Published in final edited form as:

Free Radic Biol Med. 2013 October ; 63: 30–40. doi:10.1016/j.freeradbiomed.2013.04.030.

Comparative bioenergetic study of neuronal and muscle mitochondria during aging

Hongzhi Li^{a,b,1}, Lokendra Kumar Sharma^{b,1}, Youfen Li^{b,c,1}, Peiqing Hu^b, Abimbola Idowu^b, Danhui Liu^a, Jianxin Lu^a, and Yidong Bai^{a,b,*}

^aSchool of Life Science, Wenzhou Medical College, Wenzhou, Zhejiang 325035, China

^bDepartment of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA

^cSchool of Life Science, Xi'an Jiaotong University, Xi'an, Shanxi 710049, China

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.

Keywords

Mitochondria; Aging; Skeletal muscle; Synaptosome; Cybrid; Free radicals

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. Mean-while, 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 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 MgCl₂, 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 10⁶ 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°C for 24 h. On day 2, the medium was replaced by semiselection medium, reducing the amount of uridine to 10 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 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 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 ~ 5 × 10⁶ cells in Tris-based, Mg²⁺- and Ca²⁺-deficient (TD) buffer

(0.137 M NaCl, 5 mM KCl, 0.7 mM Na₂HP0₄, 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 MgCl₂, 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 KH₂P0₄, 10 mM MgCl₂ 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*-tetra-methyl-*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.

ATP determination

The ATP Determination Kit (Invitrogen, A22066) was used. Cells were grown in a 100-mm dish to about 40% confluence and then harvested. About 2×10^6 cells were washed with TD buffer, resuspended in Boiling Buffer (100 mM Tris, 4 mM EDTA), boiled for 5 min, and then centrifuged at 10,000g for 1 min. For luciferase assays, a cell suspension was added to the standard reaction solution and luminescence was measured in a Synergy HT Multi-Detection microplate reader (Bio-Tek Instruments). The standard curve was constructed by measuring luminescence derived from ATP solutions. To measure ATP synthesis in the presence of oligomycin, cells were incubated with 15 pg/ml oligomycin for 30 min at 37 °C before harvest. ATP-to-ADP ratio measurements from cultured cells were performed using an ApoSENSOR ADP/ADP ratio kit (Biovision; Cat. No. K255-200).

ROS measurement

Production of ROS at the mitochondrial level was assessed using the MitoSOX red mitochondrial superoxide indicator (Invitrogen, M36008). Cells were first washed in TD buffer, and after the addition of 5 µM MitoSOX, the cells were incubated for 15 min at 37 °C, then washed in TD buffer, and scraped in 2 ml TD buffer with a plastic scraper. For each analysis, about 1×10^6 homogenized cells were placed into a 96-well plate and measured in a Synergy HT microplate reader at 485/590 nm.

Cell growth and viability analysis

Multiple identical samples of 10^5 cells were grown for 4 days on six-well plates in the appropriate medium: DMEM containing 4.5 mg/ml glucose and 0.11 mg/ml pyruvate or DMEM lacking glucose and containing 0.9 mg/ml galactose and 0.11 mg/ml pyruvate, both supplemented with 10% dialyzed FBS. Cells were counted daily. Cell doubling time = $72 \text{ h} \times \log 2 / [\log(\text{total cell number at 96 h}) - \log(\text{total cell number at 24 h})]$. Cell viability was analyzed with a Vi-Cell XR cell viability analyzer (Beckman Coulter).

Statistical analysis

Probability (P) values were calculated using SPSS 14.0 software. We assumed each cybrid line is a fully independent mtDNA combination derived from mitochondria of a particular tissue of a particular age group, and every cell line can be considered an independent sample. The means of groups were compared via one-way ANOVA. First homogeneity of variance was tested, if significance was > 0.10 , then P values were calculated by Tukey's HSD; otherwise, P values were calculated by Tamhane's T2 (conservative pairwise

comparisons test based on a *t* test). Differences were considered statistically significant when $P < 0.05$.

