Age-Related Changes of Mitochondrial DNA Content and Mitochondrial Genotypic and Phenotypic Alterations in Rat Hind-Limb Skeletal Muscles

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Mitochondrial DNA (mtDNA) content relative to nuclear DNA content as well as mitochondrial transcription factor A (TFAM) content was measured in four hind-limb skeletal muscles, namely soleus (S), tibialis anterior (TA), gastrocnemius (G), and extensor digitorum longus (EDL) of adult rats. Content of mtDNA in 6-month-old rats is in the rank order of S > TA > G > EDL, and TFAM content is higher in S than in the other studied muscles. After the rat is 6 months of age, the mtDNA content decreases only in S and TA, whereas the TFAM content increases only in S. Deletions in mtDNA appear quite early in life in S and later on in the other muscles. Fibers defective for mitochondrial respiratory enzymes appear in rats at 15 months of age. In the oldest animals, the highest frequencies of occurrence of mtDNA deletions as well as of mitochondrial phenotypic alterations are found in S according to its highest mtDNA content and oxidative potential.

The deep involvement of mitochondria in aging and age-related phenomena is a widely accepted idea (1–3) since the “mitochondrial theory of aging” (4–6) has gained progressively larger support among the aging theories. Mitochondria are known today as receiver and/or integrator organelles of signaling pathways (7,8) as well as potential loci of diseases (9). However, the involvement of mitochondria in aging relates particularly to their function as “power-houses of the cell” which has been shown to be decreased by different extents by aging (1). This reduced bioenergetic capability is due to an age-related decrease of some respiratory enzyme activities which has been reported in various tissues and organisms by many authors (5,10), although a few others (11–13) suggested that such changes are more due to lifestyle or diet than to aging. The age-related decrease of the mitochondrial bioenergetic capability becomes dramatic for those postmitotic cells such as neurons and skeletal muscle fibers that heavily depend on oxidative metabolism for their power supply (10,14). In the literature, several examples of the mitochondrial dysfunction occurring in the aging process of skeletal muscle fibers in human (15,16), mouse (17), and rat (18) have been reported. Such mitochondrial bioenergetic decline has been demonstrated by means of various experimental approaches including histochemical techniques (16,19,20), which unveil at the cellular level the functionality of some mitochondrial respiratory chain enzymes and lead to the identification of different cellular phenotypes. The reactions for cytochrome c oxidase (COX) (21) and succinate dehydrogenase (SDH) (22) activities have allowed the detection of COX normal (COX-positive) and COX deficient (COX-negative) fibers as well as SDH normal (SDHn) and SDH hyperreactive (SDH+++) fibers. The last type of fibers corresponds to the ragged red fibers (RRF), revealed by the Gomori modified trichrome staining (23), that exhibit a subsarcolemmal mitochondrial proliferation. The age-related mitochondrial dysfunction has been studied also by means of molecular biology techniques, used for the detection of mitochondrial DNA (mtDNA) modifications (24), point mutations (25,26) and/or deletions both at whole tissue (5,27) and at single fiber levels (25,28). In particular, a large deletion (nt 8103-12936), encompassing 4834 bp (29,30) from a genomic region of rat mtDNA similar to that of the 4977 bp human mtDNA “common deletion” (31,32), and other large deletions have been detected and demonstrated to accumulate in various tissues of aging rat (5,33). As for the origin of such mtDNA deletions, it has been suggested that the very fast rate of mitochondrial metabolism in rat might continuously produce a high level of reactive oxygen species (ROS) (34) which might cause extensive oxidative damage to mtDNA as well as to other mitochondrial molecules. Because of the physical closeness to the respiratory complexes, the exposure to a high reducing environment in the mitochondrial matrix, and the lack of a histone-like protein coverage, mtDNA is particularly prone to oxidative damage. The age-related increase of ROS production, demonstrated also in rodent mitochondria (35,36), might originate the accumulation of the oxidized adduct 8-oxo-7,8-dihydro-2′-deoxyguanosine, OH8dG, and other modified bases, with aging, in mtDNA. Oxidized adducts might then induce the quite precocious formation of the “common deletion” (34,37,38) and/or of other mtDNA deleted species through one of the different hypothesized...
mechanisms (5). It has been postulated that the age-related accumulation of mtDNA mutations in the protein-coding genes might damage the mitochondrial bioenergetic capability leading to a severe dysfunction. The age-related increased oxidative damage to mtDNA might also affect the replicative machinery of the mitochondrial genome and induce changes in the mtDNA content. In mtDNA maintenance, a very relevant role is assigned to the mitochondrial transcription factor A (TFAM). TFAM expression appears to be affected by different kinds of stimuli influencing mitochondrial proliferation (39,40). Others (41) and some of us reported increased amounts of mtDNA (19) and TFAM (42) in aged human skeletal muscle samples, as well as increased amounts of mtDNA and TFAM in several rat tissues (43).

