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A comparison among the tissue-specific effects of aging and calorie restriction on TFAM amount and TFAM-binding activity to mtDNA in rat

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ABSTRACT

Background: Mitochondrial Transcription Factor A (TFAM) is regarded as a histone-like protein of mitochondrial DNA (mtDNA), performing multiple functions for this genome. Aging affects mitochondria in a tissue-specific manner and only calorie restriction (CR) is able to delay or prevent the onset of several age-related changes also in mitochondria.
Methods: Samples of the frontal cortex and soleus skeletal muscle from 6- and 26-month-old *ad libitum*-fed and 26-month-old calorie-restricted rats and of the livers from 18- and 28-month-old *ad libitum*-fed and 28-month-old calorie-restricted rats were used to detect TFAM amount, TFAM-binding to mtDNA and mtDNA content.
Results: We found an age-related increase in TFAM amount in the frontal cortex, not affected by CR, *versus* an age-related decrease in the soleus and liver, fully prevented by CR. The semi-quantitative analysis of *in vivo* binding of TFAM to specific mtDNA regions, by mtDNA immunoprecipitation assay and following PCR, showed a marked age-dependent decrease in TFAM-binding activity in the frontal cortex, partially prevented by CR. An age-related increase in TFAM-binding to mtDNA, fully prevented by CR, was found in the soleus and liver. MtDNA content presented a common age-related decrease, completely prevented by CR in the soleus and liver, but not in the frontal cortex.
Conclusions: The modulation of TFAM expression, TFAM-binding to mtDNA and mtDNA content with aging and CR showed a trend shared by the skeletal muscle and liver, but not by the frontal cortex counterpart.
General significance: Aging and CR appear to induce similar mitochondrial molecular mechanisms in the skeletal muscle and liver, different from those elicited in the frontal cortex.

1. Introduction

Mitochondrial Transcription Factor A (TFAM) is a High Mobility Group protein, encoded by nuclear DNA and mainly directed to mitochondria [1], which was discovered and characterized initially for its function in mitochondrial transcription [2]. Several subsequent studies revealed a number of different roles performed by TFAM in the organelle [3,4]. Because of the close connection between mitochondrial DNA (mtDNA) transcription and replication [5], TFAM has become an attractive candidate for the regulation of mtDNA copy number [6]. Furthermore, TFAM is involved in the constitution of mtDNA nucleoids [7] and appears to be a likely member of a system responsible for sensing and repair of oxidative damage to mtDNA [8,9], so that it exerts histone-like functions for mtDNA. Due to the relevant involvement in the regulation of mitochondrial biogenesis and transcription, TFAM expression has been investigated in several rat tissues [10] in different physiological conditions, including aging (liver, heart, cerebellum, kidney [11,12], hind-limb muscles [13] and frontal cortex [14]). Aging is a very complex phenomenon that implies the progressive structural and functional decline of tissues also through the age-dependent dysfunction of the mitochondrial respiratory complexes, reducing the amount of synthesized adenosine triphosphate (ATP) [15]. The involvement of mitochondria in aging is tissue-specific [16] and is particularly relevant within tissues such as the brain, heart and skeletal muscle that have a high dependence on oxidative metabolism [17]. However, other metabolically very active tissues, such as the liver, are also affected by age-related mitochondrial dysfunction [15]. Another relevant and common feature of aging is an increased presence of reactive oxygen species (ROS), mainly by-products of the mitochondrial respiratory complexes, resulting in the onset of oxidative stress. This originates oxidative damage to mitochondrial proteins, lipids and DNA and contributes to the aging phenotype [18]. In particular, mtDNA can undergo several kinds of qualitative and quantitative alterations that affect its structure and function. Quantitative alterations, namely changes in mtDNA content, were reported in various tissues from humans and

