Biochimica et Biophysica Acta 1807 (2011) 1106–1113

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

OXPHOS susceptibility to oxidative modifications: The role of heart mitochondrial subcellular location

Ana Isabel Padrão [a], Rita M.P. Ferreira [a], Rui Vitorino [a], Renato M.P. Alves [a], Maria João Neuparth [b,c], José Alberto Duarte [b], Francisco Amado [a,*]

[a] QOPNA, Chemistry Department, University of Aveiro, Aveiro, Portugal
[b] CIAFEL, Faculty of Sport, University of Porto, Porto, Portugal
[c] CITS, IPSN, CESPU, Famalicão, Portugal

ARTICLE INFO

Article history:
Received 19 February 2011
Received in revised form 11 April 2011
Accepted 13 April 2011
Available online 1 May 2011

Keywords:
Intermyofibrillar mitochondria
Subsarcolemmal mitochondria
2-D BN-PAGE/MS/MS
3-Nitrotyrosine
Carbonylation
Respiratory chain complexes

ABSTRACT

In cardiac tissue two mitochondria subpopulations, the subsarcolemmal and the intermyofibrillar mitochondria, present different functional emphasis, although limited information exists about the underlying molecular mechanisms. Our study evidenced higher OXPHOS activity of intermyofibrillar compared to subsarcolemmal mitochondria, paralleled by distinct membrane proteins susceptibility to oxidative damage and not to quantitative differences of OXPHOS composition. Indeed, subsarcolemmal subunits of respiratory chain complexes were more prone to carbonylation while intermyofibrillar mitochondria were more susceptible to nitration. Among membrane protein targets to posttranslational modifications, ATP synthase subunits alpha and beta were notoriously more carbonylated in both subpopulations, although more intensely in subsarcolemmal mitochondria. Our data highlight a localization dependence of cardiac mitochondria OXPHOS activity and susceptibility to posttranslational modifications.

1. Introduction

Essential mechanisms of energy production, signaling, biosynthesis and apoptosis are contained within mitochondria, and their orchestration plays a determinant role in cell physiology [1,2]. Since mitochondria generate between 80% and 90% of all ATP produced in the cell, it is understandable that in tissues like the cardiac muscle these organelles occupy 20–30% of the cell volume, having mitochondrial function, or dysfunction, a critical role in the performance of this tissue [3]. In cardiomyocytes, like in skeletal muscle fibers, there is the added dimension of two mitochondria subpopulations located in different regions of the cell: one abuts the sarcolemma (subsarcolemmal mitochondria, SS), and the other is trapped within the contractile apparatus (intermyofibrillar mitochondria, IMF) [4–6]. These mitochondrial subpopulations possess different properties, which may contribute to their distinct capacities for adaptation to different stimuli [4,6–10]. For instance, previous studies have reported higher respiratory rates in IMF compared to SS in cardiac tissue [6,7,11], which might be related with the levels of the unwanted by-products of inefficient electron transfer within mitochondria [10,12]. As major sources of reactive oxygen and nitrogen species (RONS), mitochondria themselves, and particularly oxidative phosphorylation (OXPHOS) complexes, are especially susceptible to oxidative and nitrative damage [2,10,12]. Protein carbonylation is considered to be a major form of protein oxidation, being widely detected namely by Western blot immunoassay [13]. Among the main carbonyl products of metal-catalyzed oxidation of proteins are glutamic and aminoadipic semialdehydes [15]. The assay of these compounds involves derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), with formation of dinitrophenyl (DNP) hydrazone product. DNPH also reacts with sulfenic acid, resultant from cysteine oxidation, a modification that regulates protein function [13]. Western blot is also used for the search of 3-nitrotyrosine modification, a hallmark of reactive nitrogen species (RNS), generally associated with impaired functional and/or structural integrity of target proteins [14]. With the advancements in proteomics technology it is now possible to identify relevant protein targets of such posttranslational modifications (PTMs) in pathophysiological conditions like aging [16,17] or diabetes [7].