The goals of this study were the measurement of the mtDNA and TFAM contents in four different hind-limb skeletal muscles of adult rats (6 months old)—namely, soleus (S), tibialis anterior (TA), gastrocnemius (G), and extensor digitorum longus (EDL), the determination of their age-related changes, as well as the analysis of the lifetime evolution of some mitochondrial genotypic and phenotypic alterations. We report here that the content of mtDNA in the four hind-limb skeletal muscles of adult rat is in the rank order of S > TA > G > EDL and that the TFAM content is significantly higher in S than in the other muscles. As far as age-related changes, the mtDNA content decreases only in S and TA, whereas the TFAM content increases only in S. Quite early in life mtDNA deletions appear, and later on (at 15 months of age), fibers defective for some mitochondrial respiratory enzymes appear. In the oldest animals the highest frequencies of occurrence of mtDNA deletions as well as of fibers altered for some mitochondrial respiratory enzymes are found in S according to its highest mtDNA content and oxidative potential.

Methods

Muscle Samples

Male Wistar rats were maintained two per cage on a 12-hour light/dark cycle at 25°C, and had access to standard laboratory chow and water ad libitum. All procedures were in accordance with the guiding principles in the care and use of laboratory animals of Bari University. Thirty rats of different ages (3, 6, 12, 15, 18, and 28 months) were killed, and four hind-limb muscles (S, G, TA, and EDL) were quickly excised and immediately frozen in isopentane cooled by liquid nitrogen and stored in liquid nitrogen until analysis.

Determination of mtDNA Content

Total DNA was prepared from about 40–50 mg of skeletal muscle samples as described by Arnaudo and colleagues (44). Total DNA (5 µg) was digested with Pvu II (MBI-Fermentas, Vilnius, Lithuania) and run on a 0.35% agarose gel (Seakem Gold; FMC BioProducts, Rockland, ME). The gel was blotted onto a Hybond-N membrane (Amersham-Pharmacia, Little Chalfont, U.K.) and simultaneously hybridized with a mitochondrial and a nuclear probe. The mitochondrial probe was a 658-bp fragment obtained by polymerase chain reaction (PCR) using the primers DN (L 15758-15777) and DR (H 117-98) described by Dinardo and colleagues (43). Rat mtDNA nucleotide positions are according to Gadaleta and colleagues (45). The nuclear probe was a 413-bp fragment containing part of the 18S rRNA gene and subcloned in the TA vector (Clontech, Palo Alto, CA). Both probes were labeled by random priming (Random Primed DNA Labeling Kit; Roche, Basel, Switzerland) and used in a 10:1 ratio of the nuclear DNA (nDNA) probe to the mtDNA probe. Blotting, prehybridization, hybridization, and washings were carried out as described by Sambrook and colleagues (46). The filter was exposed to an X-ray film at −70°C with an intensifying screen, and the hybridization signals were quantified by densitometry with an LKB-Pharmacia Ultrascan-XL Laser densitometer equipped with GelScan-LX-Evaluation software (LKB-Pharmacia Biotechnology, Uppsala, Sweden).

Detection of TFAM Content

Rabbit anti-rat TFAM antiserum was a gift from Dr. H. Inagaki (Department of Chemistry, National Industrial Research Institute of Nagoya, Japan). Goat anti-rabbit immunoglobulin G–horseradish peroxidase (hrp) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-mouse actin immunoglobulin M (1:2500) and goat anti-mouse immunoglobulin M–hrp were purchased from Oncogene Research Products (Boston, MA). Total DNA was extracted with sodium dodecyl sulfate (SDS) gel-loading buffer (50 mM Tris–HCl [pH 8.0], 100 mM dithiothreitol, 2% SDS, 10% glycerol) and their amounts were quantified using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Total proteins (2.5 µg) were separated into 12% SDS-polyacrylamide slab minigels and electroblotted onto polyvinylidene difluoride membrane (Amersham-Pharmacia). The membrane was subjected to incubation with primary antiserum, washings, incubation with secondary antibodies, and final washings according to Frigeri and colleagues (47). Secondary antibodies were labeled with hrp and detection (ECL-Plus; Amersham-Pharmacia) was performed according to the supplier’s instructions. Antibodies were used at the following dilutions: the anti-TFAM primary antibody (1:2500), the antirabbit secondary antibody (1:5000), the anti-actin primary antibody (1:2000), the anti-mouse secondary antibody (1:5000). Autoradiographs were analyzed by laser densitometry with the Chemi Doc System and Quantity One software (Bio-Rad Laboratories). Preliminary titration experiments allowed us to establish the amounts of protein extracts (2.5–25 µg) which gave a signal in the linear range of the relation: densitometric value/blotted proteins. Different exposures of each western blot, in the time range between 1 second and 5 minutes, were taken to ensure the linearity of the response for both assayed proteins.

