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Original Contribution

Comparative bioenergetic study of neuronal and muscle mitochondria during aging

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ABSTRACT

Mitochondrial respiratory chain defects have been associated with various diseases and with normal aging, particularly in tissues with high energy demands, including brain and skeletal muscle. Tissue-specific manifestation of mitochondrial DNA (mtDNA) mutations and mitochondrial dysfunction are hallmarks of mitochondrial diseases although the underlying mechanisms are largely unclear. Previously, we and others have established approaches for transferring mtDNA from muscle and synaptosomes of mice at various ages to cell cultures. In this study, we carried out a comprehensive bioenergetic analysis of cells bearing mitochondria derived from young, middle-aged, and old mouse skeletal muscles and synaptosomes. Significant age-associated alterations in oxidative phosphorylation and regulation during aging were observed in cybrids carrying mitochondria from both skeletal muscle and synaptosomes. Our results also revealed that loss of oxidative phosphorylation capacity may occur at various ages in muscle and brain. These findings indicate the existence of a tissue-specific regulatory mechanism for oxidative phosphorylation.

Introduction

Aging is a multifactorial process during which physiological alterations occur in all tissues. As organisms age, all tissues start to decline in their functions and thus exhibit various associated anomalous features at various aging time points. In humans, subtle irreversible changes occur by the third and fourth decades of life in most tissues and progressively deteriorate with further aging. The rate of aging-associated decline varies among tissues [1,2]. Nevertheless, some universal molecular events occur, in particular within the same tissues.

Brain and muscle are among the most metabolically active tissues and consequently are most affected by mitochondrial dysfunction [1,3–5]. Nevertheless, they also exhibit distinctive bioenergetics and physiological phenotypes during aging. Age is the major risk factor for neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis. Autopsy and MRI studies have revealed that brain volume and weight decrease, accompanied by increases in ventricular volume and cerebrospinal fluid spaces, in individuals over 60 [6,7]. There are other changes such as loss of neural circuits, brain plasticity, and thinning of the cortex in old people. Meanwhile, notable cellular changes occur in aging brains. Neurofibrillary tangles, which are aggregates of hyperphosphorylated tau protein, and senile plaques, extracellular deposits mainly composed of small peptides containing 39–43 amino acids known as amyloid β-peptide, are neuropathological hallmarks of Alzheimer disease, which has been considered as accelerated brain aging [7,8]. It is also well established that the age-dependent decline in body mass is largely due to loss and atrophy of muscle cells [9,10]. Age-related reductions in muscle mass (sarcopenia) are a direct cause of the decline in muscle strength and resulting disability observed in older adults. Muscle mass is maintained mostly through the balance between protein synthesis and degradation, and the rates of skeletal muscle protein synthesis decline with age. Muscle changes, which usually start in the fourth decade of life, include reductions in muscle fiber numbers, decreases in the size of fast-twitch type II fibers and motor unit numbers and size, and increases in noncontractile structures such as fat and connective tissue [10,11].

Mitochondria play critical roles in regulating energy production, metabolism, signal transduction, and apoptosis [12]. The primary

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function of mitochondria, oxidative phosphorylation, is achieved by electron transfer along the respiratory chain. By-products of this process, reactive oxygen species (ROS), generated by electrons interacting with oxygen before reaching the terminal complex of the electron transfer chain, have been proposed as both important signaling molecules and hyperactive reagents that could induce damage to various cellular components. Together with alterations in apoptosis, which is another important feature under the control of mitochondria, ROS are likely to play an important role in connecting mitochondrial dysfunction and physiological phenotypes associated with aging.

To reveal the role of mitochondrial dysfunction and the resulting bioenergetic and biochemical consequences that mediate the various physiological phenotypes during aging in brain and muscles, we carried out a systematic comparative study on mitochondria derived from brain and skeletal muscles of young, middle-aged, and old mice. We took advantage of both long- and recently established approaches of transferring mitochondria from synaptosomes and skeletal muscles to mitochondrial DNA (mtDNA)-less cells to generate cybrids exclusively containing mtDNA from brain and muscles from the same mice at different ages. A series of careful studies on various aspects of mitochondrial function have been performed. We demonstrated distinctive patterns of mitochondrial anomalies associated with brain and muscle during aging.

