Biochimica et Biophysica Acta 1832 (2013) 2057–2067

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Differential expression and glycative damage affect specific mitochondrial proteins with aging in rat liver

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ARTICLE INFO

Article history:
Received 15 March 2013
Received in revised form 19 July 2013
Accepted 22 July 2013
Available online 30 July 2013

Keywords:
Aging
Mitochondria
Proteomics
2D-DIGE
Glycation
Protein expression

ABSTRACT

Aging is accompanied by the gradual deterioration of cell functions. Particularly, mitochondrial dysfunction, associated with an accumulation of damaged proteins, is of key importance due to the central role of these organelles in cellular metabolism. However, the detailed molecular mechanisms involved in such impairment have not been completely elucidated. In the present study, proteomic analyses looking at both changes at the expression level as well as to glycative modifications of the mitochondrial proteome were performed. Two-dimensional difference gel electrophoresis analysis revealed 16 differentially expressed proteins with aging. Thirteen exhibited a decreased expression and are crucial enzymes related to OXPHOS chain complex I/V components, TCA cycle or fatty acid β-oxidation reaction. On the other hand, 2 enzymes involved in fatty acid β-oxidation cycle were increased in aged mitochondria. Immunodetection and further identification of glycated proteins disclosed a set of advanced glycation endproduct-modified proteins, including 6 enzymes involved in the fatty acid β-oxidation process, and 2 enzymes of the TCA/urea cycles. A crucial antioxidant enzyme, catalase, was among the most strongly glycated proteins. In addition, several AGE-damaged enzymes (aldehyde dehydrogenase 2, medium chain acyl-CoA dehydrogenase and 3-ketoacyl-CoA dehydrogenase) exhibited a decreased activity with age. Taken together, these data suggest that liver mitochondria in old rats suffer from a decline in their capacity for energy production, due to (i) decreased expression of OXPHOS complex I/V components and (ii) glycative damage to key fatty acid β-oxidation and TCA/urea cycle enzymes.

1. Introduction

Aging is an intricate phenomenon characterized by gradual deterioration of cell functions [1,2] associated with accumulation of damaged macromolecules, particularly proteins [3]. Several lines of evidence suggest that mitochondria play a key role in this age-related cellular dysfunction, as these organelles are the site of numerous vital anabolic and catabolic pathways; that is, they play a central role in cell metabolism [4]. ATP generation via oxidative phosphorylation (OXPHOS) is the most prominent metabolic process, but also their involvement in cellular signaling, including control of apoptosis, is well established [5–7]. Malfunctions in these metabolic and signaling reaction cascades may severely affect cellular homeostasis, hence playing a central role during the aging process. It is generally believed that dysfunction in mitochondria has a causative role in aging; that is, they are triggering key steps in this process [8–10]. Importantly, these organelles are both a major source of intracellular reactive oxygen species (ROS) and the most adversely affected organelles during aging [8,11]. Indeed, studies on isolated mitochondria have shown that about 0.2% of the total oxygen consumed is used to generate 90% of ROS in cells as byproducts of aerobic respiration [12]. Numerous studies have indicated an age-related increase in the rate of mitochondrial free radical generation and in the extent of oxidative damage to mitochondrial macromolecules [9,10,13], especially enzymes involved in the respiratory chain, leading to impairment of respiratory activity [13–16].

