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Age and Ischemia Differentially Impact Mitochondrial Ultrastructure and Function in a Novel Model of Age-Associated Estrogen-Deficiency in the Female Rat Heart

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Abstract

Altered mitochondrial respiration, morphology, and quality control collectively contribute to mitochondrial dysfunction in the aged heart. Because myocardial infarction remains the leading cause of death in aged women, the present study utilized a novel rodent model to recapitulate human menopause to interrogate the combination of age and estrogen-deficiency on mitochondrial ultrastructure and function with cardiac ischemia/reperfusion (I/R) injury. Female F344 rats were ovariectomized (OVX) at 15 mo and studied at 24 mo (MO OVX; n=41) vs adult ovary-intact (6 mo; n=41). Temporal declines in estrogen concomitant with increased visceral adipose tissue were observed in MO OVX vs adult. Following *in vivo* coronary artery ligation or sham surgery, state 3 mitochondrial respiration was selectively reduced by age in subsarcolemmal mitochondria (SSM), and by I/R in interfibrillar mitochondria (IFM); left ventricular maximum dP/dt was reduced in MO OVX ($p<0.05$). Elevated cyclophilin D and exacerbated I/R-induced mitochondrial acetylation in MO OVX suggest permeability transition pore involvement and reduced protection vs adult ($p<0.05$). Mitochondrial morphology by TEM revealed an altered time course of autophagy coordinate with attenuated Drp1 and LC3BII protein levels with age-associated estrogen loss ($p<0.05$). Here, reductions in both SSM and IFM function may play an additive role in enhanced susceptibility to regional I/R injury in aged estrogen-deficient female hearts. Moreover, novel insight into altered cardiac mitochondrial quality control garnered here begins to unravel the potentially important regulatory role of mitochondrial dynamics on sustaining respiratory function in the aged female heart.

Keywords

menopause; reperfusion injury; myocardial infarction; mitochondrial quality control; senescence

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Introduction

Myocardial infarction is the leading cause of death in aged females [23], however mitochondrial mechanisms responsible for ischemic injury in the aged estrogen (E_2)-deficient female heart remain incompletely characterized. The ability to clear damaged mitochondria through general autophagy and/or mitophagy following ischemia and reperfusion (I/R) injury has emerged as a critical mechanism impacting the maintenance of mitochondrial respiration with advancing age and disease [82, 61, 53]. Moreover, cardiac mitochondrial subpopulations contribute distinctly to cell death through regulation of energy metabolism and cardiac contraction dependent on cellular location [67, 62, 74, 29]. In contrast to previously published studies in males highlighting age-related defects in interfibrillar mitochondria (IFM) [67, 31, 73, 51, 50, 21, 59, 77], we have previously reported age-dependent functional declines in subsarcolemmal mitochondria (SSM) from females [36]. Importantly, the joint effects of aging and menopausal loss of E_2 influence cardiovascular-related mortality in females, emphasizing the necessity of a more accurate model of female aging used herein [58, 57]. The interactive effects of altered mitochondrial function and morphology on age-associated vulnerability to cardiac cell death is largely unexplored in the female heart.

In the absence of ischemia (I), increased mitochondrial volume is observed with aging in male rodents [80, 16, 18]. Following ischemic periods of 30 min or longer, mitochondria undergo irreversible cell death, hallmarked by severe mitochondrial cristae and matrix disruption [27, 24]. Though the regulation of mitochondrial quality control during I/R injury is incompletely understood [55, 56], activation of mitophagy subsequent to dynamin-like GTPase related protein (Drp-1)-mediated fission is associated with cardioprotection [61, 37]. In this regard, skeletal muscle-specific E_2 receptor (ERα) depletion is associated with enlarged dysfunctional mitochondria, ROS excess and fission/fusion imbalance, in part mediated by defective Drp-1 [72]. The impact of age-associated E_2-deficiency on mitochondrial quality control and function with I/R injury in female animals is currently unknown.

Historically, achieving a precise animal model to recapitulate human menopause has been difficult, primarily due to variations in the onset of reproductive senescence in rodents [54, 48, 8]. Although middle-aged rats (12–14 mo) undergo ―estropause", variable time to anestrous influences the temporal magnitude of E_2 loss in aged ovary intact female rats [34, 35, 60, 33]. As such, the use of ovariectomy (OVX) to model human E_2 loss has been widely used in adult rodents [11]. However, a proteomic study from our laboratory revealed a specific mitochondrial response of adult rats to OVX which fails to mimic age-associated E_2-deficiency [45], as only 6 mitochondrial proteins changed similarly in adult OVX vs aged.

To inform mechanisms of cardiac cell death sensitivity in the aged female rat heart, the present study aimed to determine changes in mitochondrial subpopulation respiration and morphological responses following regional I/R injury using a novel, physiological model of age-associated E_2-deficiency. Here, female rats were OVX at middle age (15 mo) and studied at 22–24 mo (MO OVX) to better simulate systemic effects of the human

menopausal transition, standardize temporal declines in circulating E_2 with age, and assess the combined physiological effect of age-associated E_2 loss. We demonstrate for the first time, a selective cardiac mitochondrial subpopulation response (state 3 respiration) in aged female rats to ischemic insult not previously observed. Additionally, our data suggest that age-associated E_2-deficiency may negatively impact mitochondrial quality control during I/R injury, thereby undermining protective reserves.

Materials and Methods

Animals.

Female Fischer 344 (F344) rats were obtained from the NIA colony at Charles River (Wilmington, MA). Rats were housed in an AAALAC certified facility, exposed to a 12-h light/dark cycle and received a standard laboratory rodent diet (LabDiet) and water *ad libitum*. All animal experimentation described was conducted with approval from the Institutional Animal Care and Use Committee of the Pennsylvania State University. All surgical procedures were carried out under anesthesia (40 mg/kg ketamine, 12 mg/kg xylazine, i.p.), the depth of which was determined by tail reflex prior to intubation and toe pinch during the procedure. If more anesthetic was warranted, 13 mg/kg of ketamine was delivered i.p.; the analgesic buprenorphine (0.3 mg/kg, i.m.) was administered upon anesthetic reversal. Groups studied were adult (5–6 mo, n=41) and MO OVX (OVX at 15 mo and aged to 22–24 mo, n=40). Out of a total number of 90 rats, 81 were included in the study. Criteria for exclusion were age-associated weight loss and morbidity (n=2), small AAR (<30%, n=2) or early death following coronary artery ligation (CAL; n=5; 12% in MO OVX and 2% in adult).

Surgical Ovariectomy.

All rats in the MO OVX group were subjected to bilateral OVX at 15 mo; surgeries were performed by the supplier (Charles River). Blood glucose levels were determined by tail stick (One Touch Ultra). Trunk blood prior to euthanasia was used to assess serum E_2 concentrations by RIA of duplicate samples (Ultra-Sensitive E_2 RIA) with an assay sensitivity of 5 pg/ml. Uterine weight was used to confirm E_2-deficiency.

CAL procedure.

Following intubation, the chest was opened in the third rib space, the left coronary artery was visualized and ligated using 3–0 prolene suture (sham animals did not receive ligation). After 31 min of ischemia, the ligature was released and the myocardium was exposed to variable reperfusion (R) times (10 min, 6 hr, 24 hr). The heart was removed by midline thoracotomy, and perfused with Evan's Blue dye (1%) to demarcate area at risk (AAR). Additionally, visceral adipose tissue (VAT) was removed at time of sacrifice and weighed.

