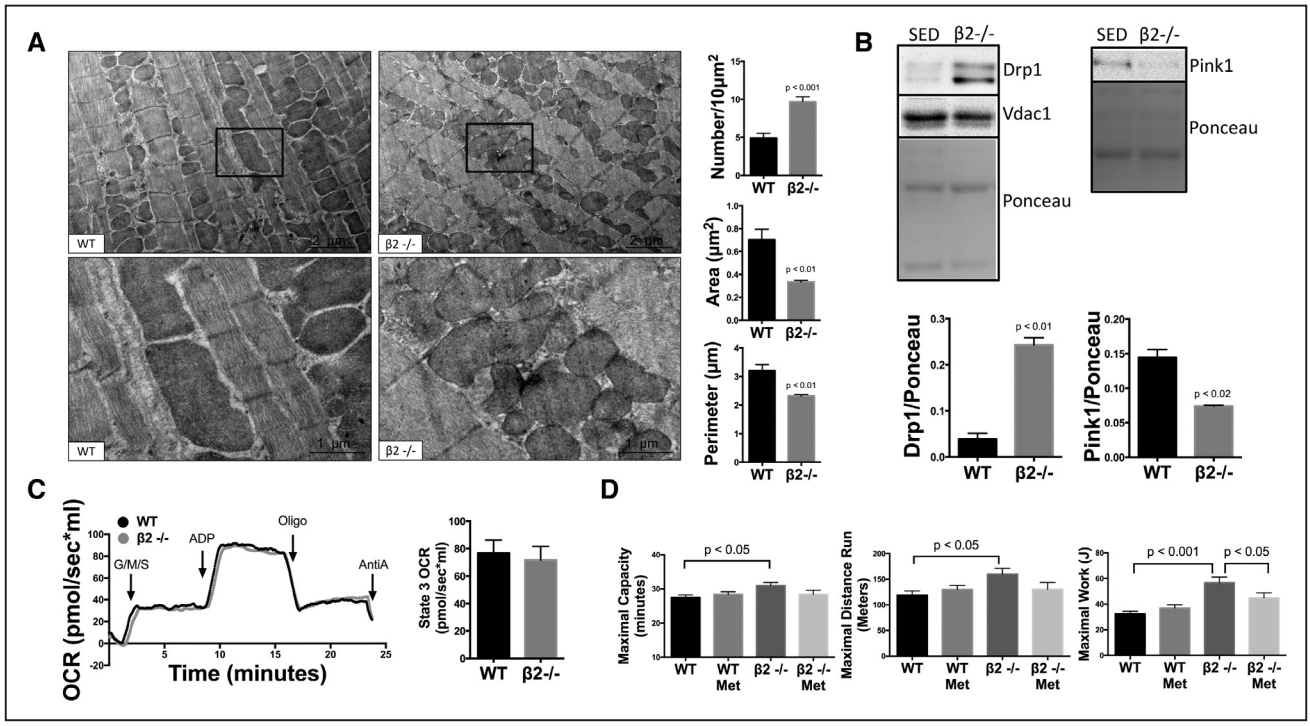

Figure 7. Ablation of the β2-adrenergic receptor (AR) results in physiological mitochondrial fission and enhanced exercise capacity. **A**, β2$^{-/-}$ mice, a model of unopposed physiological level β1-AR signaling, show dramatic mitochondrial fragmentation with increased number and decreased area and perimeter vs wild-type (WT) littermates (n=3–5/group). **B**, Drp1 (dynamin-related protein 1) mitochondrial translocation was increased and PINK1 (PTEN-induced putative kinase 1) was decreased in sedentary β2$^{-/-}$ (n=3/group). **C**, Despite marked mitochondrial fragmentation, β2$^{-/-}$ mice had normal baseline respiration by Oroboros oxygraph normalized to 50 μg of mitochondria (n=4/group). **D**, β2$^{-/-}$ mice have enhanced exercise running time, distance, and work, which is blocked with β1-AR inhibition by metoprolol (n=8/group).

(8–10 m/min for 5–10 minutes at 10° incline) and then to maximum exercise on day 3. Periods of exercise training have been shown to induce autophagy in other studies.[56] To examine whether the difference in autophagy in our study from that of He et al[36] was because of levels of exercise stress, we exercised mice to maximal capacity, showing that this also induces fission, without an increase in parkin (although unlike in our studies with submaximal exercise, parkin did not decrease) and still without evidence of autophagy (Online Figure IIIC). Thus, although there may be a tiered level of mitochondrial fission which occurs with exercise, where submaximal exercise induces physiological fragmentation without mitophagy/autophagy to enhance respiration, and extreme/maximal exercise induces a higher level of fission, we still do not see activation of autophagy in any of our models. Kruse et al[57] have shown similar findings in skeletal muscle from patients subjected to submaximal exercise (1 hour at 70% VO_2max), where Drp1 is activated immediately after exercise, but LC3 is decreased. Critically, LC3 is only increased after a 3-hour postexercise period,[57] suggesting that autophagy may be a feature of recovery from exercise. There are several other differences that could explain the divergence in our results from those of He et al.[36] Our submaximal exercise study was done on an FVB/NJ background while that of He et al[36] was done on a C57Bl/6 background, which has been shown to have reduced total exercise capacity compared with FVB/NJ,[58] so that similar exercise protocols could have led to increased stress in the C57Bl/6 mice. Importantly, we have also found that autophagy-related proteins increase rapidly (as early as 5 minutes) in the period of recovery from submaximal exercise (data not shown), thus making it necessary to harvest cardiac tissue immediately to assess the true state of autophagy during exercise. Finally, we cannot rule out the possibility of changes in autophagic flux during acute submaximal exercise because we were unable to demonstrate an increase in autophagic markers after chloroquine treatment in the absence of major additional stressors, such as ischemia or starvation. Despite this limitation, we still see no evidence of activation of mitophagy because PINK1 and parkin levels decrease with acute exercise and most importantly mitochondrial membrane potential and function are preserved.

We further show that physiological fragmentation is mediated, in part, by increased β1-AR signaling, activating Drp1, and increasing mitochondrial respiration. We confirmed this by cotreating cells with Mdivi-1 and isoproterenol, which resulted in the blockade of the isoproterenol-stimulated mitochondrial respiration. These data provide further evidence that physiological mitochondrial fragmentation is associated with enhanced, rather than degraded, mitochondrial function. Interestingly, treatment with the β2-AR antagonist ICI or the β3-AR antagonist L748 337 results in a greater increase in mitochondrial respiration above isoproterenol alone. ICI blocks both β2-AR-G stimulatory protein (Gs) as well as β2-AR-Gi signaling, thus enhancing β1-AR-Gs signaling.[59] The β3-AR has also been shown to couple to Gi.[46] Thus, treatment with isoproterenol+ICI or L748 337 results in greater stimulation

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of β1-ARs, resulting in the observed increased respiration above isoproterenol alone. Finally, β3-AR receptor signaling is known to activate mitochondrial fusion pathways in brown adipose tissue,[42] which could potentially oppose β1-AR–induced mitochondrial fission in the heart. However, β3-ARs are expressed at low levels in ventricular myocytes of mice[60] and humans.[61] Thus, the effects of β3-AR signaling may not be as important in the exercising heart as in other tissues.