Although direct oxidation of proteins and other macromolecules is believed to be the main type of endogenous damage during aging [8,17,18], recent studies have revealed that mitochondria also suffer from glycative stress [19–21], in agreement with a causal relationship between hyperglycemia-induced ROS generation and the previously reported intracellular advanced glycation endproduct (AGEs) formation [22,23]. In particular, a rise in AGE formation was shown to be due to a rapid increase in AGE-forming methylglyoxal (MGO) concentrations, mainly generated by fragmentation of glyceraldehyde-3-phosphate (G-3P) and dehydroxyacetone-phosphate (DHA-P) from the glycolytic pathway [24]. Interestingly, we have recently reported that mitochondrial

Abbreviations: 2D-DIGE, two-dimensional difference gel electrophoresis; 2D-GE, two-dimensional gel electrophoresis; MS, mass spectrometry; AGE, advanced glycation endproduct; OXPHOS, oxidative phosphorylation; PPAR-γ, Peroxisomal proliferators-activated receptor gamma; ALDH2, aldehyde dehydrogenase-2; MCAD, medium chain acyl-CoA dehydrogenase; THIM, 3-ketoacyl-CoA thiolase; MTP, mitochondrial trifunctional protein

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http://dx.doi.org/10.1016/j.bbadis.2013.07.015

matrix proteins undergo both oxidative and glycative modifications and that these altered proteins concomitantly accumulate during rat liver aging [19], and replicative senescence of fibroblasts [25]. Thus, the consequence of such a ROS-mediated attack is the accumulation of oxidatively and/or glycoxidatively damaged proteins, which may contribute to dysfunctioning of this organelle, leading *in fine* to impairment of cell function [19,20].

Impairment of mitochondrial protein synthesis is also prevalent in the elderly. Studies performed on numerous organs such as skeletal muscle in humans showed that such dysfunctioning is related to reduced mitochondrial DNA (mtDNA) abundance, a possible underlying cause of this dysfunctioning during the aging process [26]. However, recent studies conducted on Rhesus monkeys showed increased levels of mtDNA in brain [27] and skeletal muscle [28] associated with a decreased mitochondrial membrane potential [29]. Decreased synthesis of mitochondrial proteins in human skeletal muscle has been described elsewhere and is likely contributing to the decline in mitochondrial function [30], whereas in the heart, a number of different mechanisms are known to be involved in decreased mitochondrial activity [31]. Reduced protein synthesis was also associated with altered activity of oxidative metabolism enzymes in particular cytochrome c oxidase (COX) in rat skeletal muscle, liver and heart, but appeared to be tissue-specific [32].

Thus, a number of different mechanisms are thought to be involved in age-related impairment in mitochondrial function, but data documenting precise metabolic pathways affected at the molecular level remain largely elusive. The purpose of this study was to determine whether an age-related decline in rat liver mitochondrial function could be linked to changes in the expression of proteins involved in key metabolic processes, in addition to post-translationally altered proteins, especially AGE-damaged enzymes in liver mitochondrial matrix. We have previously shown a decreased activity of aconitase both in heart and liver mitochondria. However, the Lon protease activity was affected only in the liver, in parallel with an age-related accumulation of glycation-damaged matrix proteins with aging, indicating that this organ is early subjected to damage in Wistar rat [19,33]. Herein, we extended our studies on aged liver mitochondria showing that, among a set of differentially expressed proteins, several exhibited decreased expression and were crucial OXPHOS complex I/V components and key enzymes implicated in the fatty acid β-oxidation process as well as in the tricarboxylic acid (TCA) cycle. In addition, most of the fatty acid β-oxidation enzymes appeared to be AGE-modified with a subsequent decrease of their activities, whereas few of them exhibited both handicaps. Altogether, these data suggest that a reduction in expression of crucial OXPHOS complex I/V components and increased glycative damage of key fatty acid β-oxidation and TCA/urea cycle enzymes, thereby compromising their activity [20,34], may be primary determinants in the age-related decline in energy production of liver mitochondria, leading to cell dysfunction with aging in rats.

2. Materials and methods

2.1. Chemicals

Cyanine dyes for detecting protein abundance in difference gel electrophoresis (CyDye DIGE) fluor minimal dyes Cy2, Cy3 and Cy5, two-dimensional difference gel electrophoresis (2D-DIGE) materials and other 2D chemicals of analytical grade, as well as Western blot reagents, were purchased from GE Healthcare (Saclay, France). Coomassie brilliant blue G-250, the Bradford protein assay kit and chemicals for SDS-PAGE were purchased from Bio-Rad (Marnes La Coquette, France). All other reagents were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France) in the highest purity, unless specified.

