FEBS Letters 501 (2001) 74–78 FEBS 25041

Increased expression of mitochondrial transcription factor A and nuclear respiratory factor-1 in skeletal muscle from aged human subjects

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Received 6 June 2001; accepted 16 June 2001

First published online 28 June 2001

Edited by Gunnar von Heijne

Abstract The expression of two factors involved in the nuclear–mitochondrial crosstalk, namely the mitochondrial transcription factor A (TFAM) and the nuclear respiratory factor-1 (NRF-1), was studied in human skeletal muscle biopsies of young and aged subjects. Aged subjects presented a 2.6-fold and an 11-fold increase of the levels of TFAM protein and TFAM mRNA, respectively. The increased expression of TFAM was associated to the doubling of NRF-1 DNA-binding affinity and to a 6-fold increase of NRF-1 mRNA level. The upregulation of TFAM and NRF-1, in aged skeletal muscle, appears involved in the pathway leading to the age-related increase of mitochondrial DNA content.

Key words: Mitochondrion; Aging; Skeletal muscle; TFAM expression; NRF-1 expression

1. Introduction

Aging is a very complex phenomenon involving several changes both at cellular and at organism levels [1,2]. Recently, we studied phenotypic and genotypic changes of mitochondria in a large collection of skeletal muscle specimens from healthy Italian subjects of different ages. We found that skeletal muscle specimens from aged subjects were characterized by the presence of cytochrome *c* oxidase (COX)-negative/ragged red fibers (RRF), that is fibers lacking cytochrome *c* oxidase activity and with subsarcolemmal mitochondrial proliferation [3], and by an increased content of mitochondrial DNA (mtDNA) [4]. This last change might be an attempt of the cell to counteract the age-related dysfunctions of the energetic metabolism (reviewed in [1,5–7]). The increase of mtDNA content should be pursued through the action of the nucleus-encoded regulatory factors involved in the nuclear–mitochondrial crosstalk. Among such factors a relevant role is exerted by mitochondrial transcription factor A (TFAM) and nuclear respiratory factor-1 (NRF-1). TFAM is a mtDNA-binding protein, essential for maintenance, replication and transcription of mtDNA [8], whose expression is under the control of *trans*-acting factors such as NRF-1, NRF-2 and Sp1, with NRF-1 being, apparently, the major determinant [9]. NRF-1 controls not only TFAM expression but also that of many other nuclear genes involved in mitochondrial biogenesis and respiratory function: mtDNA instability and embryonic lethality were reported in the NRF-1 knockout mouse [10]. NRF-1 should, therefore, play an important role in coordinating the expression of nuclear and mitochondrial genomes in order to balance the cell energy demands and supplies [11].

Expression of TFAM was reported to be altered in skeletal muscle specimens from patients with mtDNA depletion [12,13] as well as in chemically mtDNA-depleted cells [12,14]. An increased DNA-binding activity of NRF-1 together with an increase of its mRNA level as well as mitochondrial biogenesis were associated to doubled oxygen consumption due to the induced expression of uncoupling protein (UCP)-1 in HeLa cells [15].

To evaluate the involvement of TFAM and NRF-1 in aging, we analyzed the expression of these two factors in the skeletal muscle biopsies from a group of young and aged subjects, already characterized for COX-negative/RRF and mtDNA content [4]. The data here reported show that the expression of both TFAM and NRF-1 increases in aged subjects with respect to young ones, supporting their role in the mitochondrial compensatory response to age-related energetic metabolism dysfunctions.

2. Materials and methods

2.1. Muscle samples

Skeletal muscle biopsies (200–400 mg) from *Vastus lateralis* were obtained with informed consent from four young (ages 21–33 years) and seven aged (ages 71–88 years) healthy Italian subjects, with an ordinary physical activity, undergoing routine orthopedic surgery in the opposite leg. All subjects had a normal medical history and no clinical evidence of mitochondrial pathology. The protocol of this study was approved by the Ethical Committee of the 'G. D'Annunzio' University of Chieti. Muscle specimens were immediately frozen in isopentane cooled by liquid nitrogen and stored in liquid nitrogen until analysis.