Another model of fragmentation in the absence of pathology is the β2-AR knockout, where β1-AR signaling is not increased, but is unopposed by β2-ARs, including inhibitory G_i signaling.[41] These mice have marked mitochondrial fragmentation without evidence of mitochondrial functional impairment, normal cardiac structure and function, and enhanced exercise capacity, which is blocked by β1-AR inhibition, confirming our in vitro findings of β1-AR subtype-specific regulation of mitochondrial dynamics, and providing further evidence of the process of physiological fragmentation. One limitation of this part of our study is the inability in vivo to selectively block the effects of β1-AR stimulation on mitochondrial fission as opposed to the global effects of β1-AR stimulation on cardiac function. Thus, the β1-AR receptor–mediated increase in fission that we clearly show in vitro may be only one of the pathways mediating this process in vivo, for example, as evidenced by the lack of an effect of metoprolol on total exercise capacity in wild-type mice (Figure 7D), and the normal exercise capacity we have previously shown in the $\beta1\text{-AR}^{-/-}$ mice.[62] Other pathways that may play a role in exercise-induced fission include the β3-AR and AMP-activated protein kinase signaling pathway. However, in the setting of the $\beta2\text{-AR}^{-/-}$, on a background of increased fission and fragmentation at rest, the important role of the β1-AR receptor in mediating mitochondrial fission is demonstrated.

Mitochondrial fragmentation can be viewed as an active process (cleavage of individual mitochondria) or a morphological outcome (the presence of more numerous and smaller mitochondria), and the two are not necessarily equivalent. The bizarre mitochondrial morphology (Figure 4C), which we have dubbed "puzzle piece mitochondria," induced by exercise in $Drp1^{-/-}$ mice, suggests that mitochondrial fission was being actively stimulated by exercise but that the process was interrupted before mitochondrial division. This is further evidence for a more complex role for mediators, such as Drp1.

In conclusion, we have shown that mitochondrial fragmentation is a component of the cardiac response to physiological increases in energetic demand as encountered during normal levels of exercise. In contrast to pathological fragmentation, physiological mitochondrial fragmentation enhances mitochondrial function and deactivates mitophagy, the first demonstration of a positive role for fission in normal, nonpathological, nongenetically manipulated cardiac physiology (Online Table II). Whether physiological fragmentation can be harnessed pharmacologically as a preventive or treatment of cardiovascular disease remains to be determined although some of the cardiovascular benefit of exercise could be mediated by this mechanism.

Acknowledgments

Experiments were performed by M. Coronado, G. Fajardo, K. Nguyen, M. Zhao, K. Kooiker, G. Jung, D.-Q. Hu, E. Sandoval, and A. Stotland. Experimental design and conception were performed by M. Coronado, R.A. Gottlieb, and D. Bernstein. The manuscript was written by M. Coronado and D. Bernstein, and all authors provided editorial review.

Sources of Funding

This work was supported by grants from the National Institute of Health to D. Bernstein (HL061535), R.A. Gottlieb (P01 HL112730), and M. Coronado (K99HL129474) and by a T32 Post-Doctoral Fellowship to M. Coronado and K. Kooiker (HL094274). Electron microscopy studies were supported, in part, by the ARRA (1S10RR026780-01) from the National Center for Research Resources.

Disclosures

None.

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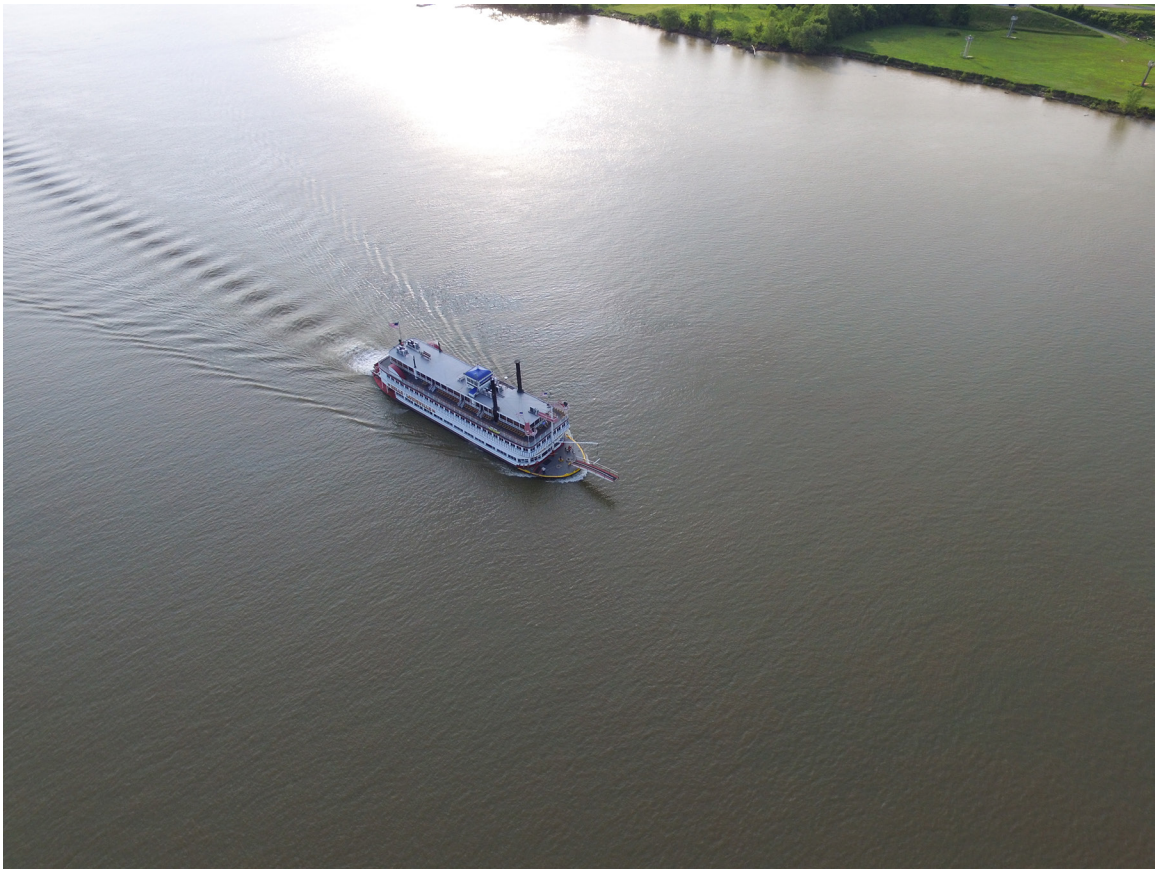

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Physiological Mitochondrial Fragmentation Is a Normal Cardiac Adaptation to Increased Energy Demand

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Circ Res. 2018;122:282-295; originally published online December 12, 2017;
doi: 10.1161/CIRCRESAHA.117.310725
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Materials

Materials and Methods

Animal Protocols:

Male 8-10 wk. old FVB/NJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were subjected to exercise on a treadmill equipped for simultaneous measurement of VO2 and VCO2 (Columbus Instruments, Columbus, OH), and distance ran and respiratory exchange ratio (RER) were determined using our established protocols [23]. Mice were exercised using two different protocols. First, sub-maximal exercise was performed for 1 h at a speed of 20 m/min with a constant grade of 5°. After 1 h mice were sacrificed and tissues prepared for assessment. Exercise capacity using this protocol was assessed by running mice to exhaustion at a speed of 20m/min at a constant grade of 5° and recording total distance ran. Second, maximal exercise capacity was assessed using a protocol that incrementally increased the degree of elevation by 2° and the speed by 2 m/min every 3 min. Mice were exercised until exhaustion while monitoring VO2 and CO2 levels according to our previously published methodology [23]. RER, a surrogate for reaching anaerobic threshold, was calculated as the ratio VCO2/VO2. Exercise time was determined as the time until exhaustion or 10 seconds while resting on the shock grid. Exercise distance was calculated as the total vertical distance traveled (meters) = Treadmill speed (meters/min) X Percent grade (Sin θ) X Exercise time (min). Work (Joules) was calculated as the body weight (kg) X total vertical distance traveled (meters) X 9.8. Myocardial infarction was induced under isoflurane anesthesia (1.5-3%). A 22-gauge catheter is used to intubate the mouse, which is placed on a Harvard small animal ventilator. A lateral incision between the fourth and fifth ribs is made to open the chest. Myocardial infarction is induced using left anterior descending (LAD) artery ligation, using a 8-0 silk suture passes with a tapered needle underneath the LAD, posterior to the tip of the normally positioned left atrium. The thoracic cavity body wall is then closed with a 7-0 silk suture, and the skin closed with a 6-0 silk suture using an interrupted

pattern. Sham mice underwent the same procedure, with the absence of LAD ligation. Mice were then assessed 24h after surgery. Protein extractions were sampled from the remote zone. Mice were also exposed to 10mg/kg of doxorubicin (NovaPlus, Bedford, OH) or saline control by tail vain injection. After 30min exposure, mice were harvested for protein analysis.