Detection of the 4834-bp-Long mtDNA Deletion

About 50 mg of skeletal muscle was used to extract nucleic acids (48). DNA (100 ng) was amplified by using the following primers: 7825-For (L 7825-7844) and 13117-Rev (H 13117-13099) for the 4834-bp-long mtDNA deletion (mtDNA4834; 459-bp product, annealing temperature 60°C), as reported in Pesce and colleagues (49). A secondary seminested PCR round, using one of the primers of the first
amplification (13117-Rev) and the new internal primer 7978-For (L 7978–7997) with a 1-μl aliquot of the first amplification, at an annealing temperature of 57°C, was performed to obtain a 306-bp product. PCR products were size-fractionated on a 1.5% agarose gel in 1× Tris-borate-EDTA buffer and visualized by ethidium bromide staining. Only those animals not showing the expected amplification products in two successive PCR rounds were considered to be not harboring the searched mtDNA deletion. The identity of the amplification products was confirmed by direct DNA sequencing as described elsewhere (50).

**Detection of Multiple mtDNA Deletions by Long Distance-PCR**

Used primers were 5881-For (L 5881–5905) and 15141-Rev (H 15141–15118) (48), and the PCR mixture contained 100 ng of DNA, 0.1 μM of each primer, 200 μM dNTPs, 1X reaction buffer (Takara Bio Inc., Shiga, Japan) and 2.5 U of Long Amplification Taq Polymerase (Takara) in a 50-μl reaction volume. The PCR conditions consisted of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 68°C for 1.5 minute for 15 cycles, followed by denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 68°C for 1.5 minutes plus 10 s/cycle for 15 cycles and a final extension of 7 minutes at 68°C in a Peltier Thermal Cycler (MJ Research, Watertown, MA). PCR products were separated on a 1.5% agarose gel (Amresco, Solon, OH) in 1X TBE buffer and visualized by ethidium bromide staining. To increase the sensibility of the technique and to exclude the possibility that the low-molecular-weight bands were due to PCR artifacts, a secondary seminested long-distance PCR (LD-PCR) round, very similar to the first, was performed on an aliquot of the first reaction mixture using the 15141-Rev primer and the new internal primer 5974-For (L 5974–5996).

**Histology**

Serial 8-μm-thick transverse sections from frozen muscles were cut with the Cryotome (HM 505 E-Microm, Walldorf, Germany), mounted on polylysine-coated glass slides, and stained for COX activity (21), SDH activity (22), and both activities. Fibers with abnormal accumulation of mitochondria, the so-called “ragged red fibers” (RRF) for their red staining in the subsarcolemmal region with the modified Gomori trichrome stain, appeared hyperreactive with the modified SDH stain (SDH<sup>++</sup>). The modified SDH stain was chosen to count the number of SDH<sup>++</sup> fibers (RRF) because it is more sensitive than the modified Gomori trichrome staining (23). Sections were viewed on a Zeiss transmitted light microscope (Carl Zeiss, Jena, Germany) and photographed using a 10× objective. In each specimen from 400 to 700 fibers of two to three representative fields were analyzed on a photomicrograph, and the percentage of those having no detectable COX activity and normal SDH activity (COXneg/SDHn) and no detectable COX activity and hyperreactive SDH activity (COXneg/SDH<sup>++</sup>) was calculated.

**Statistics**

Statistical analysis was carried out by using SPSS version 11.0 software (SPSS Inc., Chicago, IL). The comparison among the four hind-limb skeletal muscles was made using one-way analysis of variance (where data were normally distributed with equal variance). The Tukey test was used for multiple comparisons. Results in adult (6-month-old) and old (28-month-old) rats were compared using Student’s t test for unpaired data. Statistical significance for all tests was set at p < .05.