Results

Endogenous respiration of cybrids derived from mouse brain and muscle at various ages

To investigate the cybrids carrying mtDNA from muscles and synaptosomes of young, middle-aged, and old mice, respiration properties were investigated as immediate indicators of oxidative phosphorylation, the primary function of mitochondria. First, we measured the endogenous respiration activity of intact cells (Fig. 1). Although there were no differences among age groups in the cybrids containing mitochondria from synaptosomes (Fig. 1F), there was a decrease with aging of the baseline respiration in cybrids containing mitochondria from skeletal muscle, particularly a significant reduction of about 22% between the old and the young group (Fig. 1A). Next, respiration was analyzed in the presence of oligomycin A, a chemical inhibitor that can block complex V, ATP synthase, or in the presence of FCCP, which can completely uncouple oxidation from phosphorylation and raise the respiratory level to a maximum. Although no differences were detected with oligomycin-resistant respiration in both tissues (Figs. 1B and G), age-dependent decreases in the maximal respiration rates in the presence of FCCP were found in both neuronal (Fig. 1C) and skeletal muscle mitochondria (Fig. 1H). The decline in respiration started earlier and was more severe with the mitochondria from skeletal muscles (Fig. 1C).

To more carefully examine how respiration is coupled with ATP synthesis in these cybrids, we calculated the ratio of oligomycin resistance to base endogenous respiration. Although we did not see any difference among all age groups in the cybrids containing mitochondria from mouse neuronal cells (Fig. 1I), we found a 36% increase in the value of the ratio of oligomycin-resistant respiration to baseline respiration with muscle mitochondria between old and young groups, indicating less-coupled mitochondria with aging, in particular when the mice were in old age (Fig. 1D). The capacity of regulation over oxidative phosphorylation was then analyzed by calculating the ratio of FCCP-uncoupled to oligomycin-resistant respiration. As shown in Figs. 1E and J, both muscle and neuronal cybrids exhibited a progressive decline in the regulation with aging. In particular, significant decreases were found in mitochondria derived from the old mice. Again, more severe reduction was found in the mitochondria from skeletal muscles.

Complex-dependent respiration activities of cybrids derived from mouse brain and muscle at various ages

To further determine which part of the respiration process is affected during aging, we carried out complex-dependent respiration measurements in digitonin-permeabilized cells provided with specific substrates. In particular, malate/glutamate-dependent O_2 consumption rate usually reflects the rate-limiting activity of complex I; the succinate/glycerol 3-phosphate-driven respiration usually reflects the activity of complex III; and the TMPD/ascorbate-driven respiration reflects the activity of complex IV. As shown in Fig. 2, the most obvious alterations were detected in the complex I-dependent respiration as determined by malate/glutamate-driven respiration (Figs. 2A and D), in both muscle and neuronal mitochondria, with the lowest values exhibited by mitochondria derived from the old mice. No significant differences were found in complex IV-dependent respiration among the various age groups for either muscle or neuronal mitochondria (Figs. 2C and F). However, whereas a decrease in complex III-dependent respiration was detected with muscle mitochondria derived from old mice, no such reduction was found with mitochondria derived from the neuronal cells (Figs. 2B and E).