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animals [11–14,19–23]. To date, the only established experimental approach shown to delay or prevent the onset of several age-related alterations, also in mitochondria, in organisms ranging from yeast to man, is calorie restriction (CR) [24,25]. Such treatment involves the administration of a nutrient dense diet that reduces calorie intake by 40% [26] and exhibits a very marked tissue-specificity [16]. Among the likely multiple cellular mechanisms at the basis of CR efficacy, the reduction of age-related oxidative stress has obtained large credit [25,27]. Recently, however, the regulation of mitochondrial metabolism through activation of the PGC-1α-dependent cascade of mitochondrial biogenesis has also gained a wide consensus as one of the means by which CR counteracts the age-dependent dysfunction [28]. Little is known about the effects of CR on mitochondrial age-related phenotypic and genotypic alterations [29–33]. The tissue-specific effects of CR on mtDNA content [12,14,21] and TFAM amount [12,14] in aged rats have been reported. TFAM is a key factor in mtDNA replication and maintenance and the study of its possible role in mtDNA protection and repair from oxidative damage appears very interesting. Therefore, given the importance of TFAM-binding to mtDNA for various mitochondrial functions, it was very appealing for us to determine and compare TFAM amount and its binding to functionally relevant regions of mtDNA as well as mtDNA content during aging and CR in various rat tissues. We analyzed frontal cortex, soleus skeletal muscle and liver, featuring a distinct dependence on oxidative metabolism, with the aim of unveiling eventual tissue-specific differences in a situation of physiological oxidative stress, such as aging, and in CR. Our results demonstrated, as for the regulation of TFAM amount and mtDNA content and the modulation of TFAM-binding to mtDNA, a trend that was shared by the skeletal muscle and the liver but not by the frontal cortex counterpart.

2. Materials and methods

2.1. Samples

The study was approved by the Institutional Animal Care and Use Committee at the University of Florida. All procedures were performed in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. The frontal cortex, soleus skeletal muscle and liver samples used in this study were, respectively, from Fischer 344 male rats obtained from the NIH barrier-raised rodent colony (frontal cortex and soleus muscle) or from Fischer 344 × Brown Norway (F344BNF1) male rats obtained from the National Institute of Aging colony (Indianapolis, IN) (liver) and housed at the Department of Aging and Geriatric Research, Division of Biology of Aging, College of Medicine, University of Florida, Gainesville, FL. The animals consisted of the following groups: 6-month-old *ad libitum*-fed (AL-6, n = 8 for frontal cortex; n = 5 for soleus muscle), 26-month-old *ad libitum*-fed (AL-26, n = 7 for frontal cortex; n = 6 for soleus muscle), and 26-month-old calorie-restricted (CR-26, n = 7 for frontal cortex; n = 5 for soleus muscle); 18-month-old *ad libitum*-fed (AL-18, n = 6 for liver), 28-month-old *ad libitum*-fed (AL-28, n = 8 for liver) and 28-month-old calorie-restricted (CR-28, n = 7 for liver) rats. CR had been initiated at 3.5 months of age (10% restriction), raised to 25% restriction at 3.75 months, and kept at 40% restriction from 4 months until the end of each animal's life. The animals were anesthetized before being sacrificed and samples from the frontal cortex, soleus muscle, and liver were immediately removed, snap-frozen in isopentane cooled by liquid nitrogen, and stored in liquid nitrogen until further analysis.

