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Cardiac mitochondrial proteome dynamics with heavy water reveals stable rate of mitochondrial protein synthesis in heart failure despite decline in mitochondrial oxidative capacity

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ABSTRACT

We recently developed a method to measure mitochondrial proteome dynamics with heavy water (^2H_2O)-based metabolic labeling and high resolution mass spectrometry. We reported the half-lives and synthesis rates of several proteins in the two cardiac mitochondrial subpopulations, subsarcolemmal and interfibrillar (SSM and IFM), in Sprague Dawley rats. In the present study, we tested the hypothesis that the mitochondrial protein synthesis rate is reduced in heart failure, with possible differential changes in SSM versus IFM. Six to seven week old male Sprague Dawley rats underwent transverse aortic constriction (TAC) and developed moderate heart failure after 22 weeks. Heart failure and sham rats of the same age received heavy water (5% in drinking water) for up to 80 days. Cardiac SSM and IFM were isolated from both groups and the proteins were separated by 1D gel electrophoresis. Heart failure reduced protein content and increased the turnover rate of several proteins involved in fatty acid oxidation, electron transport chain and ATP synthesis, while it decreased the turnover of other proteins, including pyruvate dehydrogenase subunit in IFM, but not in SSM. Because of these bidirectional changes, the average overall half-life of proteins was not altered by heart failure in both SSM and IFM. The kinetic measurements of individual mitochondrial proteins presented in this study may contribute to a better understanding of the mechanisms responsible for mitochondrial alterations in the failing heart.

1. Introduction

Decreased mitochondrial ATP generating capacity in myocardium is a hallmark of heart failure, as evidenced by a decrease in maximal ADP-stimulated oxidative phosphorylation in intact cardiomyocytes or in isolated mitochondria [1–3]. The activity of mitochondrial oxidative enzymes in whole tissue homogenates and the yield of isolated mitochondria are decreased in models of advanced heart failure induced by chronic arterial pressure overload [2,4]. This suggests loss of mitochondrial protein content in failing myocardium, which could be due to lower rates of protein synthesis.

A new method to measure the synthesis rate of individual proteins *in vivo* was recently developed using heavy water (^2H_2O) to label the precursor amino acids and then to assess the time course of ^2H incorporation into newly synthesized proteins using advanced tandem mass spectrometry [5–8]. We recently refined this method to measure the rate of synthesis of proteins in the two spatially distinct subpopulations of cardiac mitochondria: interfibrillar (IFM, located between the myofibrils) and subsarcolemmal (SSM, found along the perimeter of the cell) [9]. In healthy rats, we identified multiple tryptic peptides from 28

Abbreviations: ^2H_2O, heavy water; IFM, interfibrillar mitochondria; SSM, subsarcolemmal mitochondria; TAC, transverse aorta constriction; ETC, electron transport chain; LV, left ventricular; LTQ, linear trap quadrupole; LC-MS/MS, liquid chromatography tandem mass spectrometry; MCAD, medium chain acyl-CoA dehydrogenase; FCR, fractional catabolic rate; FSR, fractional synthesis rate; CID, collisionally induced dissociation.

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[1] Kadambari Chandra Shekar and Ling Li contributed equally to the execution of this study.

[2] This paper is dedicated to the memory of Dr. William C. Stanley.

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proteins in both SSM and IFM, and found time-dependent increases in heavy mass isotopomers that were consistent within a given protein [9]. The rate of protein turnover was relatively slow (average half-life of 30 days, 2.4% per day), and was correlated between IFM and SSM, although it was ~15% lower in SSM than in IFM. This may have particular relevance to mitochondrial dysfunction in heart failure, as previous studies found differential effects of heart failure in SSM and IFM, which could be due to different rates of protein turnover. Several groups found that IFM are more susceptible than SSM to aging, diabetes and heart failure-induced damage [10–15]. However, the differences between the two populations in heart failure are not entirely clear. For instance, some authors described respiratory defects in IFM but not SSM in cardiomyopathic hamsters [16], although this is not a consistent finding [17,18]. Rats with advanced pressure overload- induced heart failure have greater dysfunction in IFM than SSM [4], suggesting a more impaired protein synthesis in IFM than in SSM.

The goal of this investigation was to test the hypothesis that heart failure decreases the rate of protein synthesis and therefore oxidative capacity in mitochondria, with a more pronounced effect in IFM than in SSM. We used a well-established rat model of heart failure induced by chronic aortic constriction, which results in left ventricular remodeling and dysfunction and loss of mitochondrial yield and respiratory function [1,2,4,19]. Protein synthesis was assessed using our previously established method with a heavy water tracer and advanced mass spectrometry, analysis of multiple peptide fragments within proteins of interest, and rigorous exclusion criteria applied across experimental groups and mitochondrial subpopulations.