2.2. Western blotting

Rabbit anti-human TFAM antiserum was a kind gift of Dr. R. Wiesner (Institute of Physiology, University of Koln, Germany). Goat anti-human α-actin antiserum was purchased from Santa Cruz Biotechnology. Total proteins were extracted with Trizol reagent (Gibco BRL-Life Technologies) and quantitated using the Bradford

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PII: S0014-5793(01)02628-X

method (Bio-Rad) according to the supplier's instructions. 5 μg of total proteins were separated in 12% sodium dodecyl sulfate (SDS)–polyacrylamide slab minigels and electroblotted onto PVDF membrane (Amersham-Pharmacia). The membrane was subjected to incubation with primary antiserum, washings, incubation with secondary antibodies and final washings according to Frigeri et al. [16]. Secondary antibodies were labeled with horseradish peroxidase 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 (1:5000), the anti-rabbit secondary antibody (1:2500), the anti-actin primary antibody (1:1000), the anti-goat secondary antibody (1:2500). Autoradiographs were analyzed by laser densitometry with the Ultrascan XL LKB (Pharmacia LKB Biotechnology) equipped with the GEL-SCAN XL evaluation software. Preliminary titration experiments allowed to establish the protein extracts range (2.5–25 μg) which gave a linear response of the signal. Different exposures of each Western blot, in the time range between 30 s and 2 min, were taken in order to ensure the linearity of the response for both assayed proteins.

2.3. Electrophoretic mobility shift assay (EMSA)

The nuclear protein extracts were prepared by using the method of Blough et al. [17], with an average yield of 1–2 μg of protein/mg of muscle tissue. The protein yield was similar for all assayed samples indicating no age effect on this parameter. Electrophoretic mobility shift assays were carried out in a 20 μl reaction mixture containing 50 μg nuclear protein extract, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 75 mM KCl, 0.1 mM EDTA, 1 mM DTT [18], 2 μg of sonicated salmon sperm DNA, 2 μg BSA and 100 fmol of a labeled probe containing a functional NRF-1-binding site from the δ-aminolevulinate synthase promoter [15]. Preliminary titration experiments allowed to establish the range of nuclear protein extracts (20–70 μg) giving a linear response of the signal. Competition experiments were carried out with 100 fmol of a probe containing a mutated NRF-1-binding site [15] to verify the binding specificity. Recombinant NRF-1 was obtained from a full-length NRF-1 cDNA clone in pGEM-7Zf(+) vector, kindly provided by Dr. R. Scarpulla (Northwestern University Medical School, Chicago, IL, USA).

2.4. Detection and quantitative determination of mRNAs by RT-PCR

Total RNA was isolated with Trizol reagent (Gibco BRL-Life Technologies) according to the supplier's instructions. The used primers for reversed transcription-polymerase chain reaction (RT-PCR) were: GD67A (forward 837–856) and GDMID9B (reverse 1409–1390) from [12] for glucocerebrosidase (Glace) mRNA, NRF-876F (forward 876–895) and NRF-1138R (reverse 1138–1119) from [19] for NRF-1 mRNA and TFA-563F (forward 563–586) and TFA-726R (reverse 726–705) for TFAM mRNA. In all assays 0.2 μM gene-specific primers, 200 μM dNTP, 1.2 mM magnesium sulfate and titan one tube RT-PCR system (Roche Molecular Biochemicals) were added, according to the supplier's instructions, to 150–750 ng of total RNA. A standard RT-PCR profile included: the RT phase of 30 min at 55°C and 2 min at 94°C and the PCR phase of 1 min at 94°C, 1 min at the annealing temperature of the primers couple (57°C for NRF-1, 59°C for TFAM and 60°C for Glace) and 1 min at 68°C for a number of cycles ranging from 20 to 40 depending on the template mRNA species. Dideoxy sequencing was preliminary performed on the cDNA products derived from the RT-PCR reactions. For quantitative RT-PCR the reaction mixture contained: 20 μM dNTP and 20 μCi [α-^{32}P]dATP (3000 Ci/mmol, NEN Life Science Products). Aliquots of 5 μl were taken from the mixture at determined cycle numbers and run on 5% polyacrylamide slab minigels. The specific radiolabeled bands, identified by autoradiography, were excised from the gel, dried at 80°C for 4 h in scintillation vials and counted in Maxifluor (Mallinckrodt Baker B.V.). The incorporated radioactivity was transformed in concentration (moles/μl) and plotted versus the number of cycles in a semilogarithmic graph. The extrapolation to 0 cycle of the experimental line allowed the determination of the initial concentration of the template mRNA species without need of an external or internal standard for comparisons, according to Garstka et al. [20].