**Results**

**mtDNA Content**

The mtDNA content relative to nDNA content (mtDNA/nDNA) was measured in the four hind-limb skeletal muscles to verify its eventual differences among S, TA, G, and EDL in adult rats (6-month-old rats) as well as its muscle-specific changes with age.

From 23 to 25 samples of the four hind-limb muscles (S, TA, G, and EDL), derived from rats ranging from 3 to 28 months of age, were analyzed. The analysis was performed by hybridizing Pvu II-digested total DNA with a mitochondrial and a nuclear probe. The mitochondrial probe detected a band of 16.3 Kbp corresponding to linearized mtDNA, whereas the nuclear probe detected a 12-Kbp band, corresponding to a Pvu II fragment of the nuclear 18S rDNA gene. Figure 1A shows an example of Southern blot hybridization carried out on S samples from rats 6, 15, and 28 months of age. The ratio of the intensities of the two bands was used to estimate the amount of mtDNA relative to nDNA in each sample. Comparisons among the different muscles were made, setting equal to 1 the mtDNA/nDNA value of EDL from 6-month-old rats, as reported in Figure 1B. The mtDNA content increases in all examined muscles passing from the 3-month-old animals to the 6-month-old ones confirming that the postnatal development and/or differentiation of such skeletal muscles is completed at 6 months of age (51). At this age, comparing the mtDNA/nDNA values of the analyzed hind-limb muscles with that of S (3.0), TA has a value of 2.0 (33% less mtDNA than S), G has a value of 1.5 (50% less mtDNA than S), and the glycolytic muscle EDL has a value of 1.0 (66% less mtDNA than S) (Figure 1B). As statistically significant differences, p < .05. After 6 months of age, the mtDNA content shows in S a gradual decline reaching an overall statistically significant reduction of 52% of the initial value in the 28-month-old rats. The decrease of mtDNA content begins later in life in TA, after 15 months of age, so that the mtDNA content of the 28-month-old rat is reduced by 38% with respect to the 6-month-old rat. The mtDNA content in G shows a peculiar trend because it decreases from 6 months through 15 months of age and, after such age, it increases again reaching approximately the value of the 6-month-old animal; however, such age-related changes in this muscle never reach statistical significance. In EDL, there is no age-related change of the mtDNA content.

A comparison of the mtDNA relative content among the four hind-limb muscles in the 28-month-old rats shows that no more differences exist among TA and G versus S, whereas the mtDNA content is still significantly lower in EDL than in S (~41%) (Figure 1B). As statistically significant differences, p < .05.

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TFAM Content

Because TFAM content was recently reported by some of us (43) to increase with age in several rat tissues together with the mtDNA content, we measured the TFAM content in the four hind-limb muscles from 6- and 28-month-old rats. Total proteins from the same skeletal muscle samples used for the mtDNA content determination were analyzed by SDS-polyacrylamide gel electrophoresis, blotted onto a polyvinylidene difluoride membrane, and incubated with antibodies against TFAM and α-actin. Figure 2A shows a representative western blot carried out on soleus muscle (S) samples from rats 6 and 28 months of age. Top and bottom bands show, respectively, signals from rat α-actin protein and from rat TFAM protein. The histograms show for each age group of animals the mtDNA content (mtDNA/nDNA) of the four hind-limb skeletal muscles normalized to that of the extensor digitorum longus muscle (EDL) samples from 6-month-old rats (indicated by the broken line). Bars represent the average ± standard error of values obtained from three preparations of each sample analyzed in triplicate.

mtDNA Deletions

We detected in four hind-limb muscles the presence of the mtDNA4834 deletion, by using two-rounds of PCR with a nested primer. Figure 3A shows a representative gel for the detection of the 4834-bp deletion in three different skeletal muscles (S, EDL, and TA) from the same
28-month-old rat. The ethidium bromide-stained gel shows the expected PCR products (459 bp), deriving from the primary amplification of mtDNA harboring the “common deletion” only in S and TA. The seminested PCR on the same samples was necessary to reveal the presence of such deletion as a nested PCR product of 306 bp also in EDL (Figure 3B).

The number of analyzed animals really harboring the mtDNA4834 deletion (expressed as % of occurrence) in each of the four muscles from rats belonging to different age groups is reported in the Figure 4 insets. Such percentage of occurrence increases with age in the four analyzed muscles although with muscle-specific differences. In fact, mtDNA4834 appears in one of the five analyzed S samples from the 3-month-old rats, then the percentage of occurrence gradually increases up to 28 months of age when 100% of S samples harbor mtDNA4834.

