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Mitochondrial cardiomyopathies feature increased uptake and diminished efflux of mitochondrial calcium

Salah Sommakia[a,1], Patrick R. Houlihan[b,1,2], Sadiki S. Deane[a], Judith A. Simcox[c], Natalia S. Torres[a], Mi-Young Jeong[c,d], Dennis R. Winge[c,d], Claudio J. Villanueva[c], Dipayan Chaudhuri[a,*]

[a] Nora Eccles Harrison Cardiovascular Research and Training Institute, Cardiology Division, Department of Internal Medicine, University of Utah, Salt Lake City, UT, United States
[b] Department of Cardiology, Boston Children's Hospital, Boston, MA, United States
[c] Department of Biochemistry, University of Utah, Salt Lake City, UT, United States
[d] Department of Internal Medicine, University of Utah, Salt Lake City, UT, United States

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ABSTRACT

Calcium (Ca^{2+}) influx into the mitochondrial matrix stimulates ATP synthesis. Here, we investigate whether mitochondrial Ca^{2+} transport pathways are altered in the setting of deficient mitochondrial energy synthesis, as increased matrix Ca^{2+} may provide a stimulatory boost. We focused on mitochondrial cardiomyopathies, which feature such dysfunction of oxidative phosphorylation. We study a mouse model where the main transcription factor for mitochondrial DNA (transcription factor A, mitochondrial, *Tfam*) has been disrupted selectively in cardiomyocytes. By the second postnatal week (10–15 day old mice), these mice have developed a dilated cardiomyopathy associated with impaired oxidative phosphorylation. We find evidence of increased mitochondrial Ca^{2+} during this period using imaging, electrophysiology, and biochemistry. The mitochondrial Ca^{2+} uniporter, the main portal for Ca^{2+} entry, displays enhanced activity, whereas the mitochondrial sodium-calcium (Na^{+}-Ca^{2+}) exchanger, the main portal for Ca^{2+} efflux, is inhibited. These changes in activity reflect changes in protein expression of the corresponding transporter subunits. While decreased transcription of *Nclx*, the gene encoding the Na^{+}-Ca^{2+} exchanger, explains diminished Na^{+}-Ca^{2+} exchange, the mechanism for enhanced uniporter expression appears to be post-transcriptional. Notably, such changes allow cardiac mitochondria from *Tfam* knockout animals to be far more sensitive to Ca^{2+}-induced increases in respiration. In the absence of Ca^{2+}, oxygen consumption declines to less than half of control values in these animals, but rebounds to control levels when incubated with Ca^{2+}. Thus, we demonstrate a phenotype of enhanced mitochondrial Ca^{2+} in a mitochondrial cardiomyopathy model, and show that such Ca^{2+} accumulation is capable of rescuing deficits in energy synthesis capacity in vitro.

1. Introduction

Heart failure leads to a profound reprogramming of energetic production in cardiomyocytes [1]. As heart failure progresses, energetic supply fails to meet demand, and individuals with more severe supply-demand mismatch have reduced survival [2]. Amongst the pathways altered in heart failure, our interest focuses on calcium (Ca^{2+}), which is central for excitation-contraction coupling, and is increasingly recognized as both an enhancer and inhibitor of mitochondrial function. Upon entering the mitochondrial matrix, Ca^{2+} can stimulate ATP production [3], but excess Ca^{2+} entry disrupts mitochondrial function. Because Ca^{2+} has bimodal effects, deciphering its contribution to energetic homeostasis during heart failure has remained difficult. Perhaps such difficulties arise from the relatively late occurrence of clinically-evident energetic depletion during heart failure [2], with mechanisms regulating mitochondrial Ca^{2+} becoming obscured by other homeostatic pathways triggered during heart failure progression. To identify clear examples of altered mitochondrial Ca^{2+} regulation, we sought a

Abbreviations: AA, antimycin A; CSA, cyclosporin A; ETC, Electron transport chain; IMAC, Inner membrane anion channel; OXPHOS, Oxidative phosphorylation

* Corresponding author at: 95 South 2000 East, Building 500 (CVRTI), Salt Lake City, UT 84108, United States.
E-mail address: d.chaudhuri@utah.edu (D. Chaudhuri).

[1] Contributed equally.

[2] Current address: Howard Hughes Medical Institute, Ashburn, VA, United States.

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disease model that features energetic failure as an *early* phenotype. We expected that early onset of energetic dysfunction during heart failure would allow us to examine mitochondrial Ca^{2+} regulation in a relatively isolated manner.

A spectrum of diseases featuring energetic imbalance arise from a heterogeneous group of mutations in proteins encoded in either the nuclear or mitochondrial genome (mtDNA) that are involved in oxidative phosphorylation (OXPHOS) [4]. These disorders often feature cardiac involvement, termed the mitochondrial cardiomyopathies, and can be devastating, increasing the mortality rates of children suffering from these disorders three-fold [5]. For our studies, such deficient OXPHOS provides a unique opportunity to examine how the heart modulates mitochondrial Ca^{2+} fluxes when energy production is failing. One such mouse model was created by a cardiac-specific deletion of the main transcription factor for mtDNA (transcription factor A, mitochondrial, *Tfam*) [6], leading to depletion of electron transport chain (ETC) subunits encoded by mtDNA. In humans, two infants with TFAM mutations died within months of liver failure, had mitochondrial abnormalities on muscle biopsies, and in one case developed clinical heart failure [7].