Mitochondrial Isolation.

Mitochondria were isolated from left ventricular (LV) tissue displaying a 30–40% AAR from adult and MO OVX following 31 min ischemia and 10 min reperfusion [66, 28] or sham as previously described [67]. Briefly, the LV was roughly minced in sucrose, tris, EGTA (STE) buffer containing (in mM): 300 sucrose, 10 Tris, and 2 EGTA. The

homogenate was centrifuged in STE containing 0.5% BSA. The supernatant contained the SSM, while the pellet contained the IFM. The pellet was subjected to nagarse digestion (5 mg/g wet weight of LV) and subsequently resuspended in STE following appropriate separation and washing. The final protein concentration of individual mitochondrial populations was determined using the Bradford method as previously described [7].

Mitochondrial Respiration.

Isolated mitochondria were respired using a Clarke-type electrode attached to a YSI oxygraph (Yellow Springs, OH) as described by us previously [36]. Briefly, mitochondria were incubated in a buffer containing (in mM): 125 KCl, 20 MOPS, 10 Tris, 2 $MgCl_2$, 2 KH_2PO_4, 0.5 EGTA, pH 7.2. Complex I respiration was measured in the presence of 2.5 mM α-ketoglutarate + 1 mM malate, while Complex II respiration was measured in the presence of 1 μM rotenone + 2.5 mM succinate; state 3 respiration was initiated by addition of ADP (final concentration 1 mM). Groups studied for mitochondrial isolation and respiration were adult sham (n=8), adult I/R (n=10), MO OVX sham (n=8), and MO OVX I/R (n=7).

Tissue Homogenization.

LV tissue from adult and MO OVX following 31 min ischemia and 6 hr reperfusion [15, 41, 78, 2] or sham were frozen and homogenized by glass-glass grinding and subcellular separation of the cytosolic and mitochondrial fractions was performed using differential centrifugation as described by us previously [34, 83, 46, 65, 44, 35]. All protein concentrations were determined by the Bradford method [7].

Western Blot Analysis.

Western blotting was executed according to well-established procedures in our laboratory [43, 83, 46, 65, 44, 35]. Equal amounts of mitochondrial or cytosolic protein per lane were electrophoresed on Criterion SDS-polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Millipore). After blocking, samples were probed with primary antibodies against cyclophilin D (CypD; AbCam, 1:12,000, 5 μg), SIRT3 (1:12,000, 5 μg), acetylated-lysine (1:1,000, 20 μg), LC3B (1:1,000, 20 μg), and Drp1 (Santa Cruz, 1:1,000, 20 μg). Proteins were visualized using enhanced chemiluminescence (GE Amersham) and densitometry performed using ImageJ. SYPRO Ruby staining (Invitrogen, Grand Island, NY) of the membrane was performed and visually inspected for consistency of protein loading [70]. All antibodies were purchased from Cell Signaling unless otherwise specified. Groups studied for western blotting were adult sham (n=3), adult I/R (n=5), MO OVX sham (n=4), and MO OVX I/R (n=6).

Transmission Electron Microscopy (TEM) tissue sample preparation.

After injection with Evan's blue dye, 1 mm^3 samples were taken from the AAR of adult and MO OVX following 31 min ischemia and 10 min (n=3/group), 6 hr (n=4/group), 24 hr reperfusion (n=2/group) [2], or sham surgery (n=7 in adult, n=8 in MO OVX). Samples were immersed in fixative containing 0.1M cacodylate buffer, 16% paraformaldehyde, and 25% glutaraldehyde; post-fixation in 1% osmium tetroxide was followed by 2% uranyl acetate.

Processed sample blocks were trimmed and sectioned (70 nm) using a Leica Ultracut UCT Microtome (Buffalo Grove, IL, USA) and placed on a copper TEM sample grid. Grids were double stained with uranyl acetate and lead citrate then samples were randomly imaged with a JEOL 1200 EXII TEM (Peabody, MA, USA) and Gatan camera. Mitochondrial area was determined in four randomly chosen longitudinal images per rat, using ImageJ (NIH).

Qualitative Analysis.

Qualitative analysis of mitochondrial ultrastructure was assessed according to a modified scoring system previously described [85] for electron densities, cristae disruption, and myofibrillar disarray. Cristae disruption is characterized by transition from dense tightly packed concentric cristae to the presence of tortuous cristae with matrix clearing. Myofibrillar disarray is characterized by Z-line streaming, increased spacing between myofilaments and distortion of the contractile elements. The following scale was constructed for each image: (+) present 0–25%, (++) 26–50%, (+++) 51–75%, or (++++) 76–100% of the image in 3–4 images/rat.

Left Ventricular Function.

A separate cohort of rats underwent CAL with 55 min ischemia. Following 6 hr of reperfusion, rats were intubated and the right carotid artery was isolated by blunt dissection. A small transverse slit was made and a Millar mikrotip pressure sensor (Colorado Springs, CO) was inserted and advanced into the LV. After a 15 min equilibration time, 15 min of data was collected for the calculation of maximum dP/dt (dP/dt_{max}) and heart rate measurements. Groups studied for LV function were adult sham (n=9), adult I/R (n=4), MO OVX sham (n=9), and MO OVX I/R (n=4).

Statistics.

All data are presented as means ± SEM and analyzed using Sigmastat. Data were compared using ANOVA: either one-way ANOVA (baseline measures only) or two-way ANOVA (age × I/R injury) as indicated. Significant interactions were analyzed with a Tukey post-hoc test. An α level of $p \leq 0.05$ was defined as statistically significant.

Results

Characteristics of adult and MO OVX rats upon euthanasia are displayed in Table 1. Body weight, left ventricular weight (LVW), and VAT weight were significantly increased with age, while uterine weight was significantly reduced (compared to adult; $p<0.001$). Normalized to body weight, uterine weight of MO OVX was 35% that of adult (0.74 vs. 2.09). Furthermore, serum E_2 levels were significantly reduced in MO OVX compared to adult, (12.87±1.84 vs 5.61±1.72 pg/ml; $p<0.01$; n=15 in adult and n=21 in MO OVX) verifying E_2-deficiency. VAT increased ~3-fold in MO OVX compared to adult representing a remarkable similarity to that seen in aging women [22]. Notably, blood glucose levels during the fed state were not different between groups (103.5±1.50 vs 104.8±3.74 mg/dL; n=6 per group).

Protein yield of SSM and IFM following sham or CAL (31 min I / 10 min R) of adult vs. MO OVX is displayed in Fig 1. SSM yield was unaffected by age or I/R. In contrast, IFM yield was significantly reduced by 37% in the MO OVX compared to adult ($p<0.001$) with no additional effect of I/R, indicating a decline in the IFM subpopulation with age-associated E_2 loss unrelated to an ischemic challenge. State 3 respiration rate in SSM (panel A) and IFM (panel B) is displayed in Fig 2. As expected, IFM respired at a higher rate compared to SSM ($p<0.001$). Statistically significant reductions in state 3 respiration energized by either Complex I or Complex II substrates were observed with age in SSM (8%) and with I/R in IFM (13–20%; $p<0.05$). Representative images of consistently obtained ~37% AAR are displayed in Fig 3. LV dP/dt_{max} (A) dP/dt_{min} (B) at baseline and following CAL (55 min I / 6 hr R) are displayed in Fig 4. Advancing age and I/R significantly reduce dP/dt_{max} by 21% and 27%, respectively and dP/dt_{min} by 27% and 30%, respectively ($p<0.05$). Group differences in HR were not observed (data not shown).