β-Adrenergic Signaling and Mitochondrial Dynamics Modulation:

To assess subtype-specific β-AR signaling, we utilized β2-AR knockout (-/-) mice generated on an FVB background in our laboratory [21]. To assay autophagic flux, mice were treated with Chloroquine (50mg/kg) by intraperitoneal (IP) injection 1hr prior to exercise testing. Chloroquine treated mice were exercise for 1hr at 20m/min and sacrificed immediately after exercise. To assess mitochondrial fission, mice were treated with the Drp1-Fis1 inhibitor P110, provided by Dr. Daria Mochly-Rosen, (0.5 mg/kg) or Mdivi-1 (25 mg/kg) by intraperitoneal injection, followed immediately by exercise testing. Either saline, TAT47-57 peptide (0.35 mg/kg) or 2% DMSO in saline were used as control treatments. To assess the role of β1-AR signaling, mice were treated with the β1-AR antagonist metoprolol (5mg/kg) by IP injection 90 minutes prior to maximal exercise challenge. To assess cardiac specific mitochondrial fission, $Drp1^{loxp/loxp}$ [22] C57B/6NJ mice were provided by Dr. Katsuyoshi Mihara and Dr. Masatoshi Nomura and were crossed onto the *myh6*-MER-Cre-Mer C57B/6NJ mouse line (Jackson Laboratory, Bar Harbor, ME) and gene recombination was induced by administration of tamoxifen (20mg/kg) IP for 5 days. On day 7 mice were used for experimentation. Male aged-matched Drp1+/+Cre+/+ and Drp-/-Cre-/- were treated with tamoxifen and used as controls.

Cell culture and treatment with agonists/antagonists

The HL-1 cardiac muscle and C2C12 skeletal muscle cell line were used for *in vitro* studies, maintained in Claycomb or DMEM media (Sigma-Aldrich, St Louis, MO), respectively, containing 10% FBS, 100g/mL penicillin/streptomycin, 0.1mM norepinephrine and 2mM L-glutamine. For at least one passage before experimentation, HL-1 cells were cultured in norepinephrine free media. When cells reached

100% confluence, they were treated with 10 μM isoproterenol (Sigma-Aldrich, St Louis, MO) (ISO) for 1h. Cells were pre-treated with 300 nM of the subtype specific inhibitors CGP 20712A (Sigma-Aldrich, St Louis, MO) (β1 antagonist) and ICI 118551 (Sigma-Aldrich, St Louis, MO) (β2 antagonist) and 1μM of L748,337 (Cayman Chem, Ann Arbor, MI) (β3 antagonist) and propranolol (Sigma-Aldrich, St Louis, MO) (β1&β2 antagonist) for 30 min before ISO exposure. Cells were also pretreated with 30μM of the mitochondrial division inhibitor Mdivi-1 for 30 min before ISO exposure. After ISO exposure, cells were either washed with PBS or used directly for respiration measurement. C2C12 or isolated adult cardiac myocytes were exposed to 100μM doxorubicin. After exposures, cells were either washed with PBS or used directly for morphology or respiration measurement.

Echocardiography

Serial two-dimensional echocardiographs were performed on anaesthetized mice. Images were acquired with a GE Vivid 7 ultrasound system (GE Healthcare, Milwaukee, WI) with 13-MHz transducer. Left ventricular ejection fraction was quantified by m-mode echocardiography at the level of the papillary muscles. Parasternal long axis view in systole was used to image and calculate cardiac output as HR * SV where SV = LVOT area * LVOT VTI.

Mitochondrial isolation

Mitochondria were isolated from HL-1 cells by first detaching cells with 0.05% Trypsin/EDTA for 10 min, washing with Claycomb media and resuspending cells in a minimal volume of isolation buffer. Cells were then lysed by passing through a 27-gauge syringe (15 strokes). Cell lysates were placed on ice for differential centrifugation. Cardiac mitochondria were isolated from ventricular tissue by first mincing and then homogenizing tissues using a Dounce homogenizer (20 strokes) in isolation buffer (300mM sucrose, 10mM hepes, 2mM EGTA, pH 7.2, 4°C). Tissue lysates were then placed on ice for differential centrifugation. Both cell and cardiac tissue lysates were centrifuged at 500 x g for 10 min and the resulting supernatants were centrifuged at 10,000 x g for 15 min. The mitochondrial pellet was washed once with

isolation buffer and the final pellet resuspended in a minimal volume of isolation buffer and kept on ice for functional assays. If samples were used for Western blot, mitochondria were solubilized in isolation buffer containing 1% Triton X and stored at -80°C. Purity of mitochondrial fractions are confirmed by the absence of the cytosolic protein enolase.

Flow cytometry analysis of mitochondrial size.

Cardiac mitochondria were isolated immediately after exercise as described above. After the 10,000 x g mitochondrial pellet was secured, mitochondria were resuspended in mitochondrial isolation buffer containing 200nM Mitotracker Deep Red (Thermo Ficher Scientific, Waltham, MA). Mitochondria were incubated for 10 minutes at RT and then washed 2X in mitochondrial isolation buffer. After final wash, mitochondria were resuspended in 4% PFA and incubated at RT for 5 minutes. Mitochondria were washed 3X and stored in PBS at 4°C. Mitochondrial suspensions were then analyzed by flow cytometry. Briefly, mitochondria were thresholded by Mitotracker Deep Red fluorescence to exclude non-mitochondrial particles. 10,000 events were recorded and used for analysis. Preliminary assessment of known bead sizes showed that side scatter area (SSA) was the best measure to differentiate particle sizes ranging between 0.2μm-2.0μm (Supplementary Fig. 1) on an LSRII flow cytometer (BD Biosciences, San Jose CA). Side scatter area (SSA) was then plotted against forward scatter area (FSC) to assess mitochondrial size using FlowJo (FlowJo, Ashland, OR).

Myocyte isolation and membrane potential/ROS assessment.

Adult ventricular myocytes were isolated from 3 mo old FVB mice as previously published[3]. Briefly, mice were anesthetized with pentobarbital sodium (100 mg/kg ip). The heart was removed and retrograde perfused at 37^{o}C with a Ca^{2+} free solution (120mM NaCl, 14.7mM KCl, 0.6mM KH_2PO_4, 0.6mM Na_2HPO_4, 1.2mM $MgSO_4$-$7H_2O$, 4.6mM $NaHCO_3$, 10mM Na-HEPES, 30mM taurine, 10mM BDM, 5.5mM glucose) followed by enzymatic digestion with collagenase (Worthington Biochemical Corporation, Lakewood, NJ) (1.5 mg/ml). Ventricles were cut into small pieces and further digested by gently pipetting with plastic

transfer pipettes for 3-5 min. Stop buffer (Ca^{2+} free solution + $CaCl_2$ 12.5μM + 10% bovine calf serum (Hyclone, Logan, UT) was added and the cell suspension was collected in a 15 ml tube and centrifuged at 400 rpm for 3 min. Myocytes were resuspended in stop buffer in increasing $CaCl_2$ concentrations until 1mM was achieved. Experiments were performed with freshly isolated myocytes resuspended in a HEPES-buffered solution (12.5mM $CaCl_2$, 137mM NaCl, 5.4mM KCl, 15mM dextrose, 1.3mM $MgSO_4$, 1.2mM NaH_2PO_4, 20mM HEPES, pH 7.4). Cells were loaded in 96 well plates, then stained with 5μM MitoSox for 10 min to quantify ROS and 10nM TMRM for 30 min to quantify changes in mitochondrial membrane potential. Cells were imaged in a widefield fluorescence microscope (ImageXpress Micro, Molecular Devices, Sunnyvale, CA) and changes in intensity means were quantified using Imaris software.

