Published in final edited form as:

Genet Med. 2012 June ; 9(3): 197–206. doi:10.1016/j.genm.2012.04.001.

Age-Dependent Reductions in Mitochondrial Respiration are Exacerbated by Calcium in the Female Rat Heart

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Abstract

Cardiovascular disease mortality increases rapidly following menopause by poorly defined mechanisms. Since mitochondrial function and Ca^{2+} sensitivity are important regulators of cell death following myocardial ischemia, we sought to determine if aging and/or estrogen deficiency (ovx) increased mitochondrial Ca^{2+} sensitivity. Mitochondrial respiration was measured in ventricular mitochondria isolated from adult (6mo; n=26) and aged (24mo; n=25), intact or ovariectomized female rats using the substrates: α -ketoglutarate/malate (Complex I); succinate/rotenone (Complex II); ascorbate/TMPD/Antimycin (Complex IV). State 2 and State 3 respiration was initiated by sequential addition of mitochondria and ADP. Ca^{2+} sensitivity was assessed by Ca^{2+} -induced swelling of de-energized mitochondria and reduction in state 3 respiration. Propylpyrazole triol (PPT) was administered i.p. 45 min prior to euthanasia to assess mitochondrial protective effects through estrogen receptor (ER) α activation. Aging decreased the respiratory control index (RCI; state 3/state 2) for Complexes I and II by 12% and 8%, respectively, independent of ovary status ($p < 0.05$). Of interest, Ca^{2+} induced a greater decrease (18–30%; $p < 0.05$) in Complex I state 3 respiration in aged and ovx animals, and mitochondrial swelling occurred twice as quickly in aged (vs. adult) female rats ($p < 0.05$). Pretreatment with PPT increased RCI by 8% and 7% at Complexes I and II, respectively ($p < 0.05$) but surprisingly increased Ca^{2+} sensitivity. Age-dependent decreases in RCI and sensitization to Ca^{2+} may explain in part the age-associated reductions in female ischemic tolerance; however protection afforded by ER agonism involves more complex mechanisms.

Keywords

estrogen receptors; permeability transition pore; myocardium; senescence

Introduction

Mitochondria are the main source of both ATP and reactive oxygen species (ROS) in the heart ideally positioning them as mediators of, and therapeutic targets for, ischemic cardiovascular diseases (CVD). Several lines of research have shown decreased electron

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transport chain activity at multiple complexes and increased ROS production with age,^{1–6} contributing to an age-dependent decrease in ischemic tolerance evidenced by the increase in CVD prevalence in the aged population.⁷ Although this increase in CVD prevalence is more pronounced in females, with a higher mortality in aged females than males,⁷ the majority of cardioprotection studies have been conducted in adult males.

A final common pathway in cardioprotection is preservation of mitochondrial integrity and ATP production, at least in part by GSK3 β -dependent inhibition of the mitochondrial permeability transition pore (MPTP).^{8,9} The MPTP is a large conductance, Ca²⁺-sensitive pore that allows molecules less than 1.5 kD to non-specifically diffuse between the cytosol and the mitochondrial matrix. Opening of the pore in response to ischemia/reperfusion (I/R)-induced Ca²⁺ overload and ROS production not only dissipates the mitochondrial membrane potential, halting ATP production and further increasing ROS, but also leads to matrix swelling which can rupture the outer mitochondrial membrane releasing pro-apoptotic proteins into the cytosol (10). Direct inhibition of the MPTP using Sanglifehrin A or cyclosporine has been shown to reduce infarction in many models of ischemia/reperfusion (I/R).^{11,12}

Although preserving ATP and limiting ROS production by inhibiting MPTP formation is a common strategy for cardioprotection, these strategies are often less effective in the aged;^{13–15} likely due in part to age-dependent reductions in mitochondrial respiration and disruptions in calcium handling (elucidated in male animals).^{1,5,16,17} Whether similar changes in respiration are present in aged estrogen deficient females, a mechanism potentially contributing to their decreased ischemic tolerance, remains to be determined. Interestingly, supplementation of aged female rats with the estrogen receptor (ER) α specific agonist propylpyrazole triol (PPT) increased mitochondrial localization of ER α and protected the myocardium as effectively as in adult animals (18), indicating that ER α may be an important determinant of mitochondrial-mediated cardioprotection.