Mitochondrial protein levels of cyclophilin D (CypD), SIRT3, and acetylated-lysine following CAL (31 min I / 6 hr R) are displayed in Fig 5. CypD protein was significantly increased by 42% with age independent of I/R injury (panel A; $p<0.001$). SIRT3 was significantly reduced by I/R in adult (panel B; $p<0.05$) and increased by age following I/R ($p<0.001$) leading to an interaction between age and I/R ($p<0.02$). Mitochondrial protein acetylation was also significantly increased in MO OVX by ~200% compared to adult following I/R (panel C; $p<0.01$).

Mitochondrial morphological analysis is displayed in Fig 6. Following CAL with 10 min reperfusion and relative to sham baseline control responses, mitochondrial area increased by 26% in adult ($p<0.001$) but not MO OVX. Increases in mitochondrial area persisted at 6 hr reperfusion in adult, and a 29% increase was observed in MO OVX compared to sham ($p<0.01$). Mitochondrial area decreased in adult at 24 hr reperfusion, reaching similar values observed for the sham only condition. In contrast, mitochondrial area remained increased (16%) in MO OVX at 24 hr reperfusion ($p<0.05$). Qualitative characteristics of electron densities, disrupted cristae, and myofibrillar disarray are displayed in Fig 7 (panel E). Increased prevalence of electron densities began at 10 min and 6 hr reperfusion in adult and MO OVX, respectively. Disrupted cristae were more prevalent and severe in MO OVX under conditions of sham and reperfusion at 10 min and 24 hr post ischemic insult vs adult. Greater myofibrillar disarray was also observed at 6 and 24 hr reperfusion in MO OVX compared to adult. Representative examples of electron densities (panel A), disrupted cristae (panel B), and myofibrillar disarray (panel C) from adult I/R samples are presented in Fig 7. Interestingly, autophagasomes (panel D) were detected in adult I/R, but not MO OVX I/R at 6 hr reperfusion, suggesting altered autophagic temporal responses in aged. Accordingly, mitochondrial Drp1 and cytosolic LC3BII were assessed and are displayed in Fig 8. Drp1, a marker of fission-mediated mitophagy, was significantly increased by I/R in adult (panel A; $p<0.05$). No age effect was observed in sham animals, but the response to I/R resulted in a significant decrease of 39% in MO OVX compared to adult ($p<0.05$) exposing a blunted activation of Drp1 upon I/R with age. Greater increases in LC3BII, a marker of general autophagy, were observed in adult (78%) compared to MO OVX during reperfusion following ischemia (panel B; $p<0.05$). LC3BII is undetectable in sham groups.

Discussion

The current study utilized a novel model to recapitulate human menopause in aged female rats and aimed to characterize, for the first time, cardiac mitochondrial subpopulation respiratory behavior and morphological responses to ischemic stimuli. Our results suggest a phenotype where reductions in both SSM and IFM function may play an additive role in the enhanced susceptibility to cardiac I/R injury in aged E_2-deficient females compared to adults. The main findings are as follows: 1) OVX at 15 mo in rats produced a physiologically timed decline in E_2 similar to that observed in postmenopausal women, 2) age-associated E_2-deficiency significantly reduced state 3 respiration in SSM while IFM were affected more by I/R injury, and 3) enlarged mitochondria with blunted activation of quality control proteins and greater protein acetylation were observed following I/R injury in aged vs adult females. Collectively these results are suggestive of decreased mitophagy and delayed resolution of mitochondrial damage following infarction, providing the first insight into cardiac mitochondrial health following an ischemic insult in a physiological model of age-associated E_2-deficiency.

Previous models used by our lab to evaluate age-associated E_2-deficiency include adult OVX (6 mo), aged (24 mo; ovary intact) or aged OVX (OVX at 23 mo and studied at 24 mo) female rats. Here, we further refine our approach using the MO OVX rat to more closely model human menopause, including increased VAT [47, 84, 81, 22, 42], which directly correlates with increased acute myocardial infarction (AMI) [64]. Over time, increases in abdominal fat area of 3.3-fold are observed in older (70 yrs) vs younger (20 yrs) women [22], which is nearly identical to the magnitude of increase in VAT growth observed here in MO OVX vs adult rats within a similar age context (i.e. 5–6 mo vs 23–24 mo). Previous results from our laboratory indicate a significantly lower increase in VAT with aged ovary intact vs adult (1.7-fold). Therefore, the MO OVX rat may represent more accurate timing of E_2 loss over time and allow for the development of a metabolic environment more consistent with human female aging. Yet, importantly, blood glucose level in the MO OVX rat is normal.

In the absence of ischemia, previous work in male rats is suggestive of IFM-selective dysfunction with senescence [51, 50, 59, 21, 40]. In contradistinction, we provide evidence for a decline in SSM with age-associated E_2 loss, but not IFM respiratory function in females. These findings extend our previous results indicating exacerbated swelling in SSM from the aged female heart [36], and are complimented by unchanged SSM protein content. Following I/R injury, respiratory dysfunction was specific to IFM in adult and aged females, but the long term functional impact *in vivo* is likely greater in aged due to significant reductions in IFM yield in MO OVX. Indeed, greater declines in LV dP/dt_{max} and dP/dt_{min} were observed in MO OVX vs adult following I/R injury. Given our observations that mortality in MO OVX increases by 15%, 20%, and 40% following 6 hr, 24 hr, and 3 days of reperfusion, respectively, compared to adult (data not shown), we speculate that LV function may worsen throughout reperfusion in aged vs adult in part due to IFM loss. Because ischemic injury in males has been reported to disrupt both SSM and IFM subpopulations [52, 12, 14, 13, 49, 39], our findings of an IFM specific-defect in females may also support a previously uncharacterized gender associated phenotype in ischemia-induced mitochondrial

dysfunction. Alternatively, differences amongst studies may arise from use of global vs regional ischemic injury. Here, I/R insult was induced by CAL *in vivo* whereby the full impact of the systemic inflammatory response is captured. To the best of our knowledge, all prior investigations of SSM and IFM respiration following I/R in males utilized a model of global ischemia within the context of the isolated heart [52, 12, 14, 13, 49, 39]. Indeed, unpublished observations from our laboratory indicate that global ischemia in the isolated adult female heart reduces state 3 respiration of both SSM and IFM. An issue deserving of future study includes the physiological relevance of different ischemic models to evaluate the contribution of mitochondrial dysfunction to the natural history of cardiac AMI with aging. Coordinate assessment of SSM and IFM function at varying time points during reperfusion is also indicated.