Mitochondrial respiration assays

Respiration was assessed in isolated mitochondria by measuring O_2 consumption using an Oroboros Oxygraph (Oroboros Instruments, Innsbruck, Austria). Standardized instrumental and chemical calibrations were performed to correct for back-diffusion of O_2 into the chamber from leakage, consumption by the chemical medium and sensor consumption. Measurements were taken from 50 μg of mitochondria or 120,000 isolated cardiac myocytes in suspension (2 mL) gently agitated at 37°C. State 2 respiration (mito only) was assessed with the addition of glutamate (10 mM), malate (2 mM) and succinate (10mM) as the complex I and II substrates, and then State 3 respiration (mito only) was assessed by the addition of ADP (0.5 mM). State 4 (leak) respiration was assessed with the complex V inhibitor oligomyocin (2 μg/mL). Respiration due to reactive oxygen species formation was assessed with Antimycin A (2.5 μM). O_2 flux was measured by Datlab2 software (Oroboros Instruments, Innsbruck, Austria), capable of converting nonlinear changes in the negative time derivative of the oxygen concentration signal.

Mitochondrial respiration was also assessed in plated HL-1 cells by measuring O_2 consumption using the Seahorse XF24^e Analyzer. HL-1 cells were grown on a plastic 24 well plate, cultured with Claycomb media and allowed to grow to confluence. Before experimentation, cells were washed with supplemented XF assay media (Seahorse Bioscience, North Billerica, MA) and allowed to equilibrate for

1 h. Specified cell groups were then pre-treated with the β-blockers CGP 20712A or ICI 118551 (300 nM), immediately placed into the Seahorse XF24^e Analyzer and assessed for O_2 consumption for 30 min. After pre-treatment, cells were then exposed to ISO (10 μM) and O_2 consumption measured for 1 h. Following ISO treatment, ATP production, maximal respiration and non-mitochondrial respiration were assessed by treating cells with oligomycin (1 μM), FCCP (1 μM) and a rotenone (1 μM) / antimycin (1 μM) mixture, respectively. O_2 flux was measured by Wave software (Seahorse Bioscience, North Billerica, MA).

Protein and enzyme assays

Mitochondrial fractions were measured for protein content by Western blot. Commercially available antibodies for Drp1 (Enzo, Farmingdale, NY), Phospho Drp1 ser616 (Cell Signaling, Danvers, MA), Phospho ULK1 Ser555 (Cell Signaling, Danvers, MA), Phospho AMPK Thr172 (Cell Signaling, Danvers, MA), Mfn1 (Abnova, Taipei, Taiwan), Mfn 2 (Abnova, Taipei, Taiwan) and Parkin and PINK1 (Santa Cruz Bioscience, Santa Cruz, CA) were used to identify expression changes in mitochondrial dynamic proteins. VDAC1/Porin (Abcam, Cambridge, MA) was used as a loading control for mitochondrial proteins and α-actinin (Abcam, Cambridge, MA), enolase (Santa Cruz, Santa Cruz, CA) and GAPDH (Advanced Immunochemicals, Long Beach, CA) for cytosolic proteins. 4-HNE (Alpha Diagnostic, San Antonio, TX) was used to assess lipid peroxidation. To ensure faction purity, enolase was used verify that absence of cytosolic proteins in mitochondrial fractions, while Vdac1 was used to verify the absence of mitochondrial proteins in cytosolic fractions.

ATP measurement assays

HL-1 cells were seeded into a 96 well plate at a density of 5,000 cells and used next day for ATP measurements. Cells were treated with ISO (10μM) for 1 hr. After treatments, ATP was measured using a commercially available kit by Abcam (ab65313) according to manufactures instructions.

Microarray

The Stanford mouse transcriptome microarray was used to compare β2-/- and WT mice (Stanford Functional Genomics Facility, Stanford, CA). This microarray includes 43,200 mouse cDNA probes representing ~25,000 unique genes and expressed sequence tags. LV myocardium from nine β2-/- and nine WT mice were divided into three independent pools (3 per pool). Six cDNA arrays, which represented a total of 18 hearts, were analyzed. Previous studies have validated the use of pooling when tissue samples are small[4]. Microarrays were scanned using an Agilent G2565AA scanner, and features extracted and analyzed using SpotReader software (Niles Scientific, Sacramento, CA). Details of microarray analysis are included in statistical analysis, below.

Confocal microscopy

Mitochondrial morphology was assessed in HL-1 cells utilizing the fluorescent probe MitoTracker Red CMXROS (Thermo Ficher Scientific, Waltham MA). Cells were stained with MitoTracker for 30 min and fixed with 3.7% formaldehyde for 15 min. Cells were imaged using a Nikon Spinning Disk Confocal microscope and analyzed by NIS-Elements software. Morphology was quantified using an Image J macro that traces the outlines of mitochondria in confocal images. Images were converted into 32-bit format, inverted, sharpened and with autothreshold. Mitochondrial tracings were determined using a size parameter between 10-100000000 and circularity between 0-1. Confocal images of PBS, ISO, CGP and ICI treated HL-1 cells were assessed using the tracing macro and displayed as outlines. Outlined images were used in the quantitative analysis of mitochondrial morphology.

