Skeletal Muscle of Female Rats Exhibit Higher Mitochondrial Mass and Oxidative-Phosphorylative Capacities Compared to Males

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Key Words

Mitochondria • Gender dimorphism • Skeletal muscle
• Oxidative metabolism • Mitochondrial biogenesis

could explain the higher facility of females to adapt to altered metabolic energy situations.

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Abstract

The effect of gender and caloric restriction on mitochondrial content and oxidative-phosphorylative capacities has been investigated in rat gastrocnemius muscle. Muscle protein, mitochondrial protein and DNA contents, enzymatic activities of mitochondrial oxidative and phosphorylative system, mitochondrial antioxidant enzymes, protein levels of complex IV (subunit I and IV) and ATPase, and the gene and protein expression of mitochondrial transcription factor A (TFAM), involved in mitochondrial replication and transcription, were measured in rats of both genders fed ad libitum and subjected to three months of 40% caloric restriction. Compared to males, gastrocnemius muscle of female rats showed higher mitochondrial DNA and protein contents, TFAM protein level, oxidative and phosphorylative machinery and activities, and glutathione peroxidase activity. In conclusion, the present data show a clear gender dimorphism in rat muscle mitochondrial features, which

Introduction

Several studies have reported a gender dimorphism in energy balance in basal conditions, with a higher total energy expenditure in female compared to male rats [1, 2]. However, when animals are subjected to situations where an extra energy demand is necessary, females are able to respond positively to a greater extent than males [3, 4]. One of these situations is the adaptation to caloric restriction periods, where female rats decrease their energy expenditure while preserving metabolically active organ mass [2, 5]. Caloric restriction is a normal situation in nature and, during evolution, females may have developed better mechanisms than males to face these food deprivation periods and survive, probably because they are subjected to more severe selection pressures during lack of food supply [6].

The gender differences in energy efficiency can be linked to changes in the mechanisms to obtain this energy,

which are mainly the mitochondria through the oxidative phosphorylation (OXPHOS) system [7]. Different physiological situations that induce changes in the energy demand of the organism also have an effect in mitochondria, which have to respond by remodelling their capacity to produce energy according to these changes. In this sense, skeletal muscle mitochondria have demonstrated a high plasticity and capacity to respond to several energy demand altered situations such as exercise or hypoxia by increasing their respiratory and phosphorylative capacities [8-10].

Previous works performed in our laboratory have reported that liver and brown adipose tissue mitochondria of female rats present a higher oxidative metabolism compared to males, which could be due to their higher mitochondrial content and biogenesis [1, 11, 12]. Mitochondrial biogenesis includes both mitochondrial proliferation and differentiation, where proliferation means an increase of mitochondrial population, while differentiation is defined as an improvement of the functional capabilities of preexisting mitochondria. Both processes are regulated by nuclear transcriptional activators and coactivators such as the mitochondrial transcription factor A (TFAM), which is involved in replication and expression of mitochondrial DNA [13].

On the whole, differences in the mitochondrial OXPHOS system seems to be a key factor to understanding the energy metabolism in situations with an altered energy demand. The objective of the present work was to investigate the possible existence of differences in mitochondrial machinery and bioenergetics between genders (i.e. content and activities of OXPHOS complexes, mitochondrial mass) which could explain their different capacity to adapt to altered energy situations like caloric restriction. We chose skeletal muscle - to be precise, gastrocnemius muscle - because it is a postmitotic tissue only active intermittently, which makes it more adaptable to energy altered situations. Nevertheless, gastrocnemius presents gender differences [14, 15] and has been commonly used to study these energy altered situations such as caloric restriction and fasting [16-20]. In order to tackle the aim, male and female rats were subjected to 100 days of 40% food restriction and compared to groups fed ad libitum. The total protein, mitochondrial DNA and protein levels, enzymatic activities of mitochondrial OXPHOS complexes I, III, IV and ATPase, Mn superoxide dismutase (MnSOD) and mitochondrial glutathione peroxidase (GPx) activities, the protein level of complex IV (subunit I and IV) and

ATPase, as well as the gene and protein expression of TFAM were measured in gastrocnemius skeletal muscle.