I/R injury induces calcium overload, ATP depletion, and permeability transition pore (PTP) opening [4, 30] leading to mitochondrial destruction and cell death. Because PTP opening is known to be regulated by CypD [3, 63, 20], I/R-induced reductions in IFM respiration may also be related to the observation that IFM express significantly more CypD compared to SSM in rat heart [5]. Indeed, we observed elevated CypD levels in aged which likely impact PTP opening and overall mitochondrial function *in vivo*. Additionally, deacetylation by SIRT3 can activate complex I [1] and inhibit CypD [76], fostering a cardioprotective milieu. Here, though SIRT3 protein levels were increased by age following I/R, significantly elevated mitochondrial acetylation presumably reflects reduced SIRT3 activity in the aged. Given known reductions in complex I subunits, and therefore reduced NAD+ availability with age [45], we speculate that diminished SIRT3 activity in the presence of elevated CypD may contribute to reduced mitochondrial function in IFM following an ischemic insult. The mechanisms of estrogenic action on mitochondrial function, particularly on SSM vs IFM, remain speculative. Mitochondrial respiration and ATP production are reduced by OVX in both cardiac and skeletal muscle of adult rodents, and normalized by *in vivo* E_2 supplementation [10, 71, 9]. We, among others, have previously demonstrated localization of ERα to cardiac mitochondria [65, 68, 32, 87], with significant reductions in aged vs adult females. Moreover, acute ERα agonism can ameliorate age-associated defects in cardiac SSM respiration [36]. Recently, knock down of skeletal muscle ERα levels was associated with profound reductions in mitochondrial respiratory capacity and aberrations in mitochondrial ultrastructure and dynamics [72], providing compelling evidence for a regulatory role for ER signaling on mitochondrial function. ERα activation is also involved in the transcription of major components of the electron transport chain including subunits of complex I, complex IV, and the ATP synthase [10]. Indeed, a recent proteomic screen conducted by our laboratory illustrated a mechanistic link between age-associated E_2 loss and mitochondrial oxidative phosphorylation proteins [45]. Thus, observed decrements in SSM respiration with age may be influenced by the loss of estrogenic effects on mitochondrial protein expression. Given known structural and functional differences in SSM and IFM [29], it is conceivable that the effects of E_2 are also subpopulation specific.

To inform the relationship between changes in mitochondrial function and ultrastructure with cardiac female aging, we next examined mitochondrial morphology prior to and at varying time points following ischemia. In the absence of ischemia, mitochondrial area did not change with age, and contrasts known volume increases reported previously in aged

male mouse hearts [80, 16, 18]. Increased prevalence of disrupted cristae in aged prior to ischemia is consistent with findings in adult OVX rats [89] and ERα knockout mice [88], raising interesting speculation regarding the role of E_2 signaling on mitochondrial ultrastructure. Here, I/R injury differentially affected the temporal response of mitochondrial morphology in aged vs adult. Though IFM function was similarly reduced in adult and aged following 10 min of reperfusion, increased mitochondrial size was observed in adult only, and likely associated with calcium-induced swelling [6, 69]. At 6 hr of reperfusion, enlarged mitochondria were accompanied by a notable absence of autophagic vacuoles in aged, perhaps indicating dysregulated mitochondrial quality control response in aged females [25, 17, 86]. At 24 hr of reperfusion, adult mitochondria returned to pre-ischemic size while increased mitochondrial area persisted in aged along with a greater prevalence of disrupted cristae. That Drp1-related mitophagy is associated with reduced mitochondrial size in mice under pathological conditions [75] provides an interesting mechanistic link between mitochondrial dynamism and observed changes in mitochondrial area described herein. Alternatively, prolonged mitochondrial enlargement combined with attenuated I/R-related Drp1 and LC3BII expression in aged may reflect impaired fission and aberrant autophagic flux known to foster dysfunctional mitochondrial populations [19, 38, 79, 53]. Future studies are necessary to characterize the potential influence of delayed mitophagy on mitochondrial respiration ultrastructure, and turnover in the aged female heart [82].

Conclusions

Here we utilize a unique model of age-associated E_2 loss in an attempt to better recapitulate the impact of human reproductive senescence and declines in circulating E_2 on cardiac I/R injury in aged females. The findings herein examine effects of the unique milieu produced by aging and estrogen-deficiency on the female heart. Ischemic heart disease remains the number one killer of aging postmenopausal women, and it is well known that strategies such as estrogen replacement have been insufficient to limit cardiac cell death in this population and are in fact, contraindicated [26]. Thus, use of female-specific models that are inclusive of advanced age and diminished estrogen in tandem are pathologically relevant and necessary to characterize the full mechanistic spectrum of ischemic cardiac cell death. Here, the MO OVX model shows promise as a platform to investigate the cellular processes involved in age-related vulnerability to cardiac ischemia/ reperfusion injury, and provide insight into how mechanisms differ from previous studies conducted in aged males. The data presented here also employ a physiological model of regional coronary I/R injury to induce myocardial infarction. Novel findings include adaptive functional responses in SSM and IFM to age and I/R injury in females. Though infarct size was not directly assessed, increases in mitochondrial acetylation, CypD, blunted markers of mitophagy, and altered mitochondrial ultrastructure in MO OVX rats are likely to enhance susceptibility to I/R injury and AMI in the post-menopausal female heart. These results may have important implications for therapeutic interventions and treatment of ischemic heart disease in aged women.

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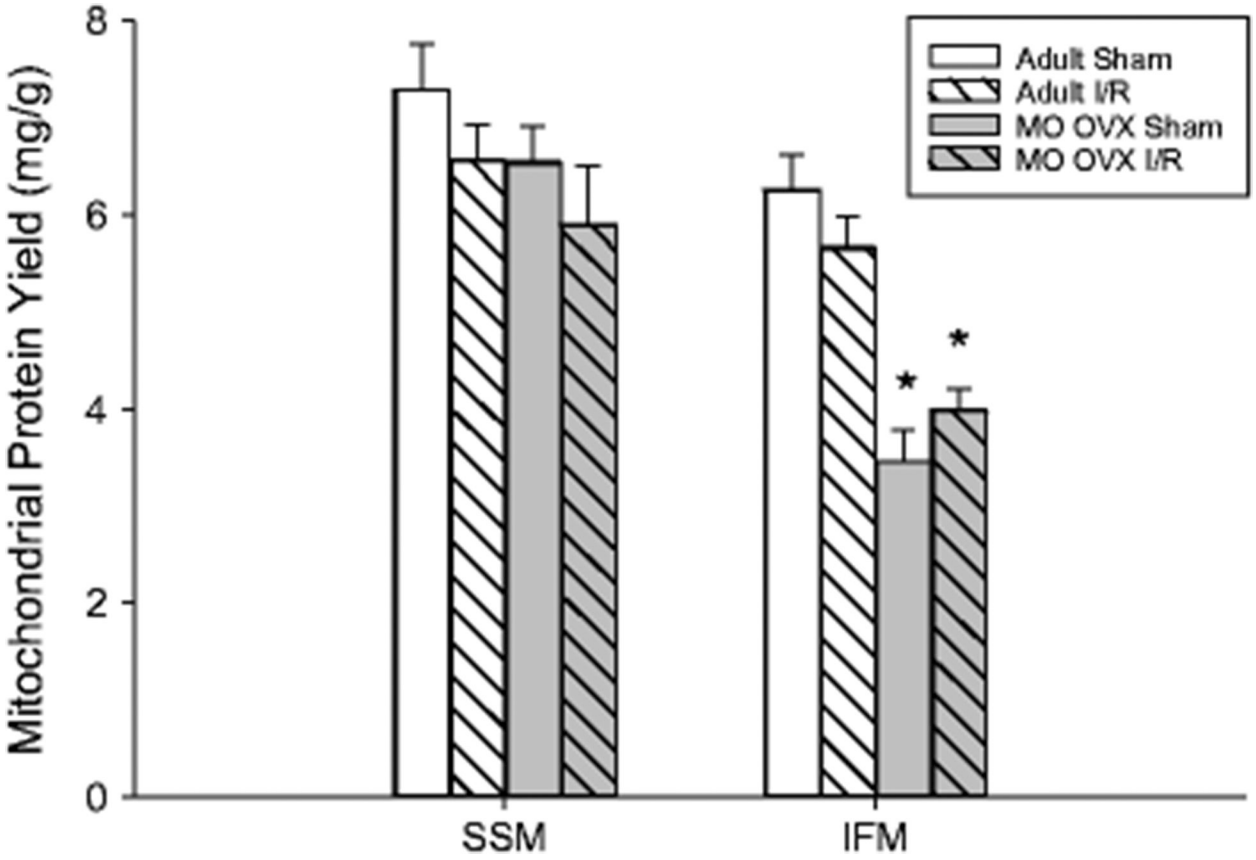