2.5. Statistics

Results in young and aged subjects were compared using Student's *t*-test for unpaired data; *P* values of less than 0.05 were considered statistically significant.

3. Results

In this study skeletal muscle specimens from young and aged healthy subjects, already characterized for COX-negative/RRF and mtDNA content [4], were analyzed for the expression of TFAM and NRF-1. The here examined aged subjects, with respect to the young ones, presented COX-negative/RRF and an average 2.5-fold higher mtDNA content.

The level of TFAM in skeletal muscle biopsies was determined by immunoblotting experiments with a human TFAM antiserum. The TFAM content of each aged subject was higher than that of all young counterparts (Fig. 1). By pooling the samples in two groups, one including the four young individuals and the other the seven aged ones, the mean value ± S.D. in the aged group was 2.5 ± 0.5; the counterpart in the young subjects group was 0.94 ± 0.3. A statistically significant ($P = 0.00052$) 2.6-fold increase of the TFAM protein level was found.

In the same skeletal muscle specimens, except those of the 29-year-old and the 75-year-old subjects not anymore available, the level of TFAM mRNA was determined by a kinetic RT-PCR method. The assay is exemplified in Fig. 2A, which shows a representative autoradiography of a gel for the 164 bp RT-PCR product of TFAM mRNA, and in Fig. 2B, that reports the derived semilogarithmic plot. In order to compare different subjects, each value was normalized with respect to the sample-specific concentration of the constitutive transcript of the glucocerebrosidase (Glace) gene [12], whose concentration was determined with the same method (data not shown). Fig. 2C shows that the ratio between TFAM and Glace mRNAs was higher in the aged subjects compared to the young ones. By pooling together the values according to subject ages as above, the mean value ± S.D. in the young subjects group was 8.2 ± 3.9; the counterpart in the aged group was 91.6 ± 48.8. A statistically significant ($P = 0.00015$) 11-fold increase of the TFAM mRNA level was found in aging.

Since a reliable estimate of the NRF-1 level by Western

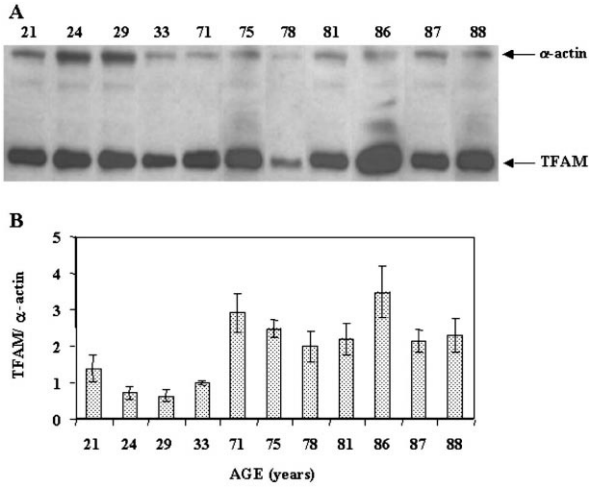

Fig. 1. Age-related increase of TFAM protein level in human skeletal muscle. 5 μg of total protein extract for each sample was subjected to Western blotting. A: Representative experiment with all assayed samples. Numbers on top of lanes indicate ages of subjects. B: Ratio between the densitometric quantitations of TFAM and α-actin in each specimen. All values are referred to that of the 33-year-old subject set equal to 1 and represent the mean ± S.D. of three independent experiments.