To better understand ischemic intolerance in aged female hearts, we recently undertook a proteomic screen of mitochondria from aged, estrogen deficient females. This screen revealed significant reductions of subunits in all five complexes of the electron transport chain suggesting a decreased ability to produce ATP and/or potential for uncoupling of the mitochondria (Lancaster et al., in review). Accordingly, in the present study we examined mitochondrial respiration in hearts from aged, estrogen-deficient rats to identify functional deficits that may be related to these proteomic changes. Given the role of Ca²⁺ overload in I/R damage, we also assessed the baseline sensitivity of mitochondria from aged, estrogen-deficient rats to a calcium challenge. Finally, we investigated whether ER α agonism could abrogate any estrogen deficiency- or age- dependent decrements in respiration or calcium tolerance.

Methods

Animal Care

Female adult (5–6 months, n = 26) and aged (23–24 months, n = 25) Fischer 344 (F344) rats were obtained from the NIA colony at Taconic (Germantown, NY). 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 of the Institutional Animal Care and Use Committee of the Pennsylvania State University.

Surgical Ovariectomy

To yield a controlled level of E₂-deficiency, a subset of adult (5mo; n=13) and aged (22mo; n = 10) F344 rats underwent surgical ovariectomy (OVX); surgeries were performed by supplier. Post-surgery OVX animals recovered for four weeks prior to experimental use and thus were age-matched to intact animals. Uterine weight was used to confirm E₂ deficiency. We have previously documented reduced circulating E₂ levels in aged, and aged OVX rats through radioimmuno-assay.^{18,19}

Mitochondrial Isolation

Rats were anesthetized with 50mg/kg pentobarbital and hearts were excised by midline thoracotomy and secured to a Langendorff apparatus. Following a brief (3min) perfusion with a modified Krebs's-Henseleit buffer containing (in mM): 1.75 CaCl₂, 117.4 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.3 KH₂PO₄, 24.7 NaHCO₃, 11.0 glucose, 0.5 pyruvate, and 0.5 EDTA, hearts were removed from the apparatus, ventricles were isolated and homogenized in 3ml of STE buffer (300mM sucrose, 10mM Tris (pH 7.4), 2mM EGTA (pH 7.4)). Homogenates were diluted in STE buffer supplemented with 0.5% BSA and centrifuged at 2000 × *g*. The resultant supernatant was centrifuged at 10,000 × *g* and the mitochondrial pellet was washed twice in STE buffer prior to resuspension in 300μl STE buffer. Protein concentration was determined by the method of Bradford.²⁰ To assess whether previously demonstrated cardioprotective effects of estrogen receptor α (ERα) agonism were mediated by changes in mitochondrial function, 5μg/kg propylpyrazole triol (PPT; Tocris, Ellisville, MO) was injected subcutaneously into a subset of animals 45min prior to euthanasia; a dose and time point previously shown to be cardioprotective.¹⁸

Mitochondrial Swelling

Measurement of MPTP opening was assessed by calcium-induced swelling of isolated, de-energized mitochondria as described previously.²¹ Briefly, 1mg mitochondrial protein was incubated in 3ml of de-energizing buffer containing (in mM): KSCN 150, MOPS 20, Tris 10, nitriloacetic 2, 0.5μM rotenone, 0.5μM antimycin A, and 2μM of the calcium ionophore A23187, all pH 7.2. The absorbance at 520nm was followed and the rate of decrease following a 200μM CaCl₂ challenge was used as a measure of Ca²⁺-induced MPTP opening.

Mitochondrial Respiration

Respiration of isolated mitochondria was measured at 30°C using a Clarke-type electrode attached to a YSI oxygraph (Yellow Springs, Ohio) as described previously (21). Briefly, mitochondria were incubated in a buffer containing (in mM): 125 KCl, 20 MOPS, 10 Tris, 2 MgCl₂, 2 KH₂PO₄, 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 + 2.5mM succinate; state 3 respiration was initiated by addition of ADP (final concentration 1mM). To assess Ca²⁺ sensitivity, the decrease in complex I state 3 respiration was measured in response to 500 μM Ca²⁺. Complex IV respiration was assessed in series following assessment of complex II in the presence of 0.5 μM antimycin A, 10mM ascorbate, and 0.3mM N,N, N', N'-tetramethyl-p-phenylenediamine (TMPD).