Fig 1:

Protein yield of subsarcolemmal mitoschondria (SSM) and interfibrillar mitochondria (IFM) following isolation from adult and MO OVX female F344 rat left ventricle (LV) following sham or CAL (31 min I/10 min R). Data are presented as a mean ± SEM (n=7–10 per group). * $p<.001$ main effect of age-associated E_2-loss within IFM.

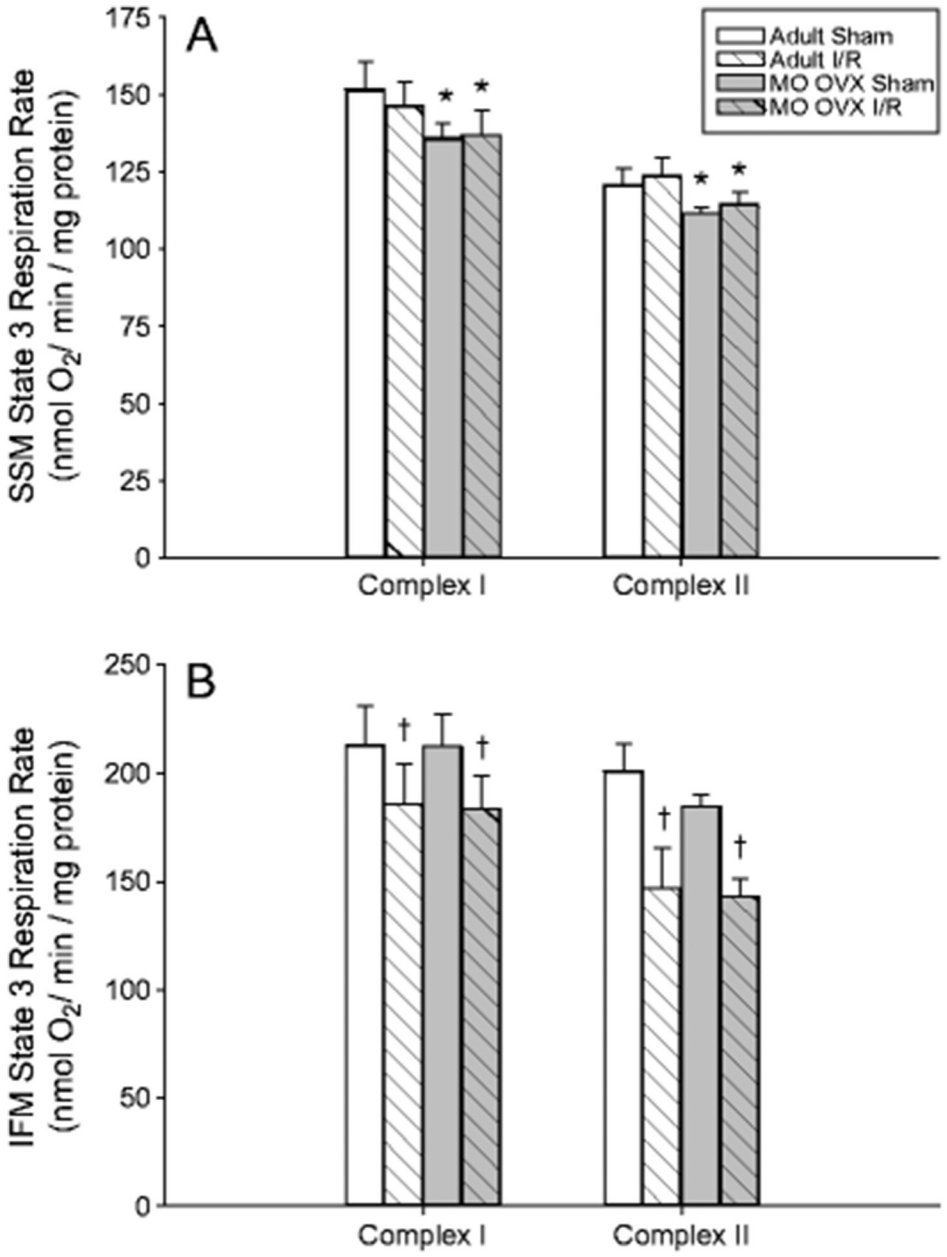


Fig 2.

State 3 mitochondrial respiration rate in adult and MO OVX female F344 rat LV tissue following sham or CAL (31 min I/10 min R). State 3 respiration at Complex I or Complex II in SSM (panel A) and IFM (panel B). Data are presented as a mean ± SEM (n=7–10 per group). * $p<0.05$ main effect of age-associated E_2-loss; † $p<0.01$ main effect of I/R; ‡ $p<.001$ main effect of population.