Materials and methods

Generation of cybrids with mitochondria from mouse skeletal muscle and brain

Female C57BL/6 mice were purchased from Charles River Laboratories. Among them, six each were age 6, 13, and 26 months (representing young, middle-aged, and old), and they were housed at the University of Texas Health Science Center at San Antonio animal facility. All animals were housed and treated as per institutional animal care committee rules, and all experimental procedures were approved by the same committee. Skeletal muscles from the whole thigh were dissected from the hindlegs [13]. Synaptosomes were derived from the brains [14]. In brief, after the skull of each animal was opened, the cerebral hemispheres were removed and the cortices were dissected out. Parts of muscle and brain as described above were cut into small pieces in a 10-mm dish on ice and rinsed several times with Hanks balanced salt solution (Invitrogen). The muscle samples were covered with 6 vol of hypotonic buffer (10.0 mM Tris, 10.0 mM KCl, 0.15 mM $MgCl_2$, pH 6.7) and processed with a Dounce type A homogenizer for about 30 strokes. The processed samples were centrifuged at 600 *g* for 3 min and the supernatant was then subjected to another centrifugation at 100*g* for 10 min. Finally, muscle mitochondria enclosed by cell membranes were collected by centrifugation at 4500*g* for 10 min. The brain pieces were rinsed with medium I [10% 0.3 M sucrose, 5 mM Tris/Cl (pH 7.5), 0.1 mM EDTA (pH 7.0)] and were further processed with a Dounce type A homogenizer for about 16 strokes. The samples were then centrifuged at 1310*g* for 5 min at 4 °C, after which the supernatant was centrifuged at 12,000*g* for 5 min. The pellet was resuspended with 1 ml medium I per gram tissue and then diluted with 4 vol of 8.5% Percoll solution [18.1 ml medium II (0.25 M sucrose, 5 mM Tris/Cl (pH 7.5), 0.1 mM EDTA (pH 7.0)) and 1.9 ml of the stock solution of isosmotic Percoll (SIP; 9 vol of Percoll solution, 1 vol of 2.5 M sucrose) for 20 ml]. Next, 2 ml of the mixture was layered onto a premade and prechilled sucrose/Percoll density gradient [2 ml 16% Percoll (1.65 ml medium II and 0.35 ml SIP) were added to the bottom of the centrifuge tube, and 2 ml of 10% Percoll (1.78 ml medium II, 0.22 ml SIP) was slowly layered on top of the 16% Percoll layer]. The gradients were centrifuged at 18,000*g* for 20 min. Synaptosomal mitochondria enclosed by a cell membrane between the 8.5 and the 10% Percoll gradient for a total of 1–2 ml were collected. The synaptosomal mitochondria were further washed with medium II and centrifuged at 18,000 *g* for 20 min.

The generation of muscle- and brain-specific cybrids was described previously [13]. In brief, approximately 10^6 ρ^0 cells [15] were mixed with freshly isolated membrane-enclosed muscle or synaptosome mitochondria prepared from about 1 g of muscle or brain sample. The mixture was centrifuged for 10 min at 2000*g*, and the pellet was resuspended in 45% PEG by mild and brief pipetting while avoiding the creation of air bubbles. After 1 min treatment with PEG, the fusion process was stopped by diluting with 10 vol of Dulbecco's modified Eagle's medium (DMEM). The fusion products were incubated with DMEM supplemented with 50 μg/ml uridine at 37°Cfor 24 h. On day 2, the medium was replaced by semiselection medium, reducing the amount of uridine to10 μg/ml. Five days later, the medium was changed again to selection medium, i.e., the same medium without uridine. Transformants were selected based on the pyrimidine auxotrophy of the ρ^0 cells [16] as the cybrids containing exogenous mitochondria from muscle or synaptosome were able to survive in the selective medium.

We obtained 24, 20, and 25 cybrid clones carrying mtDNA derived from the skeletal muscles and 29, 31, and 69 cybrid clones of synaptosome, from young, middle-aged, and old mice, respectively.

Cybrids and media

All cell lines used in this work were grown in monolayer culture. The mtDNA-less ρ^0 LL/2-m21 cell line was a derivative of mouse cell line LL/2 as described previously [15]. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen).

Oxygen consumption measurement

Measurements were carried out in two chambers of a YSI Model 5300 biological oxygen monitor. The medium (DMEM–FBS) was replaced with fresh medium 1 day before the measurements. Determination of the endogenous oxygen consumption rate in intact cells was carried out on $\sim 5 \times 10^6$ cells in Tris-based, Mg^{2+}- and Ca^{2+}-deficient (TD) buffer (0.137 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris/HCl, pH 7.4 at 25 °C). After recording the basal respiration rate, 2.5 μg/ml oligomycin was added to measure the oligomycin-inhibited rate, and then 0.5 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was added to measure the maximal respiration. For measurements of the complex-dependent oxygen consumption rate in digitonin-permeabilized cells, about 5×10^6 cells were resuspended in 1 ml of buffer A [20 mM Hepes (pH 7.1), 10 mM $MgCl_2$, 250 mM sucrose], and then 100 μg of digitonin in 1 ml of the same buffer was added. After incubation for 1 min at 25 °C, the cell suspension was diluted with 8 ml of buffer A. The cells were rapidly pelleted and resuspended in respiration buffer [20 mm Hepes (pH 7.1), 250 mM sucrose, 2 mM KH_2PO_4, 10 mM $MgCl_2$ and 1.0 mM ADP]. Substrates and inhibitors were added with Hamilton syringes. The final concentrations were as follows: malate, 5 mM; glutamate, 5 mM; succinate, 5 mM; glycerol 3-phosphate, 5 mM; ascorbate, 10 mM; *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 0.2 mM; NADH, 0.5 mM; rotenone, 100 nM; flavone, 0.5 mM; antimycin A, 20 nM; and KCN, 1 mM.