Statistics

All data are presented as means ± SEM and analyzed using Sigmapstat. Data were compared using ANOVA: a 2-way (age x ovary status) for baseline data, a 2-way repeated measures (age x ovary; pre- and post-calcium), or a 3-way (age x ovary x PPT) for PPT effects. In instances where groups were compared to a control group, data was analyzed as a natural

log of the raw data. Significant interactions were analyzed with a Tukey post-hoc test. An α level of $p = 0.05$ was defined as statistically significant.

Results

The effects of age and estrogen deficiency on baseline morphology are displayed in Table 1. As expected, body weight and ventricular weight were significantly increased in aged (compared to adult; $p < 0.05$), however ovariectomy only increased body weight and ventricular weight in adults. Ovariectomy significantly decreased uterine weights in both age groups, indicating estrogen deficiency, although remained significantly higher in the aged OVX rats ($p < 0.05$). Importantly, there was no significant difference detected in mitochondrial protein yield from any group.

Baseline respiration in adult and aged mitochondria is displayed in Figure 1. A representative respiration trace is shown in Figure 1A. State 2 respiration (Figure 1B) was unaffected by age or estrogen deficiency in response to both Complex I and Complex II substrates ($p > 0.05$). There was a tendency for state 3 respiration to decrease with age in the presence of succinate and rotenone ($p = 0.09$) but this decrease failed to reach significance. State 3 respiration was unaffected by age or estrogen deficiency in the presence of α -ketoglutarate/malate or in the presence of TMPD/ascorbate (Figure 1C). However, the ratio of state 3 to state 2 respiration (RCI; Figure 1D) was decreased with age by 12% and 8% at Complex I and II, respectively ($p < 0.01$), suggesting that mitochondria from aged hearts may be less coupled than those from adult.

Given the established role of Ca^{2+} -overload in post-ischemic damage, we sought to determine whether increased damage in the aged and estrogen-deficient heart may be due to an ischemia-independent increase in calcium sensitivity. Addition to $500\mu\text{M}$ Ca^{2+} 1 min after initiation of state 3 respiration of Complex I led to a significant decrease (20–40%; $p < 0.05$) in RCI in all groups 1 min and 3 min after Ca^{2+} addition (Figure 2A,B). This decrease was more pronounced in adult ovariectomized, and aged ovariectomized groups (30% and 24%, respectively, compared with adult control, all $p < 0.05$). To confirm an increased sensitivity to Ca^{2+} , MPTP opening in de-energized mitochondria was assessed by Ca^{2+} -induced swelling. Figure 2C shows that in response to $200\mu\text{M}$ Ca^{2+} , mitochondria from aged rat hearts swelled twice as quickly as those from adult hearts (main effect of age; $p < 0.05$) indicating increased MPTP opening in aged compared to adult hearts.

Since we have previously demonstrated a cardioprotective role of $\text{ER}\alpha$ agonism in adult and aged rats, we sought to determine whether this cardioprotection was attributable to alterations in mitochondrial function and/or Ca^{2+} sensitivity. Figure 3A shows that in response to PPT treatment 45min prior to mitochondrial isolation, a small but statistically significant increase in RCI was observed at both Complexes I and II (ANOVA analysis revealed main effect of PPT indicating 8% and 7% increase, respectively; $p < 0.05$). Interestingly, this translated into an increased sensitivity to Ca^{2+} as assessed by the decrease in Complex I respiration (Figure 3B), but not swelling (data not shown).

Discussion

The increased prevalence of cardiovascular diseases in women following menopause coupled with the failure of hormone replacement therapy to demonstrate cardioprotection, has led many researchers to re-examine mechanisms of cardioprotection and subsequent loss of this cardioprotection in advancing age with decreased estrogen.^{7,22–24} It has become clear that ovariectomizing adult animals may not accurately represent the combined effects of age and estrogen deficiency seen in the postmenopausal female. Since the mitochondria play a

critical role in survival and as mediators of cell death, the current study sought to determine the effects of age and estrogen deficiency on function and calcium sensitivity in mitochondria isolated from female rat hearts. The main findings are as follows: 1) Age significantly reduced RCI at Complexes I and II; 2) Estrogen deficiency and age sensitized the mitochondria to Ca^{2+} -overload; 3) PPT increased mitochondrial RCI but did not improve Ca^{2+} sensitivity.