Materials and Methods

Materials

Chemicals were supplied by Sigma-Aldrich (St. Louis, USA), Panreac (Barcelona, Spain) and Amersham Pharmacia Biotech (Little Chalfont, UK). Mouse antibodies against complex IV subunit I and IV were obtained respectively from Molecular Probes (A-6403) and Mitosciences (MS407), rabbit antisera against TFAM was kindly provided by Dr. H. Inagaki, and goat antibody against F1- β -ATPase was obtained from Santa Cruz Biotechnology (C-20, Sc-16690). Real-time PCR reagents and Oligonucleotide primer sequences were supplied by Roche Diagnostics (Basel, Switzerland).

Animals

Animal experiments were performed in accordance with general guidelines approved by our institutional ethics committee and the EU rules (86/609/EEC). Two-month-old Wistar rats (supplied by Charles River, Barcelona, Spain) were used at the beginning of the experiments, divided into four groups ($n = 6$ in each group): two control groups of males and females were fed ad libitum with pelleted standard diet (Panlab, Barcelona), and the other two groups of males and females were subjected to a 40% food restriction (40% less than ad libitum group) for 100 days with the same pelleted diet as groups fed ad libitum. In restricted animals, food was supplied on a daily basis at the beginning of the dark cycle, and was weekly updated compared to ad libitum rats in order to correct for growth requirements. All the animals were housed individually with free access to water in wire-bottom cages and acclimated at 22°C with a 12-h light/dark cycle.

Sacrifice and isolation of mitochondria

Animals were sacrificed by decapitation at the start of the light cycle and gastrocnemius muscle was quickly removed, washed and cut into pieces. The differential centrifugation process for subsarcolemmal mitochondria isolation was modified from previous described [21]. Briefly, gastrocnemius muscles were homogenized with a Teflon/glass homogeniser in 25mL of isolation buffer (manitol 220mM, Sucrose 40mM, EDTA 1mM, Tris-HCl 10mM, pH 7.4) adding 25mg of bovine serum albumin free fatty acids. An aliquot of the homogenate was stored at 4°C for posterior analysis and the remainder was used for mitochondria isolation. Nuclei and cell debris were first removed by centrifugation at 500xg for 10min at 4°C. Supernatant was centrifuged at 8000xg for 10min at 4°C and resuspended with 25mL of isolation buffer. Another centrifugation at 8000xg was performed and, finally, pellets were resuspended in 0.5mL of isolation buffer and kept on ice for further analysis. Mitochondrial protein was determined by the method of Bradford [22].

Gene	Forward Primer (5'-3') Reverse Primer (5'-3')	Denaturation temperature °C (time in secs)	Annealing temperature °C (time in secs)	Extension temperature °C (time in secs)	Product length	T _m
mtDNA	TAC ACG ATG AGG CAA CCAAA GGT AGG GGG TGT GTT GTG AG	95 (10)	60 (12)	72 (12)	161 bp	80.0
TFAM	GCT AAA CAC CCA GAT GCA AAA CGA GGT CTT TTT GGT TTT CC	95 (10)	60 (10)	72 (12)	269 bp	84.4
18S	GAG GTG AAA TTC TTG GAC CGG CGA ACC TCC GAC TTT CGT TCT	95 (5)	62 (8)	72 (12)	93 bp	82.2

Table 1. Oligonucleotide primer sequences and conditions used in real-time PCR amplification. mtDNA, mitochondrial DNA; TFAM, mitochondrial transcription factor A; 18S, ribosomal RNA 18S.