In mice, cardiac *Tfam* deletion recapitulates the central problem caused by the heterogeneous group of mutations causing mitochondrial cardiomyopathies: mtDNA depletion and subsequent OXPHOS dysfunction. Multiple studies have revealed that these mice develop cardiac dysfunction with many of the same clinical, biochemical, and ultrastructural features found in human mitochondrial cardiomyopathies, such as an early decline in energetic function and abnormal cytoplasmic Ca^{2+} handling [8–10]. Although these mice have been otherwise characterized, regulation of their cardiac mitochondrial Ca^{2+} remains unexplored.

Studying mitochondrial Ca^{2+} regulation in this model of mitochondrial dysfunction may also provide broader clinical insight, as mitochondrial dysfunction is widespread in heart failure. OXPHOS dysfunction and mtDNA damage are prevalent in dilated cardiomyopathies, and indicate worse prognosis [11–14]. In animal models of ischemic [15], cardiotoxic [16], diabetic [17], tachycardia-induced [18], and pressure-overload [19] cardiomyopathies, reductions in TFAM, mtDNA, and OXPHOS are common. In a proteomic analysis, TFAM was the second-most downregulated protein in failing hearts [20]. Conversely, overexpressing TFAM improves function after cardiac injury [21], and is the subject of therapies targeted towards cardioprotection [22]. However, the late occurrence of clinically evident OXPHOS deficiency has made defining regulatory mechanisms difficult in common forms of heart failure, whereas it drives heart failure in mitochondrial cardiomyopathies.

Here, we study mitochondrial Ca^{2+} transport in the second postnatal week in mice with cardiac-specific *Tfam* deletion. As OXPHOS declines, these animals develop a dilated cardiomyopathy. In these failing hearts, we find that mitochondria become far more Ca^{2+} avid, taking up Ca^{2+} at twice the rate as controls. This occurs via increased activity of the mitochondrial Ca^{2+} uniporter, the main channel transporting Ca^{2+} from cytoplasm to mitochondrial matrix. In addition, mitochondria from these mice release matrix Ca^{2+} at half the rate as controls, indicating reduced activity of the sodium-calcium (Na^{+}-Ca^{2+}) exchanger. The increase in Ca^{2+} influx and reduction in Ca^{2+} efflux reflect corresponding changes in protein levels for the mitochondrial Ca^{2+} uniporter and Na^{+}-Ca^{2+} exchanger. Though the decrease in Na^{+}-Ca^{2+} exchanger levels occurs at the transcript level, the mechanism for increased uniporter function appears to be post-transcriptional, as mRNA levels are reduced despite higher protein amounts. Finally, we find substantial inhibition of mitochondrial respiration in these mice after Ca^{2+} depletion, whereas Ca^{2+} incubation boosts respiration to levels comparable to controls.

2. Methods

Full description of methods is available in the Supplementary materials.

2.1. Animal use

The floxed *Tfam* (a kind gift of Dr. Nils-Göran Larsson) and α-myosin heavy chain-promoter driven Cre recombinase (*Myh6-Cre*) mice (Jackson Labs, Bar Harbor, ME, strain #011038) have been backcrossed into a C57BL/6J background [6,23]. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with institutional guidelines. Animals used were 10–15 days old except where noted.

2.2. Mitochondrial isolation

For all assays, knockout and littermate controls were processed in parallel. Mice were euthanized with CO_2 and hearts rapidly removed. A mitochondrial fraction was subsequently isolated by differential centrifugation [24].

2.3. Mitochondrial enzyme assays

We processed 20 μg of mitochondrial fractions using the Citrate Synthase Activity Kit (Sigma, St. Louis, MO). For subsequent assays of complex I–IV activity, we determined the citrate synthase activity in a sample of *Tfam* heterozygous mitochondria and adjusted amounts of wild-type and knockout littermate mitochondria to match that level. Complex I–IV were analyzed as detailed previously [25], using assay rates in the presence or absence of an inhibitor to determine the complex-specific activity.

2.4. Inductively coupled plasma-mass spectrometry (ICP-MS)

Mitochondria isolated by differential centrifugation were incubated in 40 μM digitonin to permeabilize compartments other than the mitochondrial matrix, prior to final pelleting for analysis. Mitochondria were processed by the ICP-MS metals lab at the University of Utah.

2.5. Fluorescent imaging of mitochondrial inner membrane voltage gradient ($\Delta\Psi$) and Ca^{2+} transport

We performed protocols as previously described using 50 μg of the mitochondrial fractions, except for Ca^{2+}-induced tetramethylrhodamine methyl ester (TMRM) depolarization, where we used 20 μg [26].

2.6. Whole-mitoplast electrophysiology

We prepared mitoplasts from mitochondrial fractions and subsequently performed voltage-clamp analysis using the Kirichok protocol [24].

2.7. Oxygen consumption analysis

Oxygen consumption was determined with a Clark electrode (Hansatech Instruments, Norfolk, UK), or with the fluorescent oxygen probe MitoXpress Xtra (Luxcel Biosciences, Cork, Ireland). For MitoXpress Xtra, we used fluorescence lifetime imaging of mineral oil-sealed wells in a 96-well microplate [27].

2.8. Statistical analysis

Microsoft Excel and R were used for data analysis. We rejected the null hypothesis for P values < 0.05. For multiple comparison tests on