2. Materials and methods

2.1. Experimental design and surgery

All experiments were performed according to the guidelines provided for the Care and Use of Laboratory Animals by the National Institutes of Health (Publication 85-23) and were approved by the Institutional Animal Care and Use Committee at the University of Maryland Baltimore School of Medicine. Male Sprague Dawley rats (6–8 weeks old, Harlan Laboratories, IN) underwent either sham surgery or transverse aorta constriction (TAC) to induce cardiac hypertrophy and heart failure ($n = 14$/group), as previously described in detail [19]. Briefly, rats were anesthetized with isoflurane, intubated, and a partial median sternotomy was performed to expose the transverse aorta. A tantalum clip (0.5 mm internal diameter) was positioned around the transverse aorta between the brachiocephalic trunk and the left common carotid artery [20]. Sham animals underwent similar surgical procedure without the insertion of the clip. All rats were fed a custom manufactured standard diet (Research Diets, New Brunswick, NJ, USA) and maintained for 22 weeks post-surgery and then euthanized to obtain cardiac mitochondria. Drinking water was supplemented with 5% heavy water (^2H_2O; Sigma-Aldrich, St. Louis, MO, USA) for either 0, 3, 10, 20, 40, 60 and 80 days before euthanasia ($n = 2$ rats per time point). Food and water were provided ad libitum. Pooled mitochondria from two sham and heart failure rats at each time points were used for isolation of SSM and IFM sub-populations and proteomics studies. Thus, $n = 7$ samples per group were analyzed by mass spectrometry. Since only negligible ^2H-labeling was detected after 3 days of ^2H_2O exposure, this time point was excluded from the kinetic analysis.

2.2. Echocardiographic measurements

Left ventricular chamber size and function were assessed 21 weeks after surgery by echocardiography using a high-resolution imaging system (Vevo 2100 High-Resolution Imaging System, MS250 transducer, VisualSonics Inc., Toronto, Canada). Animals were anesthetized with isoflurane (1.5%), and images were acquired with rats in the supine position on a warming platform and analyzed as previously described [21].

2.3. Tissue harvest and mitochondrial isolation

Cardiac tissue was harvested at 22 weeks post-surgery. Fed animals were anaesthetized with 5.0% isoflurane between 3 and 6 h after initiation of the light phase. The thorax was opened and blood was collected from the left ventricle, immediately placed on ice, and centrifuged to obtain plasma. The heart was removed, and a section of the left ventricle free wall was immediately frozen in liquid nitrogen and stored at −80 °C for later assessment of mitochondrial enzyme activities in whole tissue. The remainder of the left ventricle was used for mitochondrial isolation. The two subpopulations of mitochondria, SSM and IFM, were isolated as described previously in detail [22] with a minor modification [23]. Briefly, SSM was released by the treatment of the heart with Polytron homogenizer. To release IFM, trypsin (5 mg/g wet heart weight) was added to the remaining tissue. After rapid homogenization and centrifugation the supernatant containing the IFM fraction was isolated. To prevent protease digestion of IFM proteins, trypsin activity was immediately stopped by the soybean trypsin inhibitor (2.5 mg/g wet weight). IFM and SSM samples were stored at −80 °C for later analysis.

Mitochondrial protein content was determined by the Lowry method using bovine serum albumin as a standard [24].

2.4. Assessment of mitochondrial function

Mitochondrial respiration was measured as previously described in detail [25,26]. Briefly, 0.2 mg/ml of mitochondrial proteins were suspended in a respiration buffer containing 100 mM KCl, 50 mM MOPS, 5 mM KH_2PO_4, 1 mg/mL BSA/Fraction V and 1 mM EGTA. State 3 (ADP-stimulated) and 4 respiration (ADP-limited) were measured at 37 °C with glutamate + malate (10 mM and 5 mM respectively), and palmitoylcarnitine (40 μM). Succinate with Rotenone (20 mM and 7.5 μM respectively) was used to assess respiration through Complex II of the ETC exclusively. State 3 respiration was measured in the presence of 200 μM ADP. State 4 respiration was assessed after ADP consumption. Respiratory Control Ratio, the ratio of State 3 to State 4 was calculated to assess the control of oxygen consumption by phosphorylation. The ratio of ADP added in the chamber to the total amount of oxygen consumed in state 3 (ADP:O ratio) was calculated as an index of the efficiency of oxidative phosphorylation.

The maximal activities of the mitochondrial citric acid cycle enzyme citrate synthase and the fatty acid β-oxidation enzyme medium chain acyl-CoA dehydrogenase (MCAD), markers of mitochondrial content, were measured in whole tissue homogenates as previously described [27]. Activities were normalized to the wet mass of tissue. In addition, citrate synthase activity was measured in isolated IFM and SSM so that the extraction of mitochondria from the myocardium could be measured.

2.5. Protein separation and sample preparation

Deuterium enrichment of plasma water was assessed by ^2H-acetone exchange method previously described [9]. Cardiac mitochondrial proteins from two rats at each time point pooled together and were separated using one-dimensional gel electrophoresis using gradient gels (Biorad, Hercules, CA, USA) with 4%–12% bis–tris gels. Initial protein concentration was measured using Lowry's method of protein estimation with bovine serum albumin (BSA) as a standard. Each gel lane was loaded with 20 μg of SSM or IFM mitochondrial protein. 1D gel electrophoresis using NuPAGE running buffer (Invitrogen, Grand Island, NY, USA) was carried out for 90 min at 130–150 V and 20A and further overnight gel staining was performed by incubating the gel with Coomassie brilliant blue dye at 4 ºC.

2.6. Proteome dynamic analysis

For the analysis of protein synthesis rates and half-lives, we followed a protocol similar to the one in our previous study [9], with minor