ATP production measurements

Concurrent with electron transfer along the respiratory chain, protons are pumped across the inner membrane of mitochondria at complexes I, III, and IV, generating a cross-membrane potential that drives the ATP production. ATP content was measured by lumino-metry in boiled cells. An age-associated ATP content decrease was found in cybrids containing mitochondria from both muscle and synaptosomes (Figs. 3A and D). In contrast to the respiration properties, a progressive and more pronounced decline in ATP content was found in cybrids containing mitochondria from neuronal cells. Next, the mitochondrial oxidative phosphorylation-independent contribution to ATP production was investigated. The ATP content was measured in the presence of a complex V or ATP synthase inhibitor, oligomycin A. Whereas no obvious differences were seen in cybrids containing skeletal muscle mitochondria, an age-associated decline was observed in the cybrids containing mitochondria from neuronal cells of the old mice (Figs. 3B and E). We also measured the ATP-to-ADP ratio and found that compared to total ATP level, which was significantly lower in old muscle mitochondria, there was no significant difference in the ATP/ADP ratio of old muscle mitochondria (Fig. 3C). However, we observed a decrease in ATP/ADP ratio in old synaptosomal mitochondria, in particular compared to middle-aged (Fig. 3F).

ROS production

Oxidative stress has been suggested as a pathogenic mechanism mediating mitochondrial dysfunction linked to various physiological phenotypes associated with aging. To determine if there is an age-associated alteration in ROS production in our cybrid system, mitochondrial-specific generation of superoxide was analyzed by MitoSOX. MitoSOX is a probe that is rapidly and selectively targeted to the mitochondria, and once inside mitochondria, it is preferentially oxidized by superoxide. However, as shown in Figs. 4A and B, there were no significant changes in the cybrids containing mitochondria from muscle or from neuronal cells.

Glucose dependence and cell viability

Mammalian cells rely on both mitochondrial oxidative phosphorylation and glycolysis to generate ATP. To focus on the ATP production contributed by mitochondrial oxidative phosphorylation, the growth properties of cybrids were examined in galactose-containing medium in which cells predominantly rely on oxidative phosphorylation for ATP demands [16]. Although there were no differences in doubling time in glucose medium among age groups in cybrids containing mitochondria from both muscle and neuronal cells (Figs. 5B and E), an age-associated increase in doubling time in galactose medium was found in the cybrids carrying mitochondria from both muscle and neuronal cells, especially in the cybrids with mitochondria from old mice (Figs. 5A and D). To reduce variability in growth properties caused by other factors, the ratio of doubling time in galactose medium to that in glucose medium was calculated using the growth index formula. As shown in Figs. 5C and F, the age-associated differences persisted in both muscle and neuronal cybrids, especially the decline in the old group.

Because mitochondrial dysfunction has been associated with apoptosis, cell viability was investigated in glucose- and galactose-containing medium after 3 days of culture in the respective medium. As shown in Fig. 6, consistent with what was observed with growth properties, in the galactose medium, more cell death was observed in the old-age cybrids containing muscle and neuronal mitochondria (Figs. 6A and D). In contrast, no significant differences in cell viability were found among the various age groups in the regular glucose medium (Figs. 6B and E). The ratio of viability in galactose to that in glucose medium, expressed as the viability index, also demonstrated a significant decrease in cell viability in

the cybrids carrying mitochondria of old mice from both muscle and neuronal cells (Figs. 6C and F).

Discussion

The decline of mitochondrial function and the accumulation of mtDNA mutations have been reported in various organisms and in various tissues. However, there are few in-depth studies on the bioenergetic and biochemical implications of these alterations. In this study, we took advantage of the availability of approaches to transfer mitochondria from muscle and neuronal cells to cultured cell lines and carried out a systematic comparative investigation on both muscle and neuronal systems in the same animals to shed light on fundamental questions such as when mitochondrial dysfunction started to appear and how this alteration mediated the bioenergetic and physiological changes associated with aging in these systems. As the mitochondria used for fusion were extracted from a mixture of about equal amounts of tissues from the six mice in each group, and the cybrids from each group exhibited quite diverse mitochondrial DNA, we assumed each cybrid line is a fully independent mtDNA combination derived from mitochondria of a particular tissue of a particular age group, and every cell line can be considered an independent sample.