Enzymatic activities

The enzymatic activities of the following mitochondrial OXPHOS complexes were determined in resuspended mitochondrial pellet: NADH:ubiquinone oxidoreductase (complex I) [23], ubiquinol:ferricytochrome-c oxidoreductase (complex III) [24], ferrocytochrome-c: oxygen oxidoreductase (cytochrome c oxidase or complex IV) [25] and ATP phosphohydrolase (ATPase or complex V) [23]. Citrate synthase [26] and mitochondrial antioxidant MnSOD [27] and GPx [28] activities were also assayed. Complex IV activities were also determined in total gastrocnemius homogenates in order to calculate mitochondrial recovery.

Quantification of mitochondrial DNA (mtDNA) by real-time PCR

mtDNA extraction and semi-quantification of mitochondrial pellet suspensions were carried out as previously described [29]. Real-time PCR was performed to amplify a 162-nt region of the mitochondrial NADH dehydrogenase subunit 4 gene, which is exclusive of mitochondrial DNA (primers sequence shown in Table 1). Amplification was carried out in a LightCycler rapid thermal cycler system (Roche, Barcelona, Spain) using a total volume of 10 µL containing 0.375 µM of each primer, 3 mM MgCl₂, 1 µL LightCycler-FastStart DNA Master SYBRTM Green I and 2.5 µL of pre-treated mitochondrial sample [29]. PCR reactions were cycled 35 times after initial denaturation (95°C, 10 min) with the conditions shown in Table 1.

Real time RT-PCR analysis

Total RNA was isolated from frozen gastrocnemius samples using TriPure[®] isolation reagent and quantified using a spectrophotometer set at 260 nm. One µg of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a 10 µL volume of retro-transcription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X[®]-100, 2.5 mM MgCl₂, 2.5 µM random hexamers, 10 U RNase inhibitor and 500 µM each dNTP. Real-time PCR was carried out for TFAM and corrected by the housekeeping gene 18S ribosomal RNA (18S). Oligonucleotide primer sequences (Table 1) were obtained from Primer3 and tested with IDT OligoAnalyser 3.0. Finally, a basic local

alignment search tool (NCBI Blast) revealed that the primer sequence homology was obtained only for the target genes. Real-time PCR was performed using SYBR Green technology on a LightCycler rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Each reaction contained 1 µL LightCycler-FastStart DNA Master SYBR Green I (containing FastStart Taq DNA polymerase, dNTP mix, reaction buffer, MgCl₂ and SYBR Green I dye), 0.5 µM of the sense and antisense specific primers, 2 mM MgCl₂ and 3 µL of the cDNA dilution in a final volume of 10 µL. The amplification program consisted of a preincubation step for denaturation of the template cDNA (95°C, 10 min), followed by 40 cycles consisting of a denaturation, an annealing, and an extension step in conditions shown in Table 1. After each cycle, fluorescence was measured at 72°C. The real-time PCR efficiencies (e) were estimated on average of all sample efficiencies, which were calculated by the following formula: $e = (F/F_0)^{1/n-n_0}$, where n and n₀ were the crossing point values of F and F₀ respectively, fluorescences that belong to the linear section of the logarithmic quantification curve.

Western blot of TFAM, complex IV (subunits I and IV) and ATPase

15 µg of mitochondrial protein was electrophoresed on 14% v/v polyacrilamide SDS-PAGE gels. Proteins were electrotransferred onto a nitrocellulose filter (Biorad, California, US). A ponceau S staining was performed in order to check correct loading and electrophoretic transfer. Blots were incubated with the correspondent antibodies. Immunoblot development was performed using an enhanced chemiluminescence western blotting analysis system (Amersham, UK). Film blots were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). Apparent molecular weight was estimated using a protein molecular-mass standard (Amersham, UK) in 25, 39, 16 and 51 kDa for TFAM, complex IV (subunits I and IV) and ATPase respectively.