It is clear that mitochondria play a central role in cardioprotection and research elucidating the mechanisms of this protection is ongoing. Importantly, the vast majority of this research is being performed in adult models, rather than the population at risk for a cardiovascular event, i.e., the aged. Although several groups have investigated changes in basal mitochondrial function with age, the results are variable, likely due to differences in isolation, mitochondrial subfractions, and measurement protocols. Respiration rates have been reported to increase,^{25–26} decrease,^{1–5} or have variable effects depending on individual complexes or mitochondrial subpopulation.^{6,27–29} It is important to note that with the exception of Davies *et al*,²⁵ all of these aging studies were performed on male heart mitochondria which may not necessarily extrapolate to aging in females.

Results of the present study in females show no significant age-dependent changes in state 2 or state 3 respiration of Complexes I, II, IV (state 3 only) which are in agreement with oxygen consumption studies of similar mitochondrial populations in adult and 24 mo rats.^{3,6,29} However, in contrast to these studies, here we report for the first time, age-dependent decreases in the RCI's for Complexes I and II in females, which is consistent with increased mitochondrial uncoupling with aging. Although inhibition of ATP/ADP exchange and/or ATP synthase may also account for decreased RCI, ADP-induced respiration was not significantly decreased. Furthermore, it has been proposed that mitochondrial uncoupling may be a compensatory mechanism sacrificing ATP production efficiency to combat increased reactive oxygen species production seen with aging in tightly-coupled mitochondria.³⁰ Indeed, in a recent proteomic screen of mitochondria from aged and estrogen deficient rat hearts, we uncovered perturbations of several electron transport (ETC) proteins which could upset the stoichiometry of the ETC and contribute to increased ROS production. These changes were coincident with altered levels of the antioxidants catalase and MnSOD (Lancaster *et al*, in review), implying increased ROS production.

In addition to increased ROS production with age, it is well established that cardiac calcium handling is perturbed with senescence and that the aged myocardium is more sensitive to ROS- and Ca^{2+} - induced MPTP opening.^{16,17} The present study confirms that similar to studies in male hearts, increases in Ca^{2+} -sensitivity with aging occur in the female myocardium as evidenced by Ca^{2+} -induced decreases in Complex I respiration and swelling (Figure 2). Interestingly, the age-dependent reductions in Complex I respiration with Ca^{2+} were mirrored in adult mitochondria with ovariectomy, suggesting that estrogen may play a protective role with respect to Ca^{2+} -sensitivity in adult animals. This hypothesis is supported by the observation that female mitochondria accumulate Ca^{2+} more slowly than do male,³¹ and that estrogen supplementation reduces mitochondrial calcium accumulation.³² That ovariectomy did not worsen the age-dependent sensitization to Ca^{2+} in the present study might suggest that age and ovariectomy are sufficient to sensitize the mitochondria to Ca^{2+} to the same degree.

To further explore the role of estrogen on mitochondrial respiratory function and calcium sensitivity, rats were pretreated with the ER α agonist PPT, a treatment we have previously established as conferring cardioprotection to both adult and aged female rats.¹⁸ The finding of the present study that PPT increased RCI confirms previous studies of enhanced

respiration by estrogens^{33,34} and extends these findings to include aged and aged estrogen-deficient animals. Surprisingly however was the observation that following PPT treatment calcium sensitivity, as assessed by blunting of Complex I state 3 respiration, was enhanced by PPT. These findings are in contrast to those of Morkuniene *et al*, in which isolated hearts perfused with 17 β -estradiol were able to accumulate more Ca²⁺ prior to MPTP opening.³² Although the reasons for this discrepancy are unknown, they are likely due at least in part to differences in methodological approaches and estrogen agonists and doses. Of note, in a previous study from this group, Ca²⁺-induced swelling and -decreased state 3 respiration were unaffected by 17 β -estradiol.³⁵ It is also important to note that it is possible that PPT-induced protection from post-ischemic calcium overload relies on an interaction of estrogenic signaling and ischemia for the protection to become manifest. Alternatively, PPT-induced mitochondrial protection may require cytosolic components absent from the isolated mitochondrial preparation used here, which is an area of current research in our lab.