blotting was not obtainable because of the low amount of the protein [15], NRF-1 DNA-binding activity was evaluated: electrophoretic mobility shift assay (EMSA) of nuclear proteins extracted from the eight, still available, skeletal muscle samples was performed. By using a probe containing a functional NRF-1-binding site [15] a retarded band was visible, as shown in a representative gel shift experiment (Fig. 3A, lane 2). The band was abolished by substituting the functional probe with one containing a mutated NRF-1-binding site (Fig. 3A, lane 3) and was markedly decreased by adding a 100-fold molar excess of the unlabeled functional probe (Fig. 3A, lane 4). A NRF-1-specific retarded band, having the same mobility as that obtained with the recombinant NRF-1 (Fig. 3B, lane C), was evident in all assayed skeletal muscle specimens (Fig. 3B). Quantitation of the band shift data shows that in each aged subject the binding affinity of NRF-1 was higher than that in all young counterparts (Fig. 3C). By pooling together the results according to subject ages as above, in the aged subjects group the mean value ± S.D. was 2.5 ± 0.2; that in the young one was 1.2 ± 0.1. A statistically significant

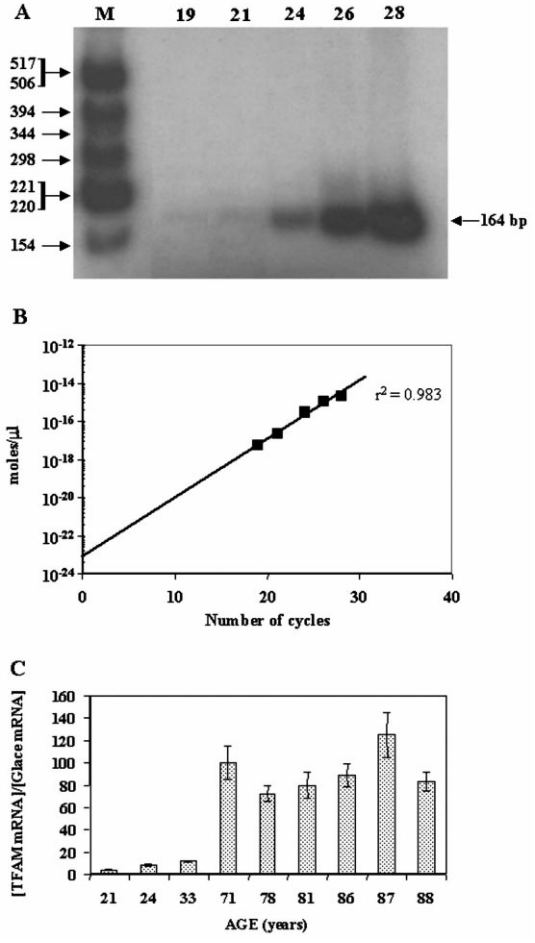

Fig. 2. Age-related increase of TFAM mRNA level in human skeletal muscle. Quantitative RT-PCR of total RNA with specific primers for TFAM. A: Representative experiment with the 78-year-old subject mRNA. Numbers on top of lanes indicate the cycles number. M (bp size marker) is [α-^{32}P]-labeled pBR322×*Hin*fI. B: Semilogarithmic plot of the signals shown in A. C: Ratio between the concentrations of TFAM mRNA and Glace mRNA in all assayed subjects. Values represent the mean ± S.D. of three independent experiments.