Statistics

Results were expressed as mean ± SEM. Differences between groups were assessed by two-way ANOVA and post-hoc Student's t-test using SPSS 12.0 (Statistical Program for

	MALE		FEMALE		statistics
	ad libitum	restricted	ad libitum	restricted	
Initial body weight (g)	279±6	297±9	177±5	182± 5	G
Final body weight (g)	482±14	354±8 ^b	261±8 ^a	194±4 ^{a b}	G, R, GxR
Gastrocnemius weight (g)	5.46±0.25	4.31±0.07	3.32 ±0.06	2.57±0.09	G, R
Total gastrocnemius protein(mg/g)	53.0± 2.8	47.3±3.8	76.8± 3.0	88.0±2.7 ^b	G, GxR
Total gastrocnemius Triglyceride(mg/g)	50.0±2.4	48.8±3.4	78.9±2.4	95.3± 3.9 ^b	G, GxR
Mitochondrial DNA (a.u./g tissue)	0.79± 0.27	1.10 ±0.28	1.75±0.37	2.38 ±0.54	G
Mitochondrial protein (mg/g tissue)	5.14± 0.69	4.87 ±0.80	8.99 ±1.32	13.1 ± 2.5	G

Table 2. Body weight, gastrocnemius muscle weight and biochemical parameters of fed ad libitum and 40% restricted male and female rats. Data are expressed as mean ± SEM and analysed by two-way ANOVA and post-hoc Student's t-test (p<0.05). G = gender, R = caloric restriction, GxR = interactive effect, ^a vs. male, ^b vs. fed ad libitum.

the Social Sciences software) for Windows package, except mRNA expression, which was performed using a Pair Wise Fixed Reallocation Randomisation Test © statistical model [30, 31]. In all cases, the statistical significance was set at p<0.05.

Results

Biometrical and biochemical parameters

Body and gastrocnemius muscle weights of male and female rats fed ad libitum compared to animals subjected to 40% caloric restricted diet are shown in Table 2. Body weight was substantially decreased by caloric restriction compared to ad libitum animals, with a similar percentage of weight loss in both genders (-26,3% in males and -25.2% in females). Gastrocnemius muscle weight was lower in females than males and decreased in food restricted animals (-22.5% and -20.4% respectively). Muscle protein and triglyceride levels were higher in females than males and caloric restriction enhanced these values only in females. There were no significant differences between groups in mitochondrial recovery, which was calculated at about 15% using homogenate and mitochondrial complex IV activities.

Mitochondrial DNA and protein

As mitochondria are the organelles in charge of producing energy in the cell, differences in muscle capacity between genders [3, 32-34] could be due to differences in muscle mitochondrial mass. To study this possibility, we measured mitochondrial DNA and protein contents (Table 2). Compared to males, muscle of females showed higher levels of both parameters, whereas caloric

restriction did not significantly modify them, in spite of a trend to increase in females.

Oxidative and phosphorylative mitochondrial machinery and activities

At the tissue level, differences in mitochondrial mass could lead to changes in mitochondrial oxidative and phosphorylative enzymatic activities, so we decided to measure the activity of mitochondrial complexes I, III, IV and ATPase (expressed as activity vs citrate synthase activities measured in the same samples, Table 3). Mitochondria from females compared to males exhibited a higher activity in all the OXPHOS enzymes studied, and an increase by caloric restriction was found only in ATPase activity.

ATPase is the complex V of the OXPHOS system in charge of producing ATP to satisfy the cellular energy demand, so the higher ATPase/complex IV activity ratio found in females could indicate their higher phosphorylative-to-oxidative capacity.

As changes in enzymatic activity can be related to changes in the enzyme content, we decided to quantify by western blot the protein levels of complex IV (subunit I and IV) and ATPase (Figure 1). Complex IV subunit I and ATPase protein levels were higher in females than males, which agrees with the different OXPHOS activities between genders. Although females also showed a higher tendency in complex IV subunit IV protein content compared to males, this parameter did not reach significant differences.