One of the main results of this study is that, among the respiration features, maximal respiration as measured in the presence of FCCP exhibited the most sensitive alteration during aging in both muscle and neuronal cells (Figs. 1C and H). Probably as a consequence, mitochondria derived from old mice in both systems also showed compromised respiratory control ratio. Nevertheless, we also found some distinctive patterns associated with each system. Overall, compared with its neuronal system counterpart, respiration associated with mitochondria from skeletal muscle started to decline early with aging, and the decline was more severe. Interestingly, although we did not detect any obvious age-associated decrease in baseline respiration with cybrids containing neuronal mitochondria, a significant decline was recorded with cybrids carrying mitochondria derived from skeletal muscle of old mice (Figs. 1A and F). It is likely that this deficiency was associated with a decrease in coupling of oxidation and phosphorylation such as we observed with the same set of cybrids. A similar age-related compromised respiration and coupling efficiency of respiration was reported previously in a large-scale investigation with skin-derived fibroblasts from human subjects with a greater age range [17,18]. To exclude the possibility that the nonmitochondrial respiration determined the age-dependent decline of respiratory capacity, we also measured the rotenone- and antimycin-resistant respiration with supplied substrates. We found such nonmitochondrial respiration generally higher in old groups and that it contributed about 3, 5, and 6% of total respiration in cybrids with synaptosomal mtDNA derived from young, middle-aged, and old mice, and 1, 4, and 4% of total respiration in cybrids with muscle mtDNA derived from young, middle-aged, and old mice. To avoid the alterations in ATP demand and mitochondrial morphology and distribution introduced by trypsin treatment, all our measurements were performed immediately after the cells were detached from the dishes.

The mitochondrial electron transfer chain consists of complexes I, II, III, and IV. Complex I is the first enzyme of the respiratory chain and the largest and most complicated component of the system [19]. Among its 45 subunits, 7 of 14 core subunits are encoded by mtDNA. Because complex I is the major entry point for electrons into the respiratory chain and is suggested to be the rate-limiting step in overall respiration, it plays a central role in energy metabolism [19,20]. Age-dependent respiratory defects have been found at complexes I, III, and IV, all containing subunits encoded by mtDNA, whereas all the nuclear DNA encoding complex II remains largely unaffected [21]. In particular, human complex I activity was found to decrease with aging in liver, muscle, and brain [22], and a brain-specific decrease

in complex I activity during aging has been reported in rats [23]. Interestingly, complex I dysfunction has also been reported in the substantia nigra and frontal cortex in sporadic neurodegenerative disorders such as Parkinson disease [24]. In our study, complex I deficiency was the most pronounced defect within the respiratory system (Figs. 2A and D). The reason for such a complex-specific deficiency is probably related to the relatively large coding capacity of mtDNA (59.2% for complex I, 7 subunits; 24.4% for complex IV, 3 subunits; and 9.3% for complex III, 1 subunit, of the total mitochondrial genome) or perhaps to the proximity of complex I (and III) to the ROS generation sites [21,25]. These results are also consistent with previous observations that complex IV was in much excess, whereas complex I was under tight control [26].

When we measured the total ATP content and ATP/ADP ratio in those cybrids, a more severe decline was observed with mitochondria derived from neuronal cells (Figs. 3D and F). At the same time, the oligomycin-resistant ATP content as revealed by measurement in the presence of an ATP synthase inhibitor, oligomycin, also decreased in the neuronal old group (Fig. 3E).

An alternative way to monitor mitochondrial function is to measure the growth capacity of cells when they are exclusively relying on oxidative phosphorylation for ATP production [20]. Again, we saw significant increases in cell doubling times during culture in galactose medium with cybrids carrying mitochondria derived from both skeletal muscle and neuronal cells of old mice (Figs. 5A and D). More severe defects have been observed with mitochondria from skeletal muscles (Fig. 5A). Because we did not detect any obvious defects when cells were cultured in regular glucose medium (Figs. 5B and E), the data suggest an upregulation of glycolysis when mitochondria are derived from older animals. It is interesting to note that such a bioenergetic switch is also a signature of cancer cells [27], because aging is the biggest risk factor for the occurrence of cancer.