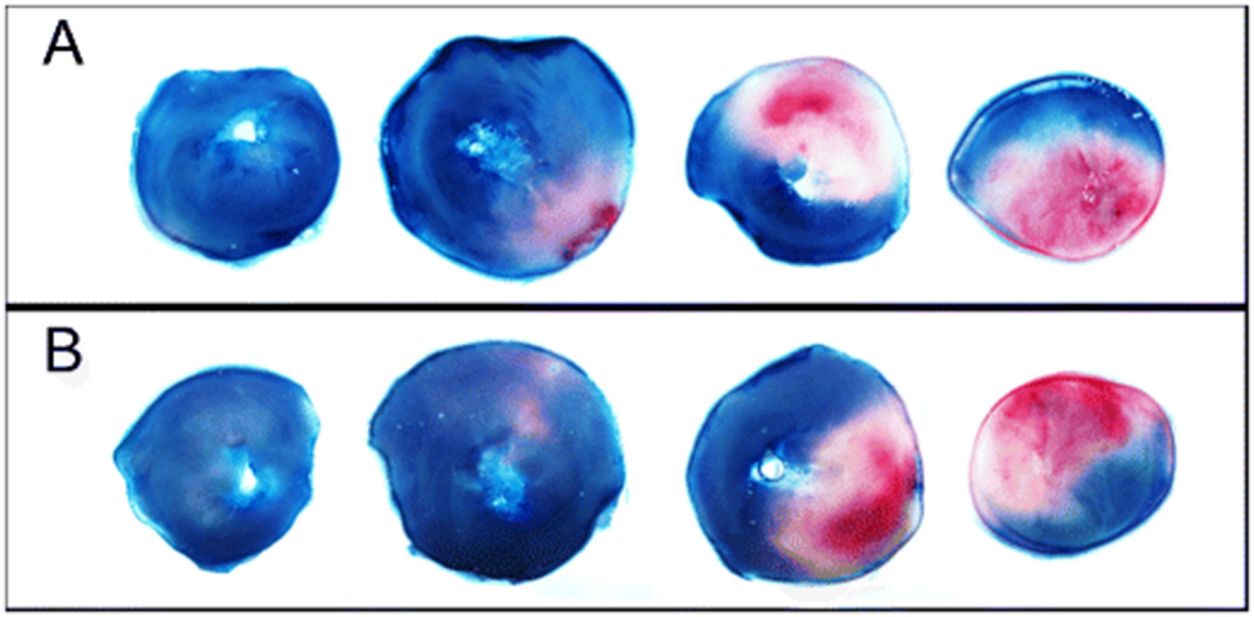


Fig 3.

Representative Evan's Blue staining of adult (A) and MO OVX (B) hearts following CAL demonstrating ~37% AAR.

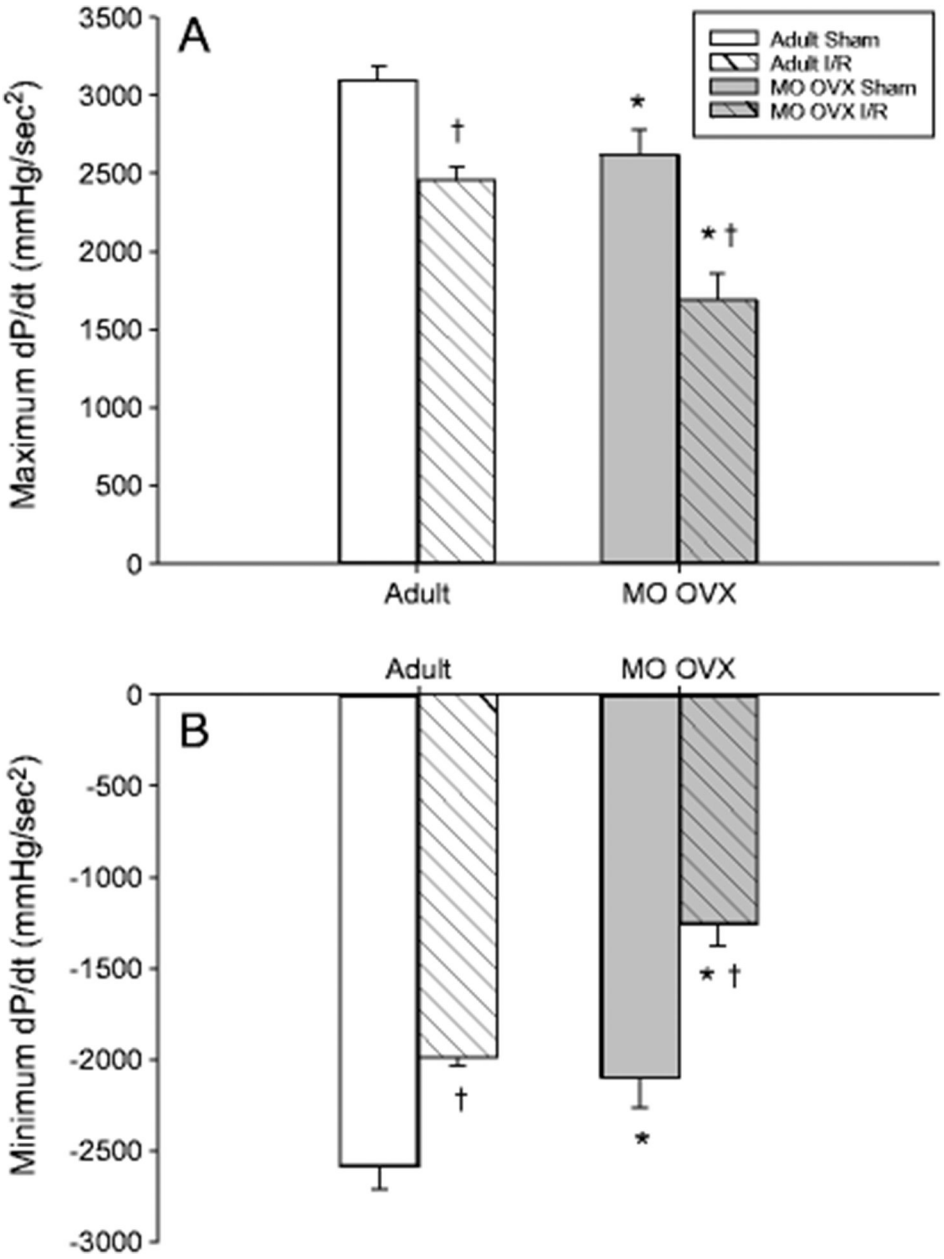


Fig 4.

LV maximum dP/dt measured *in vivo* in sham vs CAL-operated animals (55 min I/6 hr R). Data are presented as a mean ± SEM (n=4–9/group). * $p<0.05$ effect of age-associated E_2-loss; † $p<0.05$ effect of I/R.

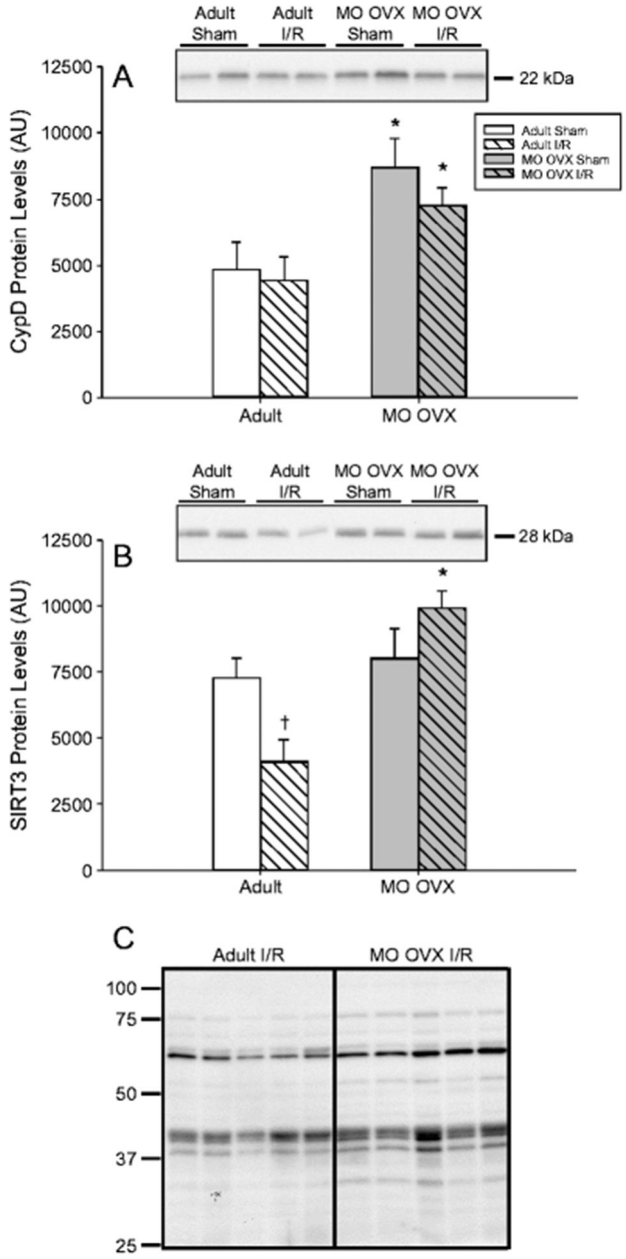


Fig 5.