In the other hind-limb muscles analyzed, mtDNA4834 appears for the first time at 6 months of age; thereafter, its occurrence increases gradually with age. In the oldest animals, a different percentage of occurrence is found in the various muscles: in fact, about 75% of analyzed animals bear such deletion in G and TA, whereas only 50% of the animals bear the deletion in EDL. G and TA show, at different ages, an intermediate percentage of occurrence between the values of S and EDL.

To obtain a more comprehensive representation of the presence of mtDNA deletions in each analyzed animal, the DNA extracted from the four hind-limb muscles was analyzed by LD-PCR (48). Figure 3C shows results of a typical LD-PCR experiment on DNA extracted from the S muscle of a 28-month-old rat. The ethidium bromide-stained gel shows multiple bands deriving from the amplification of deleted mtDNA species (Lane 1). Lane 2 shows the products deriving from the seminested LD-PCR carried out with an aliquot of the primary LD-PCR. Analyzing the gel containing 22–28 samples of muscles derived from rats of different ages, we found that each specimen has a distinctive pattern of mtDNA deletions. By counting the different bands obtained in all analyzed samples, we could detect at least 20 different mtDNA deleted species. This number is likely an underestimate, due to factors such as the resolution power of the gel, which prevented discrimination between bands having similar molecular weights. Figure 4 shows the number of mtDNA deleted species found in each analyzed specimen. All samples from rats younger than 28 months of age carried 0–2 deletions, whereas samples from 28-month-old rats had 0–11 deletions. More than one deletion was found earlier (6 months of age) in S than in the other muscles. Also, the percentage of samples harboring more than one deletion among the 28-month-old rats is muscle-specific: all samples from S, four from TA, three from G, and two from EDL of six analyzed samples for each muscle harbor more than one deletion.

**Histochemical Analysis**

To verify whether the genotypic alterations reported above were associated with the alteration of all fibers in a chosen muscle or a mosaic of fibers with different respiratory activi-

![Figure 3. Detection of the 4834-bp deletion and of multiple deletions by primary and seminested polymerase chain reaction (PCR). Gels are representative of PCR-amplified products. A, Ethidium bromide staining of a 1.5% agarose gel shows the 459-bp PCR products deriving from the primary amplification of the 4834-bp deletion in soleus (S) and tibialis anterior (TA) skeletal muscles of a 28-month-old rat. M₁ = DNA size standard (pBR DNA × Hinf I: 1631, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200 bp). C, Ethidium bromide staining of a 1.5% agarose gel shows the 306-bp products deriving from the seminested PCR on aliquots from the same reactions shown in gel A. M₂ = DNA size standard (3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200 bp). B, Ethidium bromide staining of a 1.5% agarose gel shows the 224-bp products deriving from the seminested PCR on aliquots from the same reactions shown in gel A. M₃ = DNA size standard (10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750 bp).](https://academic.oup.com/biomedgerontology/article-abstract/63/7/715/763340/719)
No COX-positive/RRF fibers were found in any of the examined muscles from each age group of animals. The overall percentage of COX-negative/RRF fibers in S, TA, and G, that is, the number of COX-negative/RRF counted in each specific sample, was around 0.5% in the oldest animals, whereas the overall percentage of COX-negative/SDHn fibers is 0.6% (not reported data).

DISCUSSION

In this study we report for the first time, to our knowledge, the mtDNA and the TFAM contents of four hind-limb skeletal muscles, namely S, TA, G, and EDL from adult Wistar rats as well as their age-related changes. Furthermore, to better characterize the aging process at mitochondrial level in these four hind-limb skeletal muscles, are reported also the age-related changes involving the percentage of occurrence of mtDNA4834, other deleted species, and some altered mitochondrial phenotypes.

It has been reported for a long time that the concentration of mtDNA is directly proportional to the oxidative capacity of mammalian striated muscles (53). More recently, He and colleagues (54) reported that the absolute copy number of mtDNA in single type I oxidative fibers of human skeletal muscle is double that in single type II glycolytic fibers. The rank order of mtDNA contents for the four hind-limb muscles in 6-month-old rats (from highest to lowest) is S > TA > G > EDL. Such order corresponds to the respective degrees of dependence on oxidative metabolism already reported for these muscles in adult Wistar and other rat strains (55–58). In particular, S has the highest mtDNA content relative to nDNA among the other age-matched muscles, in agreement with its highest mitochondrial content (56) and its fiber type composition (about 90% type I plus type IIA), whereas EDL has the lowest mtDNA content and a fiber type composition mostly glycolytic (about 70% type IIB) (55–58). TA and G have intermediate mtDNA content between S and EDL and a mixed fiber type composition (55–58).