One important notion of the mitochondrial theory of aging is the central role of oxidative stress. In fact, oxidative damage during aging has been well documented. However, in the cybrid system we established, and with MitoSOX as the probe, we failed to observe significant differences in mitochondrial superoxides across the age groups in both skeletal muscle and neuronal systems (Figs. 4A and B). Similar results have also been reported by other laboratories [28]. Nevertheless, we also observed some upregulation in uncoupling of oxidative phosphorylation during aging, particularly in the muscle system. As proposed by the “uncoupling to survive” hypothesis of aging, a mild uncoupling could serve as a protective mechanism that minimizes ROS production in adult tissues and preserves mitochondrial function with aging [1,17,29,30]. Such a protective mechanism was also reported in isolated mitochondria [31,32].

It is worthwhile to note the results of previous studies on mutator mice expressing a proofreading-deficient version of mtDNA polymerase [33,34] that exhibited a premature aging phenotype due to increased frequency of mtDNA mutations [33,35]. Although no increase in ROS or oxidative damage was detected in these mice [36], the increased mtDNA mutations were associated with enhanced apoptosis. However, it was also reported that such alterations in apoptosis were not found in the wild-type old mice, which exhibited much lower mtDNA mutation loads compared with the mutator mice. In our study, although we did not notice any changes in cell viability when cybrids were cultured in glucose medium, cybrids carrying mitochondria from both skeletal muscle and neuronal cells from old mice showed reduced cell viability when challenged by culture in galactose medium (Fig. 6).

Many structural and functional changes occur with age, in both muscle and brain [4,37], and mitochondrial dysfunction has been implicated in these processes [38,39]. Moreover,

mitochondrial dysfunction and mtDNA mutations have been associated with various neurological diseases and muscle disorders [40–42]. In this study, we found that the maximal respiratory capacity, complex I activity, overall ATP production, and viability in galactose medium all decreased in cybrids containing mitochondria of both skeletal muscle and neuronal cells from old mice. Meanwhile, the decline of baseline endogenous and complex III-driven respiration, together with reduced coupling of oxidative phosphorylation, was detected only with mitochondria derived from old skeletal muscle. Interestingly, deterioration of the above processes frequently happened earlier and more severely in the cybrids containing mitochondria from skeletal muscle. It might not be a coincidence that the weakening of physical strength during aging, which is usually associated with muscle function, often precedes the decline in cognitive activities associated with neuronal function [43].

Acknowledgments

This work was supported by grants from the National Institutes of Health (R01 AG025223) and the Morrison Trust to Y.B. We thank Drs. Anne Chomyn, Mai Zhou, and James Lechleiter for helpful discussion.