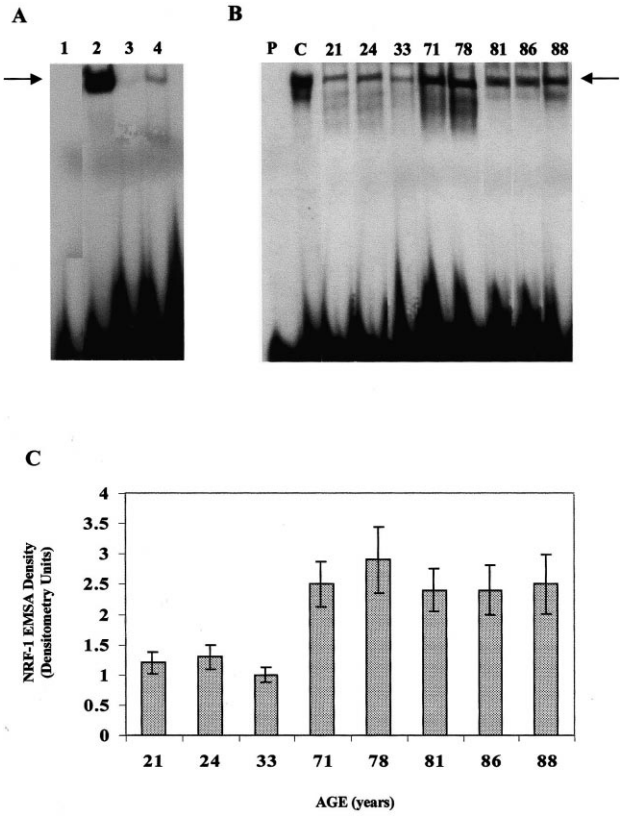

Fig. 3. Age-related increase of NRF-1-binding activity in human skeletal muscle. 50 μg of nuclear protein extract for each sample was subjected to EMSA with different probes. A: Representative competition experiments with the 78-year-old subject nuclear extract. The position of the NRF-1-specific gel shift is indicated by the arrow. From left to right are shown: the probe containing a functional NRF-1-binding site without added protein (lane 1), the same functional probe with added nuclear protein (lane 2), the probe with a mutated NRF-1-binding site with the added protein (lane 3), the functional probe diluted by a 100-fold molar excess of the same unlabeled probe with the added protein (lane 4). B: Representative experiment with all assayed samples. Numbers on top of lanes indicate ages of subjects. Lane P contains probe only, lane C contains the reaction with human recombinant NRF-1. The intense radioactive band at the bottom of the gel is free probe about to run off. C: Densitometric quantitation of NRF-1 gel shifts for each specimen. All values have been referred to that of the 33-year-old subject set equal to 1 and represent the mean ± S.D. of three independent experiments.

($P = 0.00006$) doubling of the NRF-1-binding capacity was verified.

Finally, we measured by RT-PCR the level of NRF-1 mRNA in the same nine samples assayed for TFAM mRNA level. Fig. 4A reports a representative kinetics experiment showing the cycle-dependent increase of the 262 bp product of NRF-1 mRNA, whereas the corresponding semilogarithmic plot is shown in Fig. 4B. After normalizing each value with respect to Glace mRNA it appears that NRF-1 mRNA level was higher in all aged subjects than in each young subject (Fig. 4C). By pooling together the values, the mean value ± S.D. in the young subjects group was 2.2 ± 1.6; the counterpart in the aged group was 13.7 ± 2.1. A statistically significant ($P = 0.00008$) 6-fold increase of the NRF-1 mRNA level in the aged individuals versus the young counterparts was found.