Mitochondrial protein levels of cyclophilin D (CypD; A), SIRT3 (B) and acetylated-lysine residues (C) in adult and MO OVX F344 rat LV tissue following sham or CAL (31 min I/6 hr R) with representative immunoblots. Data are presented as a mean ± SEM (n=3–6 per group); * $p<0.001$ effect of age-associated E_2-loss within I/R status; † $p<0.05$ effect of I/R within age status; age × I/R interaction in SIRT3 ($p<0.05$).

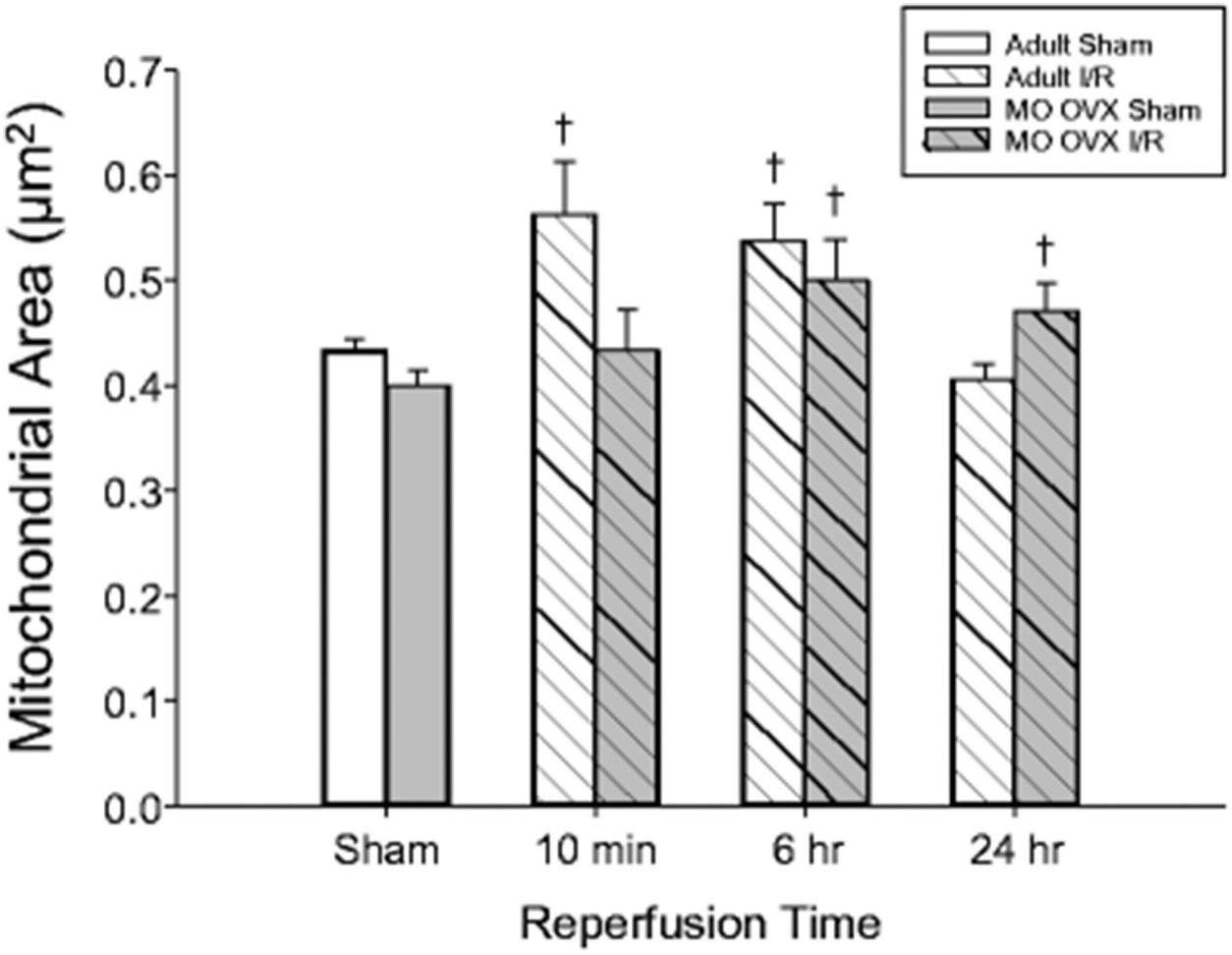
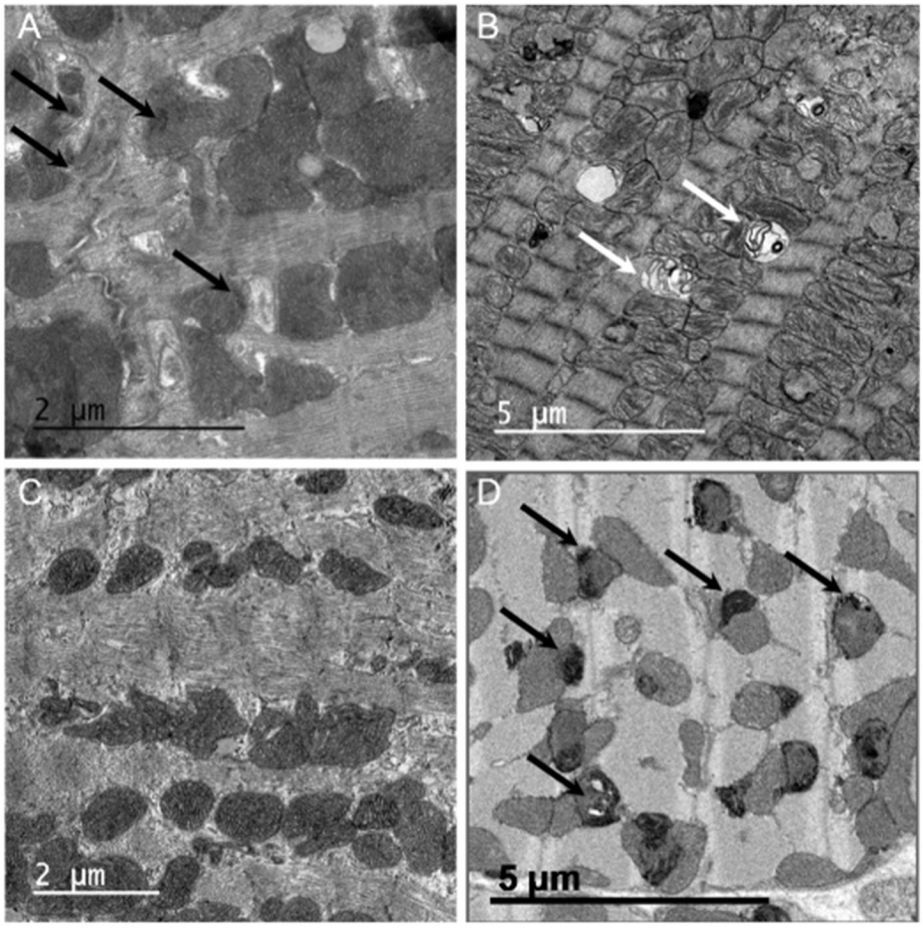


Fig 6.