Electron microscopy

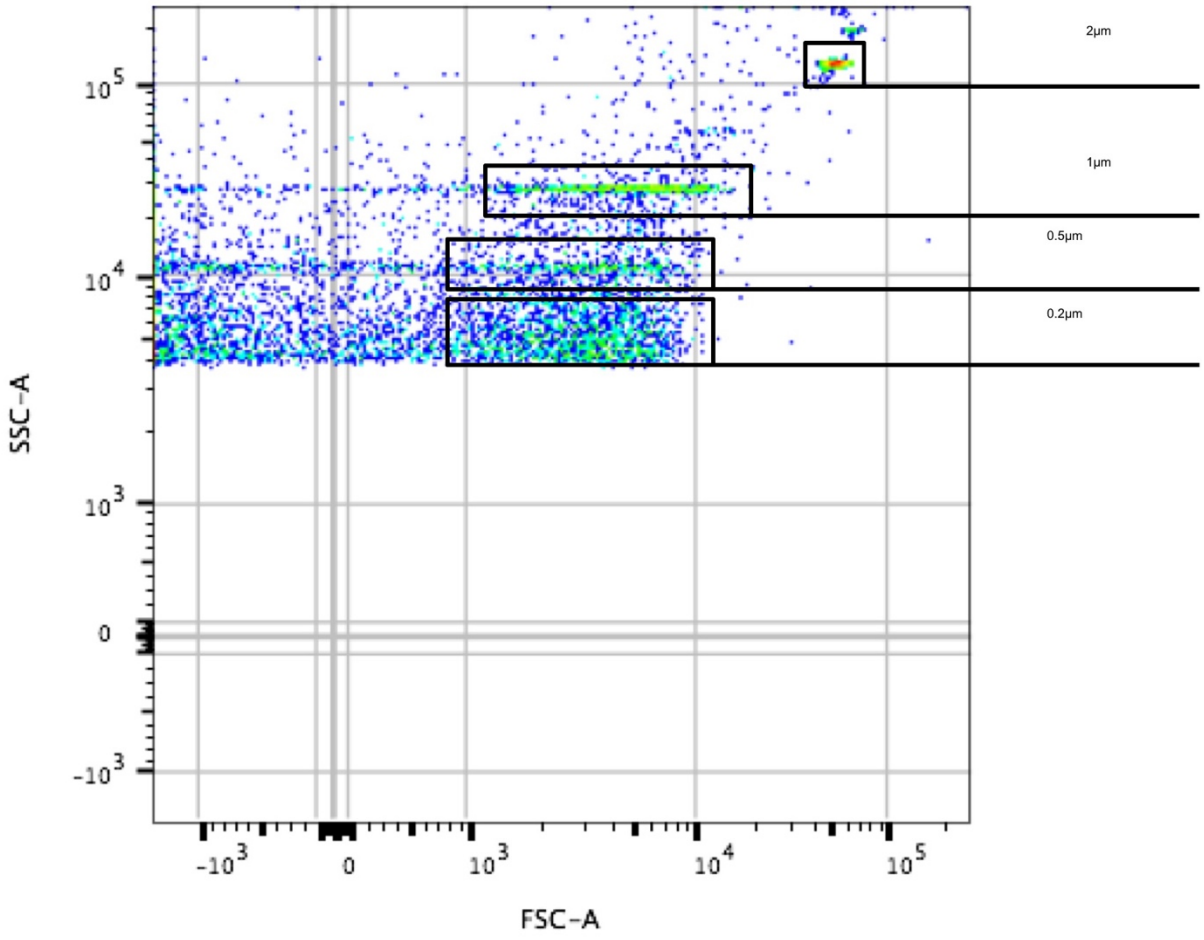
Mitochondrial morphology was assessed by transmission electron microscopy (TEM). LV cardiac tissue was excised from 3-5 age-match mice (7-8 wks) and immediately fixed in a 2% glutaraldehyde 4% paraformaldehyde sodium cacodylate buffer overnight. Samples were then stained with 2% osmium

tetroxide, dehydrated by ethanol series and embedded in an epoxy resin for 24 h. Ultrathin sections (80-150nm) were prepared and interfibrillar mitochondria were imaged at 2000X magnification on a JOEL TEM 1400 (JOEL, Tokyo, Japan) and photographed on a Gatan multiscan 791 digital camera (Gatan, Pleasanton, CA). The operator and analyzer was blinded to experimental condition, quantified mitochondrial number and morphology (number, area, perimeter) using Image J. Briefly, one tissue section was isolated from each of 3-5 mice and imaged by electron microscopy. From each tissue section 10-20 fields were captured, measured and averaged for each mouse. Statistical comparisons were then done between mice resulting in a total n of 3-5.

Statistical analysis

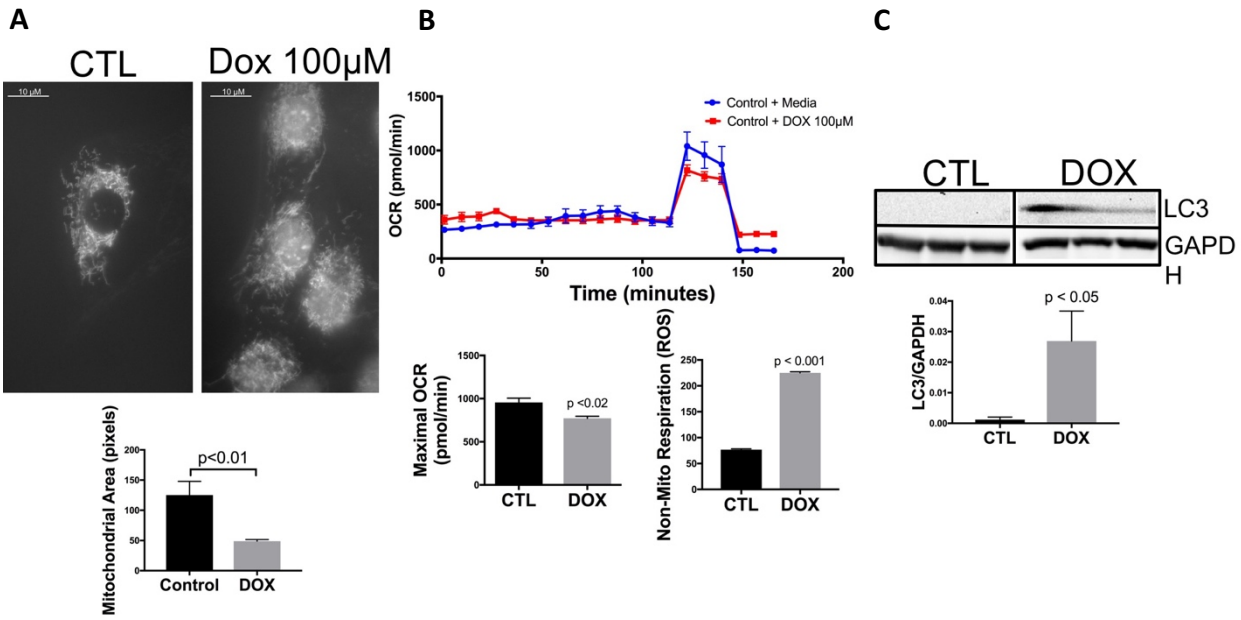
Data are expressed as the mean ± standard error of the mean. Normally distributed data comparing two groups were analyzed by Student's *t* test. Normally distributed data comparing multiple groups were analyzed by ANOVA with multiple comparisons analysis (Tukeys). Non-parametric data comparing two groups were analyzed using the Mann-Whitney U test. Statistical analysis of microarray data was performed using Stanford microarray database software and the Significance Analysis of Microarrays (SAM), Database for Annotation, Visualization, and Integrated Discovery, Hi-Throughput GOMiner and TIGR TM4 software. Multiple comparisons were accounted for by False Discovery Rate (FDR) analysis and genes were considered significantly altered with a p value less than 0.01 by Fisher's exact test. To control for multiple comparisons, we required a false discovery rate of 5%. Microarray data has been submitted to the NCBI Gene Expression Omnibus (GEO) database in MIAME format.

Online Figure I



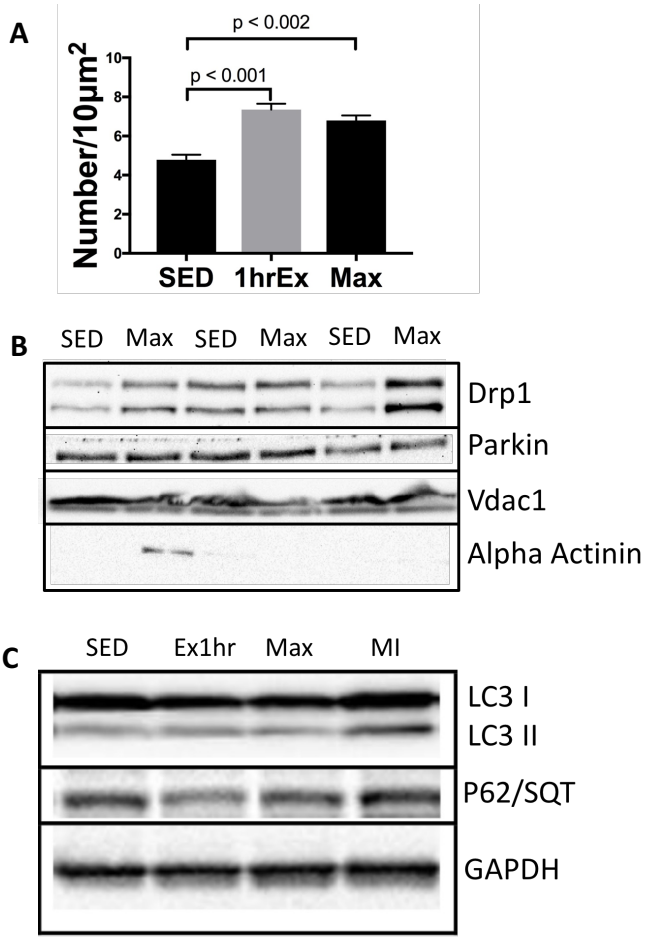
Online Figure I: Beads of known sizes were assessed by flow cytometry. Bead sizes ranged from 0.2µm, 0.5µm, 1µm, and 2µm, which fall within the size range of interfibrillar cardiac mitochondrial. Side scatter area (SSA) was selected to measure mitochondrial size because it was the only measure able to separate different bead sizes below the 1µm range.