All in all, the present data would indicate a gender dimorphism in rat muscle mitochondrial features, with

	MALE		FEMALE		statistics
	ad libitum	restricted	ad libitum	restricted	
Complex I	57.4±4.6	61.1±3.6	67.3±2.2	73.7±5.5	G
Complex III	40.3±0.7	37.1±3.0	45.3±3.8	53.2±11.1	G
Complex IV	16.3±1.8	16.6±1.3	20.0±2.9	23.4±5.4	G
ATPase	19.8±1.9	24.2±1.0	32.7±1.9	38.9±3.2	G, R
ATPase/complex IV	1.11±0.12	1.49±0.11	1.77±0.20	1.79±0.32	G
GPx	3.78±0.74	4.77±0.87	6.08±0.99	6.57±1.50	G
MnSOD	6.72±0.82	7.53±0.82	6.46±1.00	7.72±2.15	NS

Table 3. Activity of mitochondrial OXPHOS complexes and antioxidant enzymes in gastrocnemius muscle mitochondria of fed ad libitum and 40% restricted male and female rats. Enzymatic activities are expressed as mean ± SEM in I.U. (μmol/min·mg or a.u. in case of complex IV) and corrected per the citrate synthase activities measured in the same mitochondrial preparation. Values were analyzed by two-way ANOVA ($p < 0.05$). G = gender, R = caloric restriction, NS = non significant.

Fig. 1. Western blot of complex IV (subunits I and IV), β -ATPase and TFAM in gastrocnemius muscle mitochondria of fed ad libitum and 40% restricted male and female rats. Values are expressed as net intensity per g of tissue compared to control males as 100% ± SEM and analysed by two-way ANOVA ($p < 0.05$), G = gender differences, NS = non significant.

higher oxidative and phosphorylative capacities in females than males.

Mitochondrial antioxidant activities

The activities of MnSOD and GPx were assayed in mitochondrial pool (Table 3). Activity MnSOD was not different between groups and GPx was higher in females

compared to males, which could indicate a superior antioxidant defense system in female muscle mitochondria.

Mitochondrial transcription factor A

The mRNA expression of TFAM was not significantly different between groups (Table 4), and

Gene	MALE		FEMALE		statistics
	ad libitum	restricted	ad libitum	restricted	
TFAM	27.5±0.5	28.5±0.6	27.8±0.4	28.6±0.4	NS
18S	10.7±0.2	10.9±0.1	11.0±0.3	11.2±0.3	NS

Table 4. mRNA expression of mitochondrial transcription factor A (TFAM) and 18S in gastrocnemius muscle mitochondria of fed ad libitum and 40% restricted male and female rats. Data are expressed as mean ± SEM as the cycle of amplification in Real-Time PCR (crossing point) and each group represents the mean expression of six independent experiments. The statistical model used was a Pair Wise Fixed Reallocation Randomisation Test © ($p < 0.05$).

mitochondrial TFAM protein of females was two-fold higher than males (Figure 1).

Discussion

Compared to males, skeletal muscle of females showed higher total protein, mitochondrial DNA, mitochondrial protein, OXPHOS complexes and GPx activities, and protein levels of TFAM, complex IV subunit I and ATPase.

Effect of gender and caloric restriction in muscle mitochondrial bioenergetics

Rodent females have been reported to have a superior muscular capacity compared to males, with their higher locomotor activity and greater ability to run longer and farther than males [3, 32-34], which agrees with the higher muscle protein and triglyceride levels found in this work.