2.2. Experimental protocols

Experiments were performed on female Wistar (Wistar–RjHan) rats purchased from Janvier animal care (Janvier, Le Genest Saint Isle, France). Cohorts were constituted of young adults (3 months old) and elderly (20 months old) animals. These two time-points were chosen according to the previous study showing that mitochondria isolated from Wistar rat heart (postmitotic cells) or liver (slowly dividing cells), exhibited a significant decrease in the respiratory activity (the rate of net oxygen consumption and the state 3) from 10 month- until 27 month- compared with 3 month-old [33]. The animals were killed by cervical dislocation and the liver was excised for preparation of isolated mitochondria.

2.3. Isolation of liver mitochondria

Mitochondria from individual rat livers were isolated using differential centrifugation as previously described [19] and coupled with a Ficoll gradient. Briefly, a 10% (w/v) tissue homogenate was prepared with a Potter apparatus in ice-cold isolation buffer containing 220 mM mannitol, 70 mM sucrose, 0.1 mM EDTA and 2 mM Hepes (pH 7.4) supplemented with 0.5% BSA (w/v). Nuclei and cellular debris were pelleted by centrifugation for 10 min at 800 *g* at 4 °C. The supernatant was centrifuged at 8000 *g* for 10 min at 4 °C. The mitochondrial pellet was washed three times with homogenization medium and the last pellet resuspended in the same buffer and submitted to a discontinuous Ficoll gradient (10% and 7.5% from the bottom of tube) in sucrose buffer (75 mM sucrose, 1 mM EGTA and 5 mM Hepes pH 7.4) and centrifuged at 75,000 *g* for 30 min. The pellet containing free mitochondria was washed three times, resuspended in isolation buffer and contamination of preparation with lysosomes has been estimated using acid phosphatase activity as a marker as previously described [20]. Mitochondrial preparation was then stored at −80 °C.

To prepare soluble protein extract, mitochondria were resuspended in 50 mM Tris–HCl, pH 7.9, then disrupted by sonication (4 times, 10 s). The resulting suspension was centrifuged at 15,000 *g* for 10 min and then the resulting supernatant at 100,000 *g* for 45 min at 4 °C. The high-speed supernatant fraction containing the soluble protein fraction (named mitochondrial extract) was stored at −80 °C for further analysis. Total protein concentration was determined using the Bradford protein assay (Bio-Rad, München, Germany).

2.4. 2D-gel electrophoresis and Western blot of AGE-modified proteins

Mitochondrial extract samples (150 μg) from young and aged rats were mixed with 200 μl of two-dimensional sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% Pharmalytes pH 3–11). After rehydration of strips, isoelectrofocalization (IEF) of the strips was performed using the IPGphor3 system (GE Healthcare). At the end of the cycle, strips were stocked at −20 °C before the equilibration step and then submitted to second dimension gel electrophoresis (2D-GE) on an 8–18% (w/v) polyacrylamide SDS-PAGE gradient in a Ettan-DALT-6 system (GE Healthcare). Two gels were performed in parallel, one for Coomassie brillant blue staining and the second for electroblotting onto a polyvinylidene difluoride (PVDF) membrane. After transfer, the membrane was incubated for 2 h at room temperature in blocking solution and Western blot was performed using an anti-AGE monoclonal antibody (clone 6D12), as previously described [20]. The proteins were revealed with a SuperSignal West Pico chemiluminescent reagent (Perbio Science Company, Brebières, France) and densitometry analyses were performed using Image Master 2D Platinum 7 software (GE Healthcare). Colloidal Coomassie blue-stained spots matched with bands immunolabelled with anti-AGE antibodies were excised from the gel and submitted to mass spectrometry analysis as indicated below.