Online Figure II



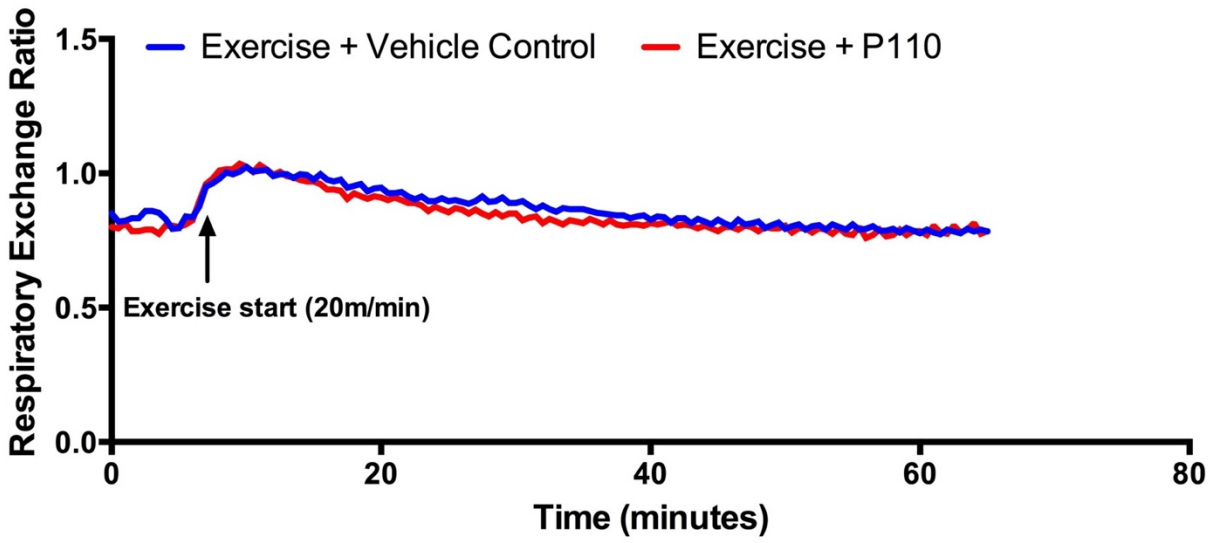
Online Figure II: Doxorubicin exposure induces pathological mitochondrial fragmentation. (A) C1C12 skeletal muscles cells exposed to 100μM of doxorubicin exhibit mitochondrial fragmentation (n = 10-20 cells/group). **(B)** Isolated cardiac myocytes exposed to 100μM of doxorubicin have decreased maximal respiration and increase ROS, as seen by increased non-mitochondrial respiration (n = 5/group). **(C)** Mice exposed to doxorubicin by tail vein injection (10mg/kg) for 30 minutes had increased LC3, indicating autophagy (n=3/group).

Online Figure III



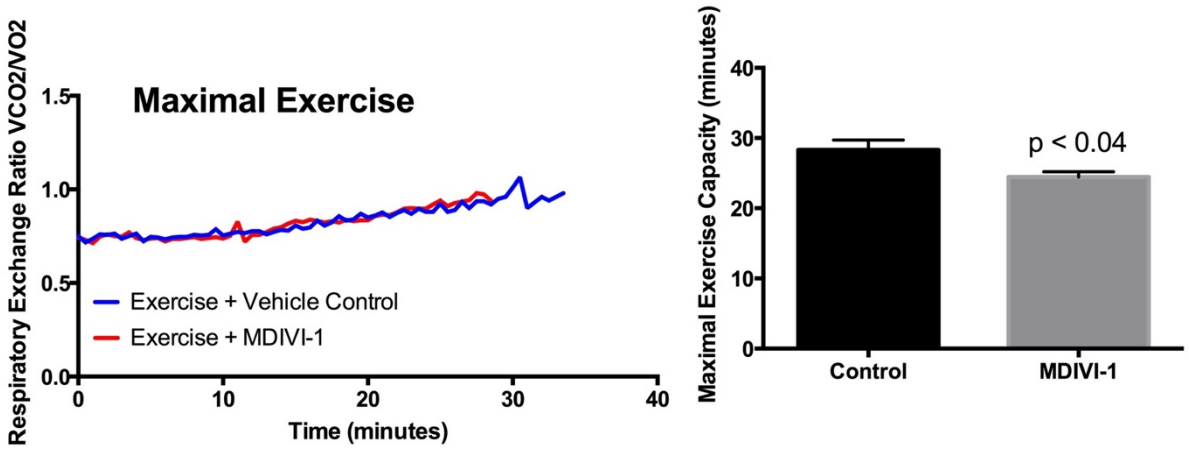
Online Figure III: Maximal and submaximal exercise both induce mitochondrial fragmentation. (A) A comparison of sub-maximal (1hr running at 20m/min) and extreme/maximal (running until exhaustion) exercise show similar mitochondrial fragmentation vs sedentary (n = 4-5/group). **(B)** Mitochondrial fractions from mice subjected to maximal exercise show Drp1 and Parkin translocation to the mitochondrial fraction (n=3/group). **(C)** Whole cell lysates of mice subjected to sub-maximal (Ex1hr) and extreme/maximal exercise (Max) show no change in LC3 or P62 (n=4/group). Mice subjected to 24hr of left anterior descending ligation were used as positive controls (MI).