Although it has been verified that mitochondria subpopulations have different functional emphases [6,10,11,18], the molecular mechanisms underlying their discrete properties in cardiac tissue are still poorly understood. As mitochondria comprise a primary locus for the formation and reactions of RONS [10,12], functional differences

Abbreviations: 2D-BN-PAGE, two-dimensional blue native polyacrylamide gel electrophoresis; Anti-DNP, anti-2,4-dinitrophenylhydrazone; Anti-3-NT, anti-3-nitrotyrosine; OXPHOS, oxidative phosphorylation; ETC, electron transport chain; IMF, intermyofibrillar mitochondria; SS, subsarcolemmal mitochondria

* Corresponding author at: Chemistry Department, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal. Tel.: +351 234370700; fax: +351 234370084.
E-mail address: famado@ua.pt (F. Amado).

0005-2728/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2011.04.002

among heart mitochondrial subpopulations' properties should be, at least partially, a consequence of the distinct susceptibility of IMF and SS mitochondrial proteins to oxidative modifications. In order to verify our assumption, fractions of SS and IMF mitochondria were isolated from heart tissue from Wistar rats following a methodological procedure recently described for skeletal muscle [18], which guarantees the high purity of mitochondrial subpopulations obtained.

2. Materials and methods

2.1. Chemicals

All reagents were obtained from Sigma-Aldrich (St. Louis, USA), unless otherwise specified. Mouse monoclonal anti-3-nitrotyrosine antibody was obtained from Chemicon (Temecula, CA, USA), rabbit polyclonal anti-DNP antibody was obtained from DakoCytomation (Hamburg, Germany), anti-ATPB antibody (cat. no. ab14730) was obtained from Abcam (Cambridge, UK) and secondary peroxidase-conjugated antibodies (anti-mouse IgG and anti-rabbit IgG) were obtained from GE Healthcare (Buckinghamshire, UK).

2.2. Animals

Adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) with an age of 12–16 weeks were used in these experiments. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*. Twenty-five rats weighing approximately 300 g were housed in collective cages before use. Out of these 25 animals, groups of 5 rats were sacrificed at different points of time within 2 weeks to have their heart muscle pooled for further analysis. All samples were used to separate their mitochondria subpopulations.

2.3. SS and IMF mitochondria isolation

Mitochondrial populations were purified from heart muscle following the protocol described by our group for skeletal muscle [18], which is based on tissue mechanical treatment and enzymatic digestion as previously reported by Palmer et al. [11]. In brief, cardiac tissue was minced with scissors in homogenization buffer (250 mM sucrose, 10 mM Tris–HCl, 0.1 mM EGTA (pH 7.4)), supplemented with 2 mM PMSF and 0.25 mg/ml trypsin (Promega, Wisconsin, USA). Following 10 minof incubation on ice, albumin fat-free was added to a final concentration of 10 mg/ml. The tissue was subsequently rinsed three times with buffer and then homogenized with a Potter homogenizer (Teflon pestle). Large cellular debris and nuclei were pelleted by centrifuging for 5 min at 1000×*g*. A mitochondria enriched fraction was obtained by centrifuging the supernatant for 20 min at 16,000×*g* and ressuspending the pellet in a small volume of homogenization buffer. Pure mitochondrial subpopulations were then obtained by ultracentrifugation at 95,000×*g* for 30 min on a density-gradient with 50% (v/v) Percoll. Two brown bands were observed; the lower band corresponded to IMF mitochondria while the upper band corresponded to SS mitochondria [18]. The mitochondrial fractions were then washed twice and were aliquoted for subsequent biochemical analysis. Protein content was determined with RC DC Protein Assay kit (Bio-Rad, Hercules, CA, USA).