Mitochondrial area garnered from TEM images from adult and MO OVX LV following sham or CAL (31 min I) and varying R times (10 min, 6 hr, 24 hr). Data are presented as a mean ± SEM (n=7–8 for sham groups and n=2–4 for I/R groups); † $p<.001$ effect of I/R vs sham in adult and $p<0.05$ effect of I/R vs sham in MO OVX.



E

Characteristic	Adult				MO OVX			
	Sham	10 min R	6 hr R	24 hr R	Sham	10 min R	6 hr R	24 hr R
Electron Densities	+	++	++	++	+	+	++	++
Disrupted Cristae	+	+	+++	+	++	+++	+++	++
Myofibrillar Disarray	+	++	++	++	++	++	+++	+++

Fig 7.

Representative TEM images of mitochondrial ultrastructure in an adult heart. Electron densities (black arrows, A), disrupted cristae and matrix clearing (white arrows, B), myofibrillar disarray (C), and autophagic vesicles (black arrows, D). E) The occurrence of ultrastructural components was assessed qualitatively in adult and MO OVX mitochondria at varying reperfusion times; +, observed in 25%; ++, observed in 50%; +++, observed in 75% (n=2–8 per group).

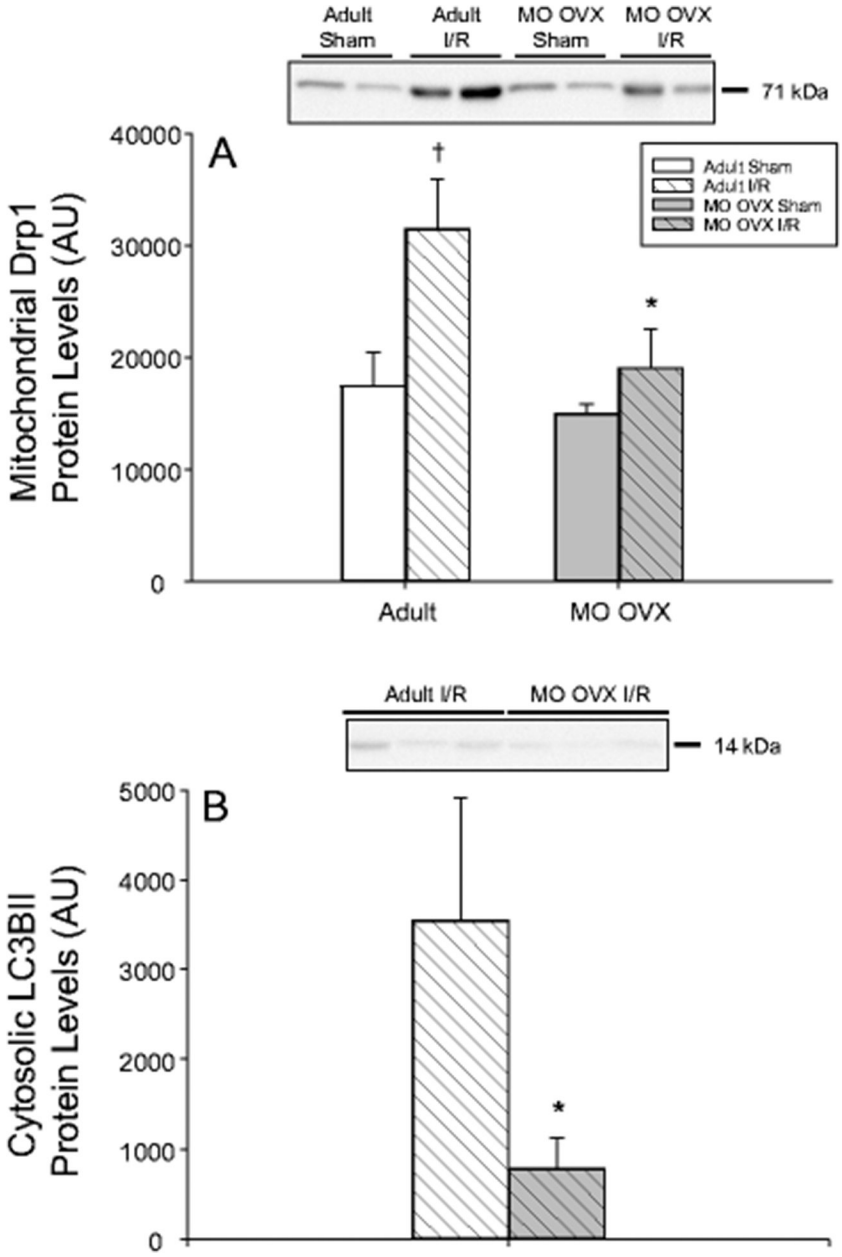


Fig 8.

Protein levels of mitochondrial Drp1 (A) and cytosolic LC3BII (B) in adult and MO OVX F344 rat LV tissue following sham or CAL (31 min I/6 hr R) with representative immunoblots. Data are presented as a mean ± SEM (n=3–6 per group); * $p<0.05$ effect of age-associated E_2-loss within I/R status; † $p<0.05$ effect of I/R within age status.

Table 1.

Physiological characteristics of adult and MO OVX F344 raats that underwent CAL (31 min I and 10 min, 6 hr, or 24 hr R) or sham surgery.

	Adult		MO OVX	
Characteristic	**Sham (n=11)**	**I/R (n=17)**	**Sham (n=12)**	**I/R (n=15)**
Body Weight (g)	203.3 ± 2.90	203.5 ± 2.08	313.5 ± 3.68 *	321.9 ± 5.15 *
LVW (mg)	456.1 ± 4.86	474.7 ± 8.77	617.0 ± 13.8 *	652.5 ± 8.61 *†
Uterine Weight (g)	0.463 ± 0.03	0.399 ± 0.04	0.282 ± 0.05 *	0.213 ± 0.02 *
Uterine Weight/Body Weight (mg/g)	2.280 ± 0.12	1.966 ± 0.21	0.840 ± 0.16 *	0.665 ± 0.06 *
VAT (g)	7.466 ± 0.68	6.664 ± 0.39	17.64 ± 0.77 *	18.93 ± 0.64 *

Results are presented as means ± SEM. MO OVX, 24 mo female rats ovariectomized at 15 mo; LVW, left ventricular weight; VAT, visceral adipose tissue.

* $p<0.001$ effect of age-associated E_2-loss.

† $p<0.05$ effect of I/R within age status.