Online Figure IV



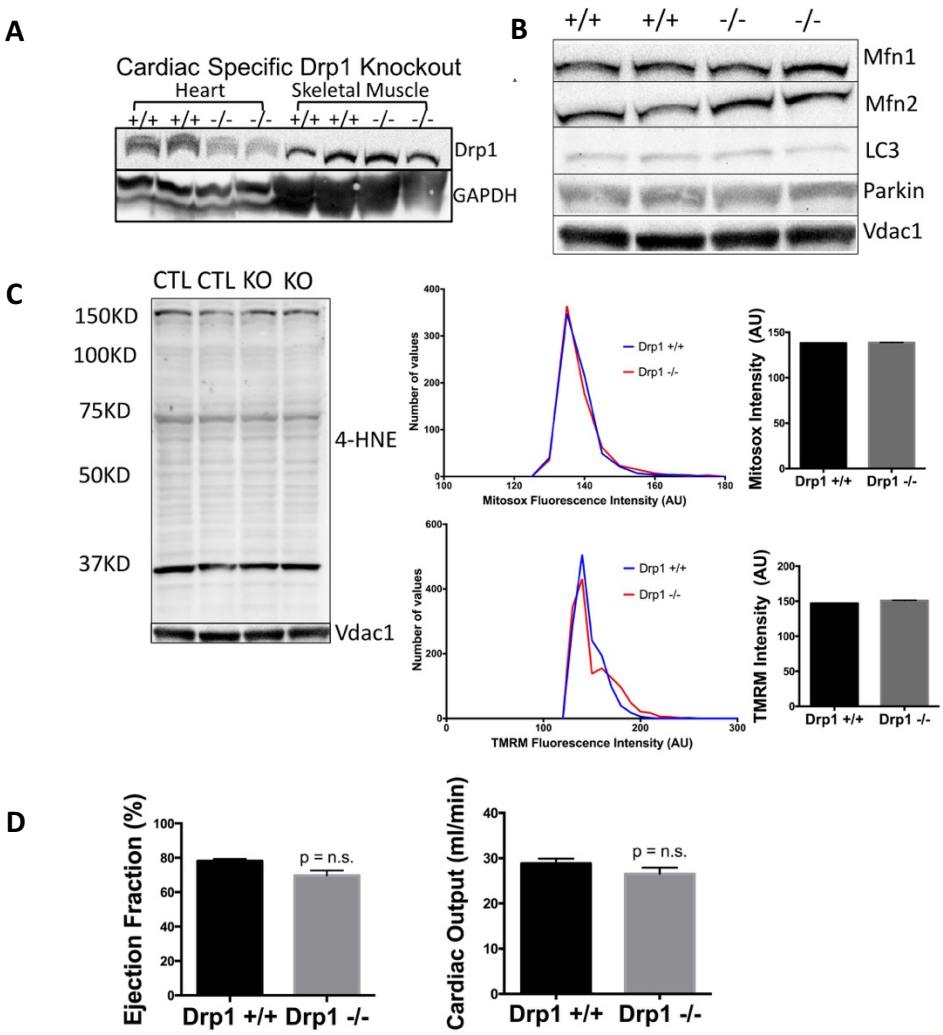
Online Figure IV: The early response to exercise is not affected by Drp1 inhibitor treatment. Mice were treated with control TAT peptide or P110 and placed on a treadmill for 1h at a speed of 20 m/sec. VO2 and CO2 levels were measured and RER (CO2/VO2) was calculated (n=3 per group).

Online Figure V



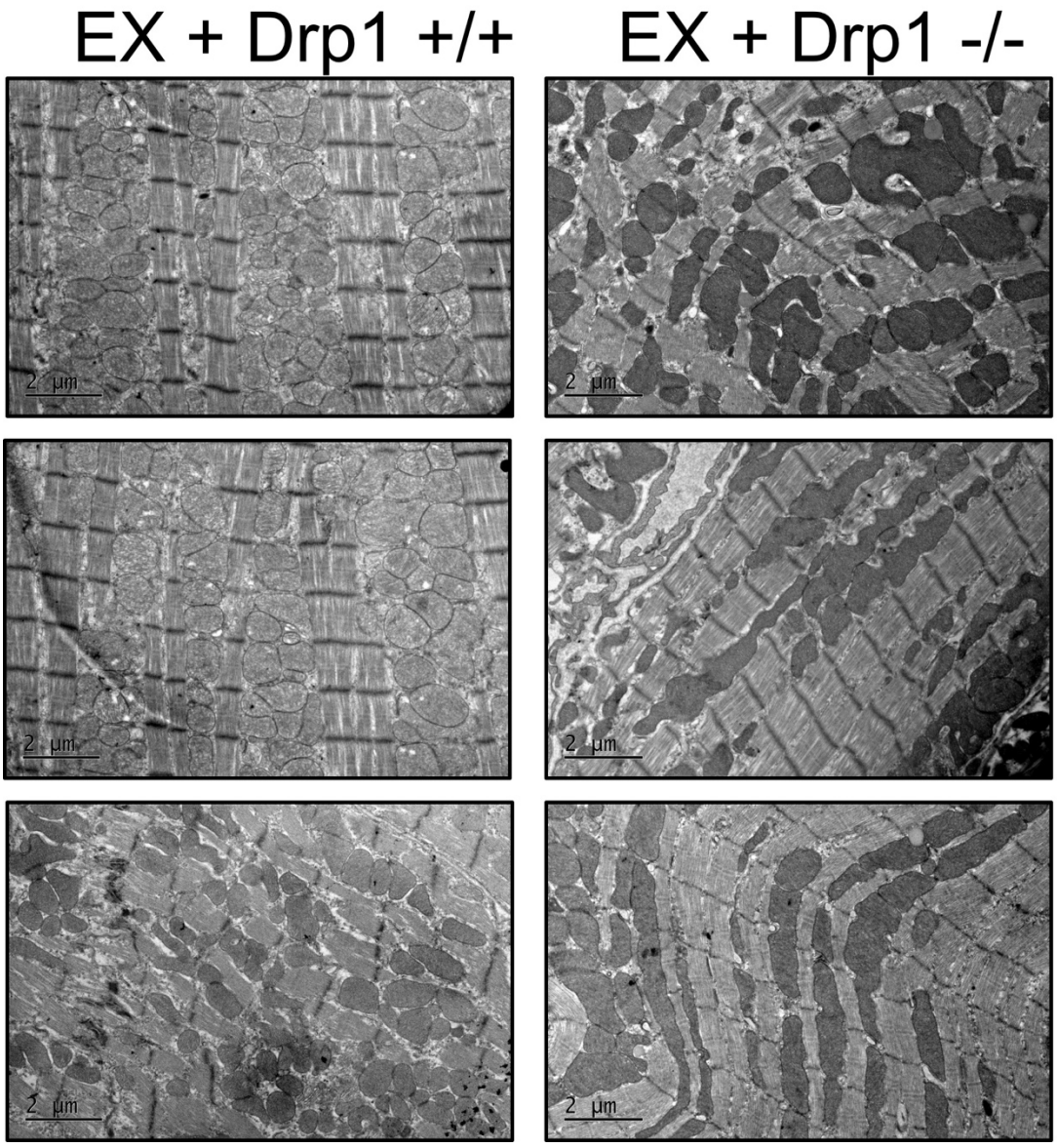
Online Figure V: Similar to P110, the Drp1 GTPase inhibitor, Mdivi-1, also decreases maximal exercise capacity. Mice pretreated with the Mdivi-1 (25 mg/kg i.p.) showed decreased maximal exercise capacity compared to vehicle control (2% DMSO in saline) (n=4/group).