References

1. Lesnefsky EJ, Hoppel CL. Oxidative phosphorylation and aging. *Ageing Res. Rev.* 2006; 5:402–33. [PubMed: 16831573]
2. Boss GR, Seegmiller JE. Age-related physiological changes and their clinical significance. *West. J. Med.* 1981; 135:434–40. [PubMed: 7336713]
3. Hiona A, Leeuwenburgh C. The role of mitochondrial DNA mutations in aging and sarcopenia: implications for the mitochondrial vicious cycle theory of aging. *Exp. Gerontol.* 2008; 43:24–33. [PubMed: 17997255]
4. Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghava-kaimal S, Nair KS. Decline in skeletal muscle mitochondrial function with aging in humans. *Proc. Natl. Acad. Sci. USA.* 2005; 102:5618–5623. [PubMed: 15800038]
5. Chabi B, Mousson de Camaret B, Chevrollier A, Boisgard S, Stepien G. Random mtDNA deletions and functional consequence in aged human skeletal muscle. *Biochem. Biophys. Res. Commun.* 2005; 332:542–549. [PubMed: 15896715]
6. Witelson SE, Beresh H, Kigar DL. Intelligence and brain size in 100 postmortem brains: sex, lateralization and age factors. *Brain.* 2006; 129:386–398. [PubMed: 16339797]
7. Anderton BH. Ageing of the brain. *Mech. Ageing Dev.* 2002; 123:811–817. [PubMed: 11869738]
8. Parihar MS, Hemnani T. Alzheimer's disease pathogenesis and therapeutic interventions *J. Clin. Neurosci.* 2004; 11:456–467.
9. Lushaj EB, Johnson JK, McKenzie D, Aiken JM. Sarcopenia accelerates at advanced ages in Fisher 344xBrown Norway rats. *J. Gerontol. A Biol. Sci. Med. Sci.* 2008; 63:921–927. [PubMed: 18840796]
10. Nair KS. Aging muscle. *Am. J. Clin. Nutr.* 2005; 81:953–963. [PubMed: 15883415]
11. Rossi P, Marzani B, Giardina S, Negro M, Marzatico F. Human skeletal muscle aging and the oxidative system: cellular events. *Curr. Aging Sci.* 2008; 1:182–191. [PubMed: 20021391]
12. Hermeking H, Rago C, Schuhmacher M, Li Q, Barrett JE, Obaya AJ, O'Connell BC, Mateyak MK, Tarn W, Kohlhuber E, Dang CV, Sedivy JM, Eick D, Vogelstein B, Kinzler KW. Identification of CDK4 as a target of c-MYC. *Proc. Natl. Acad. Sci. USA.* 2000; 97:2229–2234. [PubMed: 10688915]
13. Li Y, Li HZ, Hu P, Deng J, Banoei MM, Sharma LK, Bai Y. Generation and bioenergetic analysis of cybrids containing mitochondrial DNA from mouse skeletal muscle during aging. *Nucleic Acids Res.* 1921; 38:1913–1921. [PubMed: 20022917]
14. Polosa PL, Attardi G. Distinctive pattern and translational control of mitochondrial protein synthesis in rat brain synaptic endings. *J. Biol. Chem.* 1991; 266:10011–10017. [PubMed: 2033035]

