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Bioenergetic consequences of compromised mitochondrial DNA repair in the mouse heart

Kelsey L. McLaughlin [a, b], Joseph M. McClung [a, b], Kelsey H. Fisher-Wellman [a, b, *]

[a] Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, NC, 27834, USA
[b] East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, NC, 27834, USA

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ABSTRACT

The progeroid phenotype of mitochondrial DNA (mtDNA) mutator mice has been nebulously attributed to general mitochondrial 'dysfunction', though few studies have rigorously defined the bioenergetic consequences of accumulating mtDNA mutations. Comprehensive mitochondrial diagnostics was employed to interrogate the bioenergetic properties of isolated cardiac mitochondria from mtDNA mutator mice and wild type littermates. Assessment of respiratory flux in conjunction with parallel measurements of mitochondrial free energy all point to the cause of respiratory flux limitations observed in mtDNA mutator mouse mitochondria being due to impairments within the energy transduction step catalyzed by the electron transport system in which NADH/NAD^+ free energy is transduced to the proton motive force (ΔP). The primary bioenergetic consequence of this limitation appears to be hyper-reduction of NAD(P)H/$NAD(P)^+$ redox poise across multiple substrate conditions, particularly evident at moderate to high respiration rates. This hyper-reduced phenotype appears to result from specific reductions in both complex I and complex IV expression, presumably due to compromised mtDNA integrity. Translation of these findings to the working heart would suggest that the primary biological consequence of accumulated mtDNA damage is accelerated electron leak driven by an increase in electron redox pressure for a given rate of oxygen consumption.

1. Introduction[1]

Although the vast majority of proteins involved in mitochondrial function are encoded within the nuclear genome of the mammalian cell, the mitochondria maintain a separate genome, consisting of thousands of copies of circular mitochondrial DNA (mtDNA). In mice and humans, each double-stranded circle of mtDNA encodes 13 protein subunits of the electron transport system (ETS), including key subunits of both complex I (CI) and complex IV (CIV). This genome is replicated continuously through mechanisms separate from the cell cycle, using a single, unique, nuclear-encoded DNA polymerase (DNA polymerase gamma; Polg) [1]. The catalytic subunit of Polg (Polga) has DNA polymerase, 3′-5′ exonuclease, and 5′ deoxyribose phosphate lyase activities, making it the sole subunit responsible for synthesizing and proofreading new mtDNA.

The importance of preserving the integrity of the mitochondrial genome was first recognized through the identification of human diseases such as Leber's hereditary optic neuropathy, the etiology of which was traced to a specific mtDNA substitution mutation [2]. Later, two separate labs bred transgenic mice overexpressing an exonuclease-deficient version of Polg ($Polg^{m/m}$) in which the catalytic residue of the Polga exonuclease domain was mutated from aspartic acid to alanine (D257A) [3,4]. Loss of proofreading capabilities lead to the accumulation of single base substitution and deletion mutations randomly within the mitochondrial genome of the $Polg^{m/m}$ line, inspiring the name "mtDNA mutator" mouse. The resultant phenotype was akin to accelerated aging, including

* Corresponding author. East Carolina Diabetes and Obesity Institute, 115 Heart Drive, Greenville, NC, 27834, USA.
E-mail address: fisherwellmank17@ecu.edu (K.H. Fisher-Wellman).

[1] Abbreviations: AKGDH, alpha-ketoglutarate dehydrogenase complex; BCKDH, branched-chain keto-acid dehydrogenase complex; BSA, bovine serum albumin; CI, complex I; CIV, complex IV; CV, complex V; CK, creatine kinase; Cr, creatine; $\Delta\Psi$, membrane potential; ΔG_{ATP}, free energy of ATP hydrolysis; DDT, dithiothreitol; ETS, electron transport system; JNAD(P)H, rate of NAD(P)H production; JO_2, oxygen consumption rate; M, malate; mtDNA, mitochondrial DNA; Pc, palmitoyl-carnitine; PCr, phosphocreatine; PDH, pyruvate dehydrogenase complex; Polg, DNA polymerase gamma; $Polg^{m/m}$, homozygous transgenic mtDNA mutator mouse line; Polga, catalytic subunit of Polg; Pyr, pyruvate; R, rotenone; ROS, reactive oxygen species; Succ, succinate.

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premature hair loss and wrinkled skin, osteoporosis, sarcopenia, anemia, cardiomyopathy, presbycusis, and a reduced lifespan [3,4].

In the literature, the $Polg^{m/m}$ phenotype has been nebulously attributed to general mitochondrial 'dysfunction'. Evidence of this 'dysfunction' includes reported abnormalities in the size and shape of the mitochondria [3], reduced maximal oxidative ATP production [3], elevated apoptotic activity [4], and increased production of reactive oxygen species (ROS) [5,6]. In particular, increased ROS generation appears to contribute significantly to the progeroid phenotype of $Polg^{m/m}$ mice, as increasing the redox scavenging capacity of the mitochondria has been shown to lengthen their lifespan, slow the accumulation of mtDNA deletions, as well as rescue some aspects of cardiac function [6,7]. However, no study to date has directly identified the source(s) of ROS in $Polg^{m/m}$ mice, nor sufficiently characterized how an increased frequency of mtDNA mutations may alter the underlying bioenergetic efficiency of $Polg^{m/m}$ mitochondria in favor of accelerated ROS production (i.e., electron leak).