2.2. Western blotting

Total proteins were extracted from all tissue samples obtained from AL-6, AL-18, AL-26, AL-28, CR-26, and CR-28 animals. Approximately 100 mg of each frozen sample was grounded and suspended in 600 μl of lysis buffer (220 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 5 mM EGTA, and 1 mM EDTA). Cell lysates were pre-cleared by centrifugation in an Eppendorf microfuge at 12,000 rpm for 10 min and the supernatant fraction containing proteins was recovered. Proteins were quantified with the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the supplier's instructions. Total proteins (10 μg) were separated by gel electrophoresis on 4–12% Bis–Tris Criterion XT precast gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) and electroblotted onto PVDF membranes (Amersham-Pharmacia Biotech Inc., Piscataway, NJ, USA). The membranes were blocked for 1 h in 5% milk in 1×-PBS/Tween 20 (0.15 M NaCl, 0.1 mM KH_2PO_4, 3 mM Na_2HPO_4, 0.1% Tween 20) and probed with TFAM (1:25,000) and β-actin (1:10,000; Sigma-Aldrich Corp., St. Louis, MO, USA). The antibody against TFAM was custom-made using as antigen in rabbit the protein expressed from the clone containing the peptide fraction corresponding to aa 35 to 201 of the rat protein and donated by Dr. H. Hinagaki (Department of Chemistry, National Industrial Research Institute of Nagoya, Japan). Membranes were then incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) at a dilution of 1:10,000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membranes were washed in PBS (3 × 15 min) and proteins were subsequently visualized with an enhanced chemiluminescence kit (ECL-Plus; Amersham-Pharmacia Biotech Inc., Piscataway, NJ, USA). Autoradiographs were analyzed by laser densitometry with the Chemi Doc System and Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). The densitometric value of OD units of each TFAM band was then related to the OD unit number of the respective band of the β-actin (in the corresponding lane).

2.3. Mitochondrial DNA immunoprecipitation (mIP)

The binding *in vivo* of TFAM to specific regions of mtDNA was analyzed using mitochondrial DNA immunoprecipitation (mIP) following the procedure described by Picca et al. [14]. 250 μl of a cold formaldehyde cross-linking solution 1× (1% formaldehyde, 5 mM HEPES pH 8.0, 10 mM NaCl, 0.1 mM EDTA pH 8.0, 0.05 mM EGTA) was added to 100 mg of frozen tissue. Cross-linking was terminated by adding 0.125 M glycine and incubating at room temperature for 10 min. Tissues were briefly homogenized and centrifuged twice at 3000 rpm for 5 min. Each pellet was washed with 1 ml of RB (Resuspending Buffer, 135 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4 pH 7.4), suspended with 1 ml of homogenization buffer (100 mM Tris–HCl pH 8.0, 100 mM NaCl, 30 mM $MgCl_2$, 0.1% NP-40, 0.1 mM PMSF), and manually homogenized. The sample was centrifuged at 3000 rpm for 5 min and the pellet was incubated in lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris–HCl pH 7.4, containing 100 μg/ml leupeptin and 200 μM PMSF), for 10 min at RT. Cellular DNA was sheared by sonication and the size of DNA fragments, ranging between 500 and 900 base pairs, was checked by electrophoresis on an ethidium bromide-stained 1.2% agarose gel in 1× TAE buffer (20 mM Tris acetate, 50 mM EDTA pH 8.3). Each sample was diluted with FSB buffer (5 mM EDTA, 20 mM Tris–HCl pH 7.5, 50 mM NaCl) and pre-cleared with 75 μl of protein A-agarose/Salmon sperm 50% DNA (Millipore Corporate Headquarters, Billerica, MA, USA) for 2 ml of sample on a rotator at 4 °C for 30 min. After a centrifugation at 1000 rpm for 1 min, each sample was divided into four aliquots: the first one (100 μl) was not immunoprecipitated (input, stored at −80 °C until further use); the other three aliquots (100 μl each) were incubated overnight at 4 °C, respectively, with a rabbit anti-TFAM antibody (1:50 dilution), a non-specific rabbit anti-β-actin antibody (1:100 dilution) and without antibody (−Ab). On the following day, 15 μl of protein A-agarose was added to each sample for 2 h at 4 °C to isolate protein–DNA complexes. The samples were centrifuged at 1000 rpm for 1 min, and the pellets were washed: three times with 1 ml of 140 mM NaCl RIPA buffer (10 mM Tris–HCl pH 8.0, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate, 1 mM EDTA, 140 mM NaCl); three times with 1 ml of 500 mM NaCl RIPA buffer (as above but with 500 mM NaCl); three times with 1 ml of LiCl buffer (10 mM Tris–HCl pH 8.0,