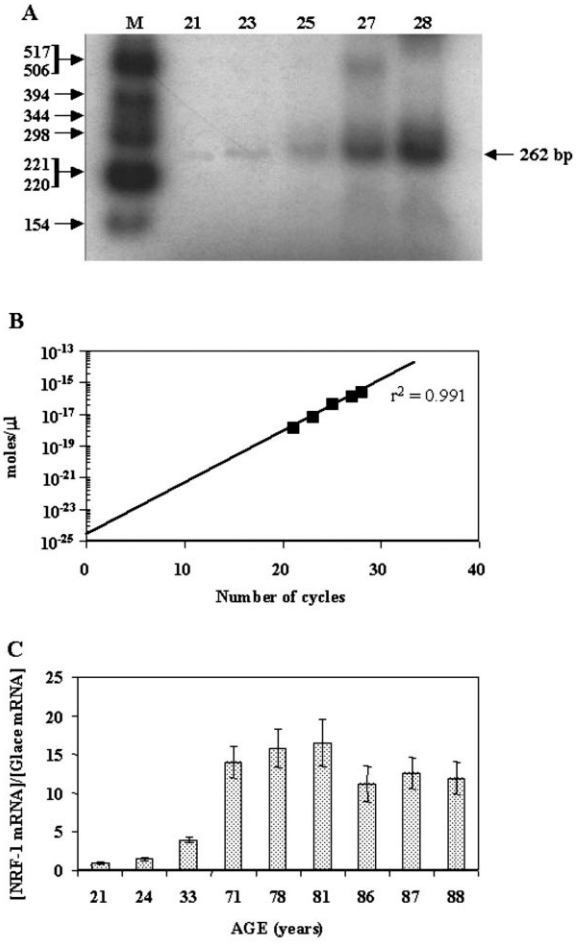

Fig. 4. Age-related increase of NRF-1 mRNA level in human skeletal muscle. Quantitative RT-PCR of total RNA with specific primers for NRF-1. A: Representative experiment with the 86-year-old subject mRNA. Numbers on top of lanes indicate the cycles number of RT-PCR. M (bp size marker) is [α-^{32}P]-labeled pBR322×*Hin*fI. B: Semilogarithmic plot of the signals shown in A. C: Ratio between the concentrations of NRF-1 mRNA and Glace mRNA in all assayed subjects. Values represent the mean ± S.D. of three independent experiments.

4. Discussion

This is the first report about the expression of two *trans*-acting factors deeply involved in the regulation of mitochondrial biogenesis in a physiological situation as aging. The results here reported demonstrate an increased expression of TFAM and NRF-1 in the same aged skeletal muscle samples where COX-negative/RRF and an increased mtDNA copy number were found. The 2.6-fold increase of the TFAM amount in aged subjects with respect to young ones closely corresponds to the average increase of the mtDNA content in the same specimens (2.5-fold); this suggests that the ratio between mtDNA and TFAM levels is maintained constant in skeletal muscle during aging. Such a close relationship between TFAM and mtDNA was already demonstrated in the TFAM knockout mouse [8] and in pathologies with mtDNA depletion [12,13]. The increase with age of TFAM expression seems to be transcriptionally regulated because of the increased TFAM mRNA level. However, the difference in the magnitude of mRNA and protein increases suggests that the amount of TFAM during aging might be regulated also by a post-transcriptional mechanism as changes in translation rate or in protein turnover. The upregulation of the TFAM gene appears to be dependent on an increased activity of NRF-1, as suggested by its increased DNA-binding affinity in aged subjects. Such an increase might be due to changes in the phosphorylation state of this factor with age. A phosphorylation of specific serine residues within the DNA-binding domain of NRF-1 has been, in fact, reported, resulting in a marked stimulation of the protein-binding activity [21]. However, the simultaneous increase with age of NRF-1 mRNA level suggests that the raised NRF-1-binding activity might also depend on an increased amount of the corresponding protein. The level of NRF-1 mRNA is lower than that of TFAM mRNA both in young and in aged subjects. This is what should be expected, being NRF-1 positioned upwards to TFAM in the cascade of regulatory steps leading to mitochondrial proliferation [9,11].

The data here reported raise the question about the signals triggering, in skeletal muscle of aged subjects, the upregulation of NRF-1 and of TFAM and, consequently, the increase of mtDNA copy number and, eventually, the mitochondrial proliferation in COX-negative/RRF. A large number of evidences link the production of ROS with aging [1,5–7]. Various stress responsive genes, including oxidative stress-inducible genes, have been reported to be induced in aging mouse skeletal muscle [22]. It is possible that, similarly to what occurs with other ROS-sensitive transcription factors as NF-κB, HSF and YAP1 [23], ROS themselves, mainly as H_2O_2, might be the molecular message that, escaping from defective mitochondria, reaches the nucleus activating mitochondrial biogenesis [24,25]. In addition to this, as already suggested, mitochondrial uncoupling, that might occur as a preliminary response of mitochondria to an increased ROS level [26], by raising the intracellular calcium level, might activate calcium-regulated kinases that could phosphorylate NRF-1 and induce the mtDNA content increase and, eventually, mitochondrial proliferation [15].

Acknowledgements: We thank Dr. R. Wiesner for anti-human TFAM antiserum; Dr. R. Scarpulla for anti-human NRF-1 antiserum and recombinant NRF-1 clone in pGEM-7Zf(+) vector. We are grateful to Ms R. Longo for word processing and S. Cortellino and L. Valletta for technical assistance. The work was supported by grants from Sigma Tau Research Program 'Nuovi Farmaci per neuropatie periferiche età-dipendenti e di natura patologica e neurotossica' and from CNR-Italy Research Program no. 97.2877.14.

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