The objective of the present study was to evaluate the bioenergetic properties of isolated cardiac mitochondria from $Polg^{m/m}$ mice using a mitochondrial diagnostics assay platform [8]. The beauty of this platform lies in its ability to provide unbiased diagnostic coverage of a given mitochondrial network at a level approaching that of traditional molecular *omics*. In brief, multiple distinct bioenergetic readouts are produced from a single mitochondrial preparation using a modified version of the creatine kinase energetic clamp. These include mitochondrial respiratory control, membrane potential, NAD(P)H/$NAD(P)^+$, and dehydrogenase flux/enzyme activity. With the entire collection of assays, a given change in respiratory control across disparate mitochondrial populations can be reasonably assigned to one of three main bioenergetic control nodes: 1. Matrix Dehydrogenases, 2. Electron transport system (ETS), 3. ATP synthesis. Additional interrogation within each control node is made possible by comparing results across different substrate combinations (each substrate mix activates a unique set of dehydrogenases and ETS complexes), as well as the additional measurements of dehydrogenase flux and enzyme activity.

2. Materials and methods

All animal studies were approved by the East Carolina University Institutional Animal Care and Use Committee. $Polg^{m/m}$ ($Polga^{D257A/D257A}$) and wildtype (WT) littermates were aged to ~13 months prior to experimentation. All mice were housed in a temperature (22 °C) and light controlled (12 h light/12 h dark) room and given free access to food and water. At the time of tissue harvest, mice were anesthetized with isofluorane, and hearts (complete left and right ventricles) were removed and subjected to mitochondrial isolation.

2.1. Chemical & reagents

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. Tris salts of phosphocreatine (Cat #P1937) and ATP (Cat #A9062) were purchased from Sigma-Aldrich. Potassium pyruvate was purchased from Combi-Blocks (Cat #QA-1116). Potassium $NADP^+$ was purchased from Ark-Pharm (Cat #AK671068). Amplex UltraRed (Cat #A36006), and Tetramethylrhodamine methyl ester (Cat #T6428; TMRM) were purchased from Thermo Fisher Scientific.

2.2. Mitochondrial isolation

Differential centrifugation was employed to prepare isolated mitochondria from heart tissue. The following buffers were utilized for all isolations: Buffer A - MOPS (50 mM; pH = 7.1), KCl (100 mM), EGTA (1 mM), MgSO4 (5 mM); Buffer B - Buffer A, supplemented with bovine serum albumin (BSA; 2 g/L). Hearts were excised and immediately placed in ice-cold Buffer B. All tissues were minced and then homogenized via a Teflon pestle and borosilicate glass vessel. Tissue homogenates were centrifuged at 600 × g for 10 min at 4 °C. Supernatant from each tissue was then filtered through thin layers of gauze and subjected to an additional centrifugation at 10,000 × g for 10 min at 4 °C. Mitochondrial pellets were washed in Buffer A, transferred to microcentrifuge tubes and centrifuged at 10,000 × g for 10 min at 4 °C. Buffer A was aspirated from each tube and final mitochondrial pellets were suspended in 100–200 μL of Buffer A. Protein content was determined via the Pierce BCA protein assay. Functional assays involving isolated mitochondria were carried out in the following buffers: Buffer C - Potassium-MES (105 mM; pH = 7.2), KCl (30 mM), KH_2PO_4 (10 mM), $MgCl_2$ (5 mM), EGTA (1 mM), BSA (2.5 g/L); Buffer D - HEPES (20 mM; pH = 8.0), KCl (100 mM), KH_2PO_4 (2.5 mM), $MgCl_2$ (2.5 mM), Glycerol (1%).

2.3. Preparation of mouse mitochondrial pellets for western blotting

A portion of each mitochondrial preparation was aliquoted and subsequently lysed in CelLytic M (Sigma-Aldrich; C2978) supplemented with protease inhibitor cocktail. The lysate was then separated into two aliquots (one for enzyme activity assays and one for Western blotting). Following centrifugation at 10,000 × g for 10 min at 4 °C, the supernatant from the Western blot lysate was subjected to sonication. Protein concentration was determined and the samples were diluted in Laemmli's loading buffer, supplemented with dithiothreitol (DDT; 5 mM). Thirty micrograms of protein were resolved by SDS-PAGE, transferred to nitrocellulose, blocked for ~1 h in 5% milk prepared with TBS followed by Western blotting with specific antibodies. Antibodies employed herein were: OXPHOS cocktail (abcam: #ab110413). Band intensity was normalized to total protein via the use of TGX Stain-Free precast gels (Bio-Rad).

2.4. Mitochondrial respiratory control

High-resolution O_2 consumption measurements were conducted using the Oroboros Oxygraph-2K (Oroboros Instruments). All experiments were carried out at 37 °C in a 2 mL reaction volume. Steady-state oxygen consumption rates (JO_2) were determined within individual experiments using a modified version of the creatine kinase energetic clamp technique [9,10]. In this assay, the free energy of ATP hydrolysis ($\Delta G'_{ATP}$, depicted throughout the manuscript as ΔG_{ATP}) can be calculated based on known amounts of creatine (Cr), phosphocreatine (PCr) and ATP in combination with excess amounts of creatine kinase (CK) and the equilibrium constant for the CK reaction (i.e., K_{CK}). Calculation of $\Delta G'_{ATP}$ was performed according to the following formula:

$$\Delta G'_{ATP} = \Delta G'^{\circ}_{ATP} + RT \ln \frac{[Cr][P_i]}{[PCr][K'_{CK}]}$$

where $\Delta G'^{\circ}_{ATP}$ is the standard apparent transformed Gibbs energy (under a specified pH, ionic strength, free magnesium and pressure), R is the gas constant (8.3145 J/kmol) and T is temperature in kelvin (310.15). Given that experiments were performed via sequential additions of PCr, both the $\Delta G'^{\circ}_{ATP}$ and K'_{CK} were determined at each titration step based on the changes in buffer ionic strength and free magnesium, as previously described [11,12]. For complete details regarding the calculation of $\Delta G'_{ATP}$ at each

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