15. Bai Y, Shakeley RM, Attardi G. Tight control of respiration by NADH dehydrogenase ND5 subunit gene expression in mouse mitochondria. *Mol. Cell. Biol.* 2000; 20:805–815. [PubMed: 10629037]
16. Bai Y, Park JS, Deng JH, Li Y, Hu P. Restoration of mitochondrial function in cells with complex I deficiency. *Ann. N. Y. Acad. Sci.* 2005; 1042:25–35. [PubMed: 15965042]
17. Greco M, Villani G, Mazzucchelli E, Bresolin N, Papa S, Attardi G. Marked aging-related decline in efficiency of oxidative phosphorylation in human skin fibroblasts. *FASEB J.* 2003; 17:1706–1708. [PubMed: 12958183]
18. Laderman KA, Penny JR, Mazzucchelli E, Bresolin N, Scarlato G, Attardi G. Aging-dependent functional alterations of mitochondrial DNA (mtDNA) from human fibroblasts transferred into mtDNA-less cells. *J. Biol. Chem.* 1996; 271:15891–15897. [PubMed: 8663253]
19. Sharma LK, Lu J, Bai Y. Mitochondrial respiratory complex I: structure, function and implication in human diseases. *Curr. Med. Chem.* 2009; 16:1266–1277. [PubMed: 19355884]
20. Bai Y, Hu P, Park JS, Deng JH, Song X, Chomyn A, Yagi T, Attardi G. Genetic and functional analysis of mitochondrial DNA-encoded complex I genes. *Ann. N. Y. Acad. Sci.* 2004; 1011:272–283. [PubMed: 15126303]
21. Van Remmen H, Richardson A. Oxidative damage to mitochondria and aging. *Exp. Gerontol.* 2001; 36:957–968. [PubMed: 11404044]
22. Hutter E, Renner K, Pfister G, Stockl P, Jansen-Durr P, Gnaiger E. Senescence-associated changes in respiration and oxidative phosphorylation in primary human fibroblasts. *Biochem. J.* 2004; 380:919–928. [PubMed: 15018610]
23. Cocco T, Sgobbo P, Clemente M, Lopriore B, Grattagliano I, Di Paola M, Villani G. Tissue-specific changes of mitochondrial functions in aged rats: effect of a long-term dietary treatment with N-acetylcysteine. *Free Radic. Biol. Med.* 2005; 38:796–805. [PubMed: 15721990]
24. Navarro A, Boveris A. Brain mitochondrial dysfunction and oxidative damage in Parkinson's. *J. Bioenerg. Biomembr.* 2009; 41:517–521. [PubMed: 19915964]
25. Ozawa T. Genetic and functional changes in mitochondria associated with aging. *Physiol. Rev.* 1997; 77:425–64. [PubMed: 9114820]
26. Rossignol R, Faustin B, Rocher C, Malgat M, Mazat JP, Letellier T. Mitochondrial threshold effects. *Biochem. J.* 2003; 370:751–762. [PubMed: 12467494]
27. Lu J, Sharma LK, Bai Y. Implications of mitochondrial DNA mutations and mitochondrial dysfunction in tumorigenesis. *Cell Res.* 2009; 19:802–815. [PubMed: 19532122]
28. Yao YG, Ellison EM, McCoy JP, Chen J, Young NS. Age-dependent accumulation of mtDNA mutations in murine hematopoietic stem cells is modulated by the nuclear genetic background. *Hum. Mol. Genet.* 2007; 16:286–294. [PubMed: 17185390]
29. Brand MD. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Gerontol.* 2000; 35:811–820. [PubMed: 11053672]
30. Speakman JR, Talbot DA, Selman C, Snart S, McLaren JS, Redman P, Krol E, Jackson DM, Johnson MS, Brand MD. Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell.* 2004; 3:87–95. [PubMed: 15153176]
31. Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD. Superoxide activates mitochondrial uncoupling proteins. *Nature.* 2002; 415:96–99. [PubMed: 11780125]
32. Brand MD, Buckingham JA, Esteves TC, Green K, Lambert AJ, Miwa S, Murphy MP, Pakay JL, Talbot DA, Echtay KS. Mitochondrial superoxide and aging: uncoupling-protein activity and superoxide production. *Biochem. Soc. Symp.* 2004; 71:203–213. [PubMed: 15777023]
33. Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM, Yamasoba T, Tanokura M, Weindrich R, Leeuwenburgh C, Prolla TA. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science.* 2005; 309:481–84. [PubMed: 16020738]
34. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly YM, Gidlöf S, Oldfors A, Wibom R, Tornell J, Jacobs HT, Larsson NG. Premature ageing in mice