2.4. Respiratory chain complexes activity

Mitochondrial fractions were disrupted by a combination of freeze–thawing in hypotonic media (25 mM potassium phosphate, pH 7.2) to allow free access to substrates for all assays [19]. Complex I activity was measured by following the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm for 4 min, after which rotenone was added and the absorbance was measured again for 4 min[20]. Complex II activity was determined according to Birch-Machin et al. [21]. In brief, the enzyme catalyzed reduction of DCIP was followed at 600 nm for 3 min after addition of 65 μM ubiquinone. The antimycin A-sensitive complex III activity was assayed at 550 nm as described [16]. The specific activity of cytochrome *c*oxidase was measured by following the oxidation of cytochrome *c* (II) at 550 nm [21]. ATP synthase activity was measured according to Simon et al. [22]. The phosphate produced by hydrolysis of ATP reacts with ammonium molybdate in the presence of reducing agents to form a blue-colour complex, the intensity of which is proportional to the concentration of phosphate in solution. Oligomycin was used as an inhibitor of mitochondrial ATPase activity.

2.5. Blue-native PAGE separation of mitochondria membrane complexes

BN-PAGE was performed using the method described by Schagger and von Jagow [23] with minor modifications. Mitochondria (400 μg of protein) from each subpopulation were pelleted by centrifugation at 20,000×*g* for 10 min and then resuspended in solubilization buffer (50 mM NaCl, 50 mM Imidazole, 2 mM ε-amino n-caproic acid, 1 mM EDTA pH 7.0) with 1% (w/v) digitonin. After 10 min on ice, insoluble material was removed by centrifugation at 20,000×*g* for 30 min at 4 °C. Soluble components were combined with 0.5% (w/v) Coomassie Blue G250, 50 mM ε-amino n-caproic acid, 4% (w/v) glycerol and separated on a 4–13% gradient acrylamide gradient gel with 3.5% sample gel on top. Anode buffer contained 25 mM Imidazole pH 7.0. Cathode buffer (50 mM tricine and 7.5 mM Imidazole pH 7.0) containing 0.02% (w/v) Coomassie Blue G250 was used during 1 h at 70 V, the time needed for the dye front reach approximately one-third of the gel. Cathode buffer was then replaced with one containing only 0.002% (w/v) Coomassie Blue G250 and the native complexes were separated at 200 V for 4 h at 4 °C. A native protein standard HMW-native marker (GE Healthcare, Buckinghamshire, UK) was used. The gels were stained with Coomassie Colloidal for protein visualization and scanned with Gel Doc XR System (Bio-Rad, Hercules, CA, USA). Band detection, quantification and matching were performed using QuantityOne Imaging software (v4.6.3, Bio-Rad).

2.6. In-gel activity of complexes IV and V

The in-gel activity and histochemical staining assays of complexes IV and V were determined using the methods described by Zerbetto et al. [24] with minor modifications. Complex IV-specific heme stain in BN-PAGE gels was determined using 10 μl horse heart cytochrome *c* (5 mM) and 0.5 mg diaminobenzidine (DAB) dissolved in 1 ml 50 mM sodium-phosphate, pH 7.2. The reaction was stopped by 50% (v/v) methanol, 10% (v/v) acetic acid, and the gels were then transferred to water. ATP hydrolysis activity of complex V was analyzed by incubating the native gels with 35 mM Tris, 270 mM glycine buffer, pH 8.3 at 37 °C, that had been supplemented with 14 mM $MgSO_4$, 0.2% (w/v) $Pb(NO_3)_2$, and 8 mM ATP. Lead phosphate precipitation that is proportional to the enzymatic ATP hydrolysis activity was stopped by 50% (v/v) methanol (30 min), and the gels were then transferred to water.

2.7. 2-D BN-PAGE separation of respiratory complexes subunits

Each lane of the first dimension gel was excised and was subsequently incubated in equilibration buffer (2% (w/v) SDS, 6 M urea, 30% glycerol, 0.05 M Tris–HCl pH 8,8 and 20 mg/ml DTT) for 30 min at room temperature to induce dissociation of the protein complexes. Next, the lanes were placed on top of a 4% stacking SDS gel polymerized over a 5–20% gradient SDS-PAGE (18×16 cm) and this gel was run at 100 V during 1 h and then at 200 V during 3–4 h. Gel was stained with Coomassie Colloidal for protein visualization or was blotted onto a nitrocellulose membrane (Whatman®, Protan®) for subsequent 3-nitrotyrosine and carbonyl groups detection by