TFAM content in the four muscles from adult (6-month-old) rats seems to correspond well to the respective mtDNA content. In fact, the TFAM amount shows the highest value in S, and progressively decreases in the age-matched muscles from TA to G to EDL. This further confirms the tight relationship already reported between the mtDNA content and the level of the transacting factor TFAM (39,59,60).

With aging, a decrease of mtDNA content begins earlier in S (from 6 months on) to progressively reach the lowest value in the 28-month-old samples. The results reported here for S muscle from Wistar rats are consistent with the age-related
decrease of the relative mtDNA content (from 6 months to 27 months) demonstrated in the same S muscle from Fisher rats by Barazzoni and colleagues (61). Furthermore, a more recent study reported the age-related changes of molecular and fiber composition in rat S (62). In that study, consistent with the results reported here, a loss of full-size mtDNA and an increase of deleted species as well as a decrease of COX activity in S of 28-month-old rat were found. Furthermore, the same authors reported a high variability in old rat S of the fiber type composition and atrophic changes accompanied by the slow–to-fast fiber type transition. The fiber type transition can explain the loss of mtDNA in S reported here. The mixed fiber type muscle TA also shows an age-related progressive decrease of its mtDNA content. However, mtDNA loss begins later in life (from 15 months on) in this muscle and proceeds faster than in the slow-twitch predominant S. In the above-cited article by Barazzoni and colleagues (61), the reported decrease of mtDNA content with aging in both separated portions of G was smaller than that in S and was explained as a common trend of the skeletal muscle group. Our data about the mtDNA content in G come from the joined portions of muscle isolated from a rat strain different from that used in (61); furthermore, our data were determined at six distinct ages, unveiling a more complex trend in mtDNA content changes during the life span that, however, never reached statistical significance.

As far as TFAM content is concerned in aging rats, an increase of both mtDNA and TFAM contents in rat cerebellum, kidney, and liver (but not in heart) has been found by some of us (43), whereas in the muscles studied here the muscle-specific differences prevail over a common trend (Figure 2). In fact, in the 28-month-old samples from S,
there is an age-related increase of the protein that apparently is in contrast with the contemporaneous decrease of the mtDNA content. Although TFAM content was measured in the homogenate of the four muscles and the COX-negative/RRF fibers were reported as percentage of occurrence in the examined samples, one possible explanation of the level of TFAM in 28-month-old rat could be that the gradual decrease of mtDNA with age in S induces TFAM expression to compensate for the loss of mtDNA with mitochondrial proliferation. However, because such proliferating mitochondria should contain mainly mutated mtDNA molecules (33), all examined samples of S from 28-month-old rats show COX-negative/RRF. A similar hypothesis might explain the age-related increase of TFAM in G from the 28-month-old animals and the decrease of the TFAM amount in age-matched TA, only a few samples of which harbor COX-negative/RRF fibers. As for EDL, again, there is consistency between the lack of any age-related change both in the mtDNA content and in the TFAM amount and the total absence of COX-negative/RRF fibers.

Conclusion
A good consistency between the mtDNA content and the respective dependence on oxidative metabolism, according to the literature (55–58), in different hind-limb muscles from adult rats has been found as well as between mtDNA content and TFAM content. An age-related trend common in the analyzed muscles includes (a) the increase in the occurrence of mtDNA$^{1834}$ and of multiple mtDNA deletions that might be responsible for the decreased activity of COX shown by the histochemical results and (b) the increase in the occurrence of COX-negative/SDHn and COX-negative/RRF phenotypes. Muscle-specific differences with aging seem to be linked essentially to the mtDNA and TFAM contents of the examined skeletal muscles. In agreement with the mitochondrial theory of aging, the S muscle, which contains more mtDNA than the other three examined muscles do, seems to be more exposed to oxidative stress and ROS production, whereas EDL seems to be almost unaffected by the age-related oxidative stress, and TA and G have an intermediate behavior. On the whole, it should be noted that the occurrence of mitochondrial genotypic and phenotypic alterations is very low in our oldest rats (28-month-old), whereas the loss of mtDNA seems to be the major age-linked change. If this is the final outcome of a remote process that has caused the life-long continuous loss of damaged fibers leading to sarcopenia or the real picture of a small entity process in aging rat skeletal muscle still needs to be conclusively discovered through further research.

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REFERENCES


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