- expressing defective mitochondrial DNA polymerase. *Nature*. 2004; 429:417–423. [PubMed: 15164064]
35. Trifunovic A, Hansson A, Wredenberg A, Rovio AT, Dufour E, Khvorostov I, Spelbrink JN, Wibom R, Jacobs HT, Larsson NG. Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. *Proc. Natl. Acad. Sci. USA*. 2005; 102:17993–17998. [PubMed: 16332961]
 36. Edgar D, Trifunovic A. The mtDNA mutator mouse: dissecting mitochondrial involvement in aging. *Aging (Albany NY)*. 2009; 1:1028–1032. [PubMed: 20157586]
 37. Bishop NA, Lu T, Yankner BA. Neural mechanisms of ageing and cognitive decline. *Nature*. 464:529–535. [PubMed: 20336135]
 38. Alexeyev MF. Is there more to aging than mitochondrial DNA and reactive oxygen species? *FEBS J*. 2009; 276:5768–5787. [PubMed: 19796285]
 39. Wei YH, Lee HC. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. *Exp. Biol. Med.* (Maywood NJ). 2002; 227:671–682.
 40. Enriquez JA, Cabezas-Herrera J, Bayona-Bafaluy MP, Attardi G. Very rare complementation between mitochondria carrying different mitochondrial DNA mutations points to intrinsic genetic autonomy of the organelles in cultured human cells. *J. Biol. Chem.* 2000; 275:11207–11215.
 41. Montoya J, Lopez-Gallardo E, Herrero-Martin MD, Martinez-Romero I, Gomez-Duran A, Pacheu D, Carreras M, Diez-Sanchez C, Lopez-Perez MJ, Ruiz-Pesini E. Diseases of the human mitochondrial oxidative phosphorylation system. *Adv. Exp. Med. Biol.* 2009; 652:47–67. [PubMed: 20225019]
 42. Montoya J, Lopez-Gallardo E, Diez-Sanchez C, Lopez-Perez MJ, Ruiz-Pesini E. 20 years of human mtDNA pathologic point mutations: carefully reading the pathogenicity criteria. *Biochim. Biophys. Acta*. 2009; 1787:476–483. [PubMed: 18840399]
 43. Herndon LA, Schmeissner PJ, Dudaronek JM, Brown PA, Listner KM, Sakano Y, Paupard MC, Hall DH, Driscoll M. Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. *Nature*. 2002; 419:808–814. [PubMed: 12397350]

Fig. 1.

Endogenous respiration measurement. Young, middle, and old represent 24, 20, and 22 muscle (left) or 29, 31, and 68 synaptosomal (right) cell lines in different cybrid groups. Respiration was measured (A, F) in intact cells and in cells treated with (B, G) oligomycin or with (C, H) FCCP. The activities were determined using about 5×10^6 cells. The ratios of various respiration rates were calculated as indicated in (D, E, I, J). (K) A representative trace of oxygen measurement to demonstrate how we obtained the base, uncoupled, and maximal respiration rates.

Fig. 2. The complex-dependent respiration measurement. Young, middle, and old represent 10,10, and 10 muscle (left) or 10,10, and 15 synaptosomal (right) cell lines in different cybrid groups, respectively. About 5×10^6 cells were treated with digitonin before measurement. The substrates for measuring (A, D) complex I-dependent, (B, E) complex III-dependent, and (C, F) complex IV-dependent respirations are described under Materials and methods.

Fig. 3. ATP content analysis. Young, middle, and old represent (A, B) 20, 18, and 19 muscle or (D, E) 19, 20, and 20 synaptosomal cell lines in different cybrid groups, respectively. The (A, D) total and (B, E) oligomycin-resistant ATP content was measured. The ATP/ADP ratio was also measured in a separate experiment. Young, middle, and old represent (C) 10, 10, and 10 muscle or (F) 9, 9, and 9 synaptosomal cell lines in different cybrid groups, respectively. Three determinations were made.

Fig. 4. ROS measurement. Young, middle, and old represent (A) 20,18, and 19 muscle or (B) 19, 20, and 20 synaptosomal cell lines in different cybrid groups, respectively. Mitochondrial superoxide level was measured with MitoSOX red mitochondrial superoxide indicator (A, B). Three determinations were made.

Fig. 5. Growth test. Young, middle, and old represent 24,20, and 25 muscle (left) or 29,31, and 69 synaptosomal (right) cell lines in different cybrid groups. Cells were grown in glucose DMEM for 24 h and then (A, D) in galactose medium or (B, E) in glucose DMEM for another 72 h. (C, F) The growth index was calculated as indicated.

Fig. 6. Cell viability determination. Young, middle, and old represent 24, 20, and 25 muscle (left) or 29, 31, and 69 synaptosomal (right) cell lines in different cybrid groups. Cells were grown in glucose DMEM for 24 h and then (A, D) in galactose medium or (B, E) in glucose DMEM for another 72 h. (C, F) The viability index was calculated as indicated.