Conclusions

In summary, the results of the current investigation provide evidence that age-dependent reductions in baseline mitochondrial respiration previously documented in male animals also occur in females. These alterations have important ramifications for understanding the increased morbidity and mortality due to ischemic cardiovascular disease seen in post-menopausal females. Furthermore, understanding the functional perturbations that occur in respiration and Ca²⁺ sensitivity with age and estrogen deficiency is necessary to develop effective treatments targeted to the post- or peri-menopausal population.

Acknowledgments

The authors would like to thank Dr. Sabzali Javadov (University of Puerto Rico) for his expert methodological advice. This work was supported by National Heart, Lung, and Blood Institute (RO1 HL091097 (DHK), RO1 HL091097-01A2S1 (DHK)); and National Institute on Alcohol Abuse and Alcoholism (RC2 AA019403 (DHK)) at the National Institutes of Health.

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Figure 1.

Baseline mitochondrial respiration in adult and aged female F344 rat hearts with ovaries intact or following ovx surgery. Respiration of mitochondria isolated from both left and right ventricles (representative tracings are displayed in Panel A) was determined in the absence (state 2, Panel B), and presence (state 3, Panel C) of 1mM ADP with the following substrates: 2.5mM α -ketoglutarate + 1 mM malate (Complex I), 1 μ M rotenone + 2.5mM succinate (Complex II), and 0.5 μ M antimycin A, 10mM ascorbate, and 0.3mM TMPD (Complex IV). The ratio of state 3 to state 2 (RCI) is displayed in Panel D. All values are means $\pm$ SEM from at least 6 independent experiments. * p <0.05 vs. adult within ovary status.

Figure 2.

Calcium sensitivity in mitochondria from adult and aged female F344 rat heart ventricles with ovaries intact or following ovx surgery. 5003M Ca^{2+} was added 1min following initiation of state 3 respiration of Complex I and followed for 4 minutes as indicated in Panel A. Quantification of inhibition (Panel B) is presented as the ratio of RCI in the presence of Ca^{2+} to the RCI in the absence of Ca^{2+} run in parallel and is presented as mean $\pm$ SEM from at least 6 independent experiments. Mitochondrial swelling induced by 200 μM Ca^{2+} (Panel C) is presented as a mean $\pm$ SEM ratio to adult intact group from at least 4 independent experiments. ‡ p<0.05 compared to adult, intact group; ¶ p<0.05 main effect of age.

Figure 3.

Effect of PPT on baseline mitochondrial respiration and calcium sensitivity in mitochondria from adult and aged female F344 rat hearts with ovaries intact or following ovx surgery. PPT (5 μ g/kg SC) was injected 45min prior to isolation of mitochondria from both left and right ventricles. Respiration (Panel A) and Ca²⁺ sensitivity (Panel B) were assessed as described previously. Data are presented as mean $\pm$ SEM ratio to control (non-PPT treated animals of the same age and ovary status; n=3/group). ‡p<0.05 compared to adult ovary intact females; § p<0.05 main effect of PPT.

Table 1**Rat Morphometrics and Mitochondrial Protein Yield**

Characteristic	Adult	Adult OVX	Aged	Aged OVX
N=	13	13	15	10
Body Weight (g)	208.7 ± 2.3	240.9 ± 5.9 [†]	291.3 ± 3.8 [*]	282.3 ± 10.1 [*]
Ventricular Wt (mg)	626 ± 6	679 ± 11 [†]	816 ± 19 [*]	798 ± 21 [*]
Uterine Weight (mg)	516 ± 28	171 ± 43 [†]	602 ± 38 [*]	291 ± 20 ^{†*}
Mitochondrial Protein Yield (mg/g Ventricle)	4.62 ± 0.09	4.48 ± 0.14	4.63 ± 0.22	5.06 ± 0.20

^{*} p<0.05 compared with Adult within ovary status;

[†] p<0.05 vs. ovaries intact within age status