This sex dimorphism in muscular capacity should be accompanied by differences in oxidative and/or phosphorylative capacities. These changes would be due to differences in mitochondrial components and/or activities, and would result in more differentiated mitochondria [11, 12, 29] in order to make the ATP synthesis as efficient as possible [35-37]. Previous studies have shown gender differences in gastrocnemius fiber type morphometry, size and number, which could be due to the different sex-related hormonal milieu [14, 15]. Furthermore, measuring ATP contents, creatine phosphokinase and myokinase enzyme activities, Ramamani et al [38] found differences in skeletal muscle metabolism between genders, which were also linked to the hormonal status. The present results show that the

activity of skeletal muscle mitochondrial oxidative-phosphorylative complexes are higher in females, which could be indicative of a more efficient muscle energy metabolism compared to males, although further studies including mitochondrial function would be necessary to clarify this point.

A different rate of respiration is linked to the content in OXPHOS components [37], where the most important are complex IV and ATPase as the main regulators of mitochondrial respiration. We observed in this work that complex IV subunit I and ATPase protein levels were about two-fold higher in females than males, in agreement with the superior OXPHOS activities of females. These results, together with the higher ATPase/complex IV activity ratios, suggest that muscle of females exhibit a more differentiated mitochondrial pool, and could explain the ability of female rats to increase their energy efficiency in response to altered energy demand situations like caloric restriction [2], and their better energy metabolic adaptation to exercise compared to males [3, 4]. These differences in mitochondrial energy efficiency could also be related to mitochondrial uncoupling and UCPs protein levels. In this sense, UCP3 expression has been described to be different between genders and modified in response to restricted caloric intake [39, 40]. However, the role of UCPs in skeletal muscle remains a matter of debate and their physiological functions have yet to be determined [40-43].

As mitochondria are the main free radical generators in cells, the higher mitochondrial capacity of females could also produce higher levels of these reactive molecules. However, the antioxidant enzymatic activity of GPx is also elevated in females, which would help them prevent oxidative damage in case of increased free radical production.

Effect of gender and caloric restriction in muscle mitochondrial mass

The increase in mitochondrial energy capacity due to changes in OXPHOS activities or components (discussed above), could also be linked to a modification of mitochondrial number, size or density per cell [11]. The gender differences found in mitochondrial protein and mitochondrial DNA contents are similar to those described in liver and brown adipose tissue [1, 11, 12], and would indicate a higher mitochondrial mass in muscle of females.

The nuclear control of mitochondrial biogenesis involves different activators and coactivators which control the expression of both nuclear and mitochondrial genes encoding mitochondrial proteins [13]. TFAM plays a key role in this process as a direct regulator of mitochondrial DNA replication/transcription [44]. In this work, we found a two-fold higher TFAM protein in muscle mitochondria of females than males, whereas TFAM mRNA expression was not different between genders. These apparently contradictory results agree with those previously reported [45] in embryo mitochondria, and could indicate a post-transcriptional regulation of TFAM. As TFAM regulates mitochondrial transcription of proteins necessary for a functional respiratory chain and phosphorylative system, the higher levels of TFAM protein in females would explain their greater Complex IV subunit I and ATPase protein levels, as well as their higher

OXPHOS activities compared to males. On the whole, the higher TFAM protein in female mitochondria would explain the mechanism by which female muscle shows a higher mitochondrial bioenergetic capacity and mass when compared to males.

By way of conclusion, we demonstrate a clear gender dimorphism in rat muscle mitochondrial features, with higher mitochondrial mass and oxidative-phosphorylative capacities in females than males, which could explain the ability of females compared to males for better adaptation to energy demand situations.

Acknowledgements

The authors thank A. Català, E. Thomàs, B. Bibiloni and E. Pujol for their help in the preparation and carrying out of experiments. We are grateful to Dr. Hidetoshi Inagaki, from the National Industrial Research Institute of Nagoya (Japan), for kindly providing the rabbit antiserum against TFAM protein. This work was supported by the Spanish Government ("Fondo de Investigación Sanitaria", FIS PIO21339, FIS PIO42377 and FIS PIO42294). B. Colom, M.P. Alcolea and A. Valle were supported by a grant of the "Ministerio de Educación y Ciencia" of the Spanish Government.

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