Online Figure VI



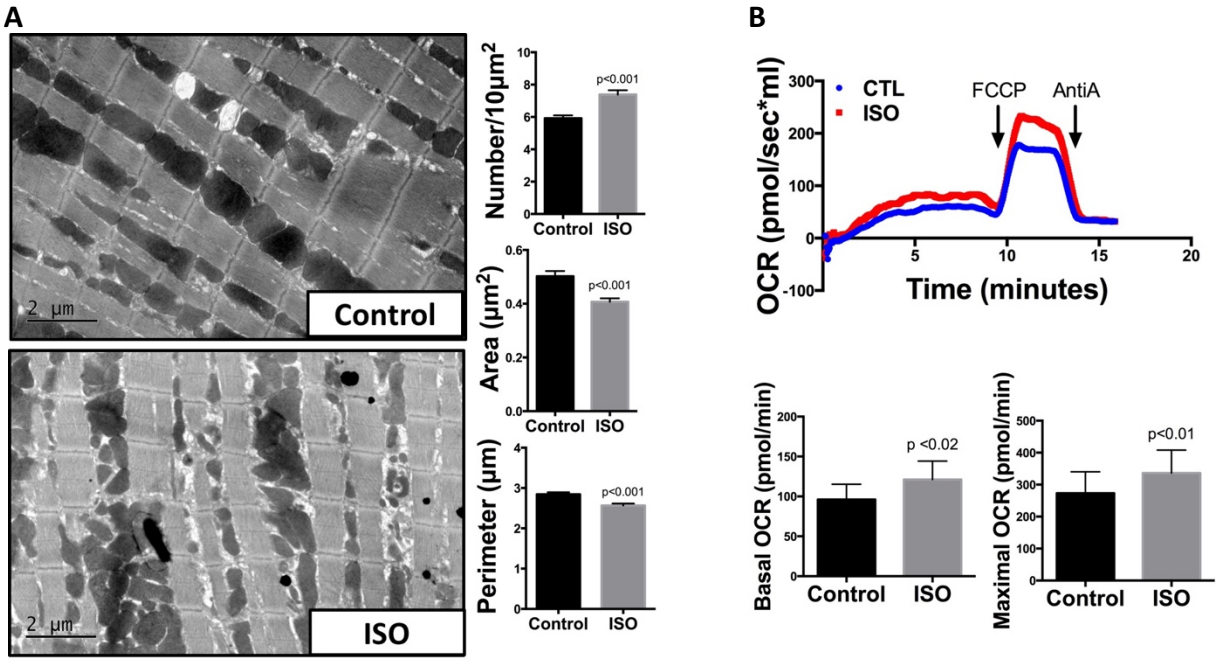
Online Figure VI: Cardiac specific Drp1 knockout results in no change in mitochondrial reactive oxygen species formation and normal cardiac function. (A) Mice were treated with tamoxifen (20mg/kg) for 5 days i.p. and on day 7 mice were used for experimentation. Male aged-matched Drp1+/+Cre+/+ and Drp-/-Cre-/- were treated with tamoxifen and used as controls for all experiments (n=2/group). **(B)** Mfns 1 & 2, LC3 and Parkin remained unchanged in the Drp1 -/- mice (n=3-4/group). **(C)** Reactive oxygen species was assessed by 4-HNE in isolated mitochondria and MitoSox in isolated cardiac myocytes, while membrane potential was assessed by TMRE in isolated cardiac myocytes. (n=3/group). **(D)** Cardiac function (ejection fraction and cardiac output) was assessed by echocardiography (n=3/group).

Online Figure VII



Online Figure VII: Abnormal mitochondria in exercised Drp1-/- mice. Additional images displaying abnormally large, elongated or bizzare-shaped mitochondria in Drp1-/- mice subjected to exercise.

Online Figure VIII



Online Figure VIII: β-AR signaling induces mitochondrial fragmentation and enhanced respiration in isolated cardiac myocytes. **(A)** Isolated cardiac myocytes treated with ISO (10µM) for 1hr were imaged by electron microscopy and showed significant mitochondrial fragmentation with increased number and decreased area and perimeter. **(B)** ISO treatment also increased both basal and maximal mitochondrial function, measured as oxygen consumption rate (OCR) by Oroboros oxygraph normalized to 120,000 cells. (n=4/group).

Online Table I

upregulated in β2 Adrenergic receptor deficient mice compared to WT FVB controls

Gene Name	Symbol	Gene sequence	Genbank ID	Fold Change
Cytochrome p450, family 2, subfamily b, polypeptide 10	cyp2b10	AV054284	NM_009999	2.56595
Prolylcarboxypeptidase (angiotensinase C)	prcp	AV108774	NM_028243	2.38771
Cytochrome b-5	cyb5	AV077886	NM_025797	1.98741
Desmin	des	AV088720	NM_010043	1.62071
Isocitrate dehydrogenase 2 (NADP+), mitochondrial	ldh2	BG068167	NM_173011	1.55985
Histidine triad nucleotide binding protein 3	hint3	AV056903	NM_025798	1.46587
Isocitrate dehydrogenase 2 (NADP+), mitochondrial	ldh2	AV140565	NM_173011	1.44434
Immediate early response 3	ier3	AV104326	NM_133662	1.42981
Nicotinamide phosphoribosyltransferase	nampt	AV108470	NM_021524	1.40950
Alkylation repair homolog 7	alkbh7	BG074929	NM_025538	1.38233
Ubiquitin C	ubc	AV032177	NM_019639	1.37561
Isocitrate dehydrogenase 2 (NADP+), mitochondrial	ldh2	AV089252	NM_173011	1.37240
Clusterin	clu	BG072209	NM_013492	1.36581
Glutathione S-transferase, mu 2	gstm2	BG070501	NM_008183	1.35842
Mitochondrial ribosomal protein L54	mrpl54	AV088357	NM_025317	1.25277
Carnitine palmitoyltransferase 1, muscle	cpt1b	AV006274	NM_009948	1.24645

downregulated in β2 Adrenergic receptor deficient mice compared to WT FVB controls

Gene Name	Symbol	Gene sequence	Genbank ID	Fold Change
Apolipoprotein A-IV	apoa4	AA064294	NM_007468	-3.86802305
L-threonine dehydrogenase	tdh	AV093742	NM_021480	-3.19468405
Chloride channel calcium activated 3	clca3	AV074056	NM_017474	-2.79025642
6-pyruvoyl-tetrahydropterin synthase	pts	AV088699	NM_011220	-2.59571707
Nuclear receptor subfamily 4, group A, member 1	nr4a3	NM_010444	NM_015743	-2.51439491
Jun-B oncogene	junb	AA608298	NM_008416	-2.19959088
ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	atp2a1	AV082969	NM_007504	-2.17112834
Proteasome (prosome, macropain) subunit, alpha type 4	psma4	AV095052	NM_011966	-2.08064583
Mitochondrial ribosomal protein L49	mrpl49	AV011359	NM_026246	-1.76962962
S100 protein, beta polypeptide, neural	s100b	AV031395	NM_009115	-1.71083472
Homer homolog 3	homer3	AV041850	NM_001146153	-1.66958845
Aconitase 1	aco1	AV001502	NM_007386	-1.62250742
Beclin 1, autophagy related	becn1	AV049038	NM_019584	-1.55537928
ATPase, Na+/K+ transporting, beta 3 polypeptide	atp1b1	AV038364	NM_009721	-1.46875229
Mitochondrial ribosomal protein L40	mrpl40	AV093751	NM_010922	-1.43624508
Mitochondrial ribosomal protein L44	mrpl44	BG073050	NM_001081210	-1.35832654
Upregulated during skeletal muscle growth 5	usmg5	AV088589	NM_023211	-1.30340711
Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	nfkb1	AA388801	NM_008689	-1.2534784
Mitochondrial trans-2-enoyl-coa reductase	mecr	BG064053	NM_025297	-1.22625661

Online Table I: Microarray analysis of gene expression, comparing β2-/- vs WT. 35 mitochondrial-related genes were significantly up- or down-regulated with β2-AR ablation (n=9-10/group). Data were analyzed using the mouse transcriptome array and Stanford microarray database software and Significance Analysis of Microarrays (SAM). Gene ontology (GO) was used to characterize up- or down-regulated genes. Genes were considered significantly altered with a p value less than 0.01 by Fisher's exact test. To control for multiple comparisons, we required an estimated false discovery rate of 5%.

Online Table II

	Physiologic fragmentation (exercise)	Pathologic fragmentation (ischemia)
Drp1	↑	↑
Mfn2	–	↓
ROS	–	↑
Δψm	–	↓
Pink1	↓	↑
Parkin	↓	↑
Respiration	↑	↓

Online Table II: Comparison of physiologic and pathologic fragmentation. Physiological fragmentation is characterized by increased mitochondrial fission, enhanced mitochondrial function, no increase in ROS, preserved Δψm and inhibition of mitophagy. In contrast, pathological fragmentation, also characterized by increased mitochondrial fission, is associated with decreased mitochondrial function, increased ROS, decreased Δψm and activation of mitophagy.

Supplemental References

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