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Long-term High-fat-diet Feeding Impairs Mitochondrial Biogenesis in Liver of Male and Female Rats

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Key Words

Gender differences • High fat diet • Liver • Mitochondrial function • Obesity • Oxidative stress • Rat

Abstract

Background/Aims: Mitochondrial biogenesis includes both mitochondrial proliferation and differentiation and its regulation under different physiological conditions is not clear. Given the sexual dimorphism previously found in mitochondrial function, the aim of this study was to investigate the gender-dependent effect of chronic high-fat-diet (HFD) feeding on rat liver mitochondrial function and biogenesis. Methods: Ten-week old male and female rats were fed a HFD (26% fat) or a control diet (2.9% fat) for 26 weeks. Mitochondrial morphology was studied. Mitochondrial DNA and protein content, hydrogen peroxide production, oxidative capacity, antioxidant defenses, as well as markers of oxidative damage and mitochondrial biogenesis were analyzed. Results: Female rats showed higher levels of mitochondrial protein and an enhanced oxidative capacity per mitochondrion than males. In both genders, HFD feeding increased mtDNA content and decreased mitochondrial differentiation markers. Conclusion: In comparison to male rats, females show higher oxidative capacity as a consequence of their greater mitochondrial differentiation under both control and obese status. In response to HFD feeding, the oxidative capacity of the whole mitochondrial population is maintained in both genders. This is obtained by means of an enhancement of mitochondrial proliferation, which counteracts the diet-induced impairment of the function of each mitochondrion.

Introduction

Liver is a vital organ of crucial importance in maintaining the overall metabolism, thus alterations in hepatic function have an impact on the health of the organism [1]. Liver diseases associated with accumulation of lipids represent the hepatic component of the metabolic syndrome and its prevalence is closely linked to both obesity and a variety of second insults, such as oxidative damage and mitochondrial dysfunction [2].

Oxidative stress is the consequence of an imbalance between oxidants and antioxidant systems in the favour of the former. Reactive oxygen species (ROS) can cause oxidative damage in mitochondrial components in the form of lipid peroxidation, protein modifications, and DNA

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mutation [3], leading to mitochondrial dysfunction, which contributes to a wide range of human pathologies, including neurodegenerative and cardiovascular diseases, diabetes and the cumulative degeneration associated with ageing [4, 5].

A close association has been described between the obese state induced by high fat diets (HFD) and oxidative stress [6]. HFD feeding increases the amount of substrate available for oxidation and the electron flux through the mitochondrial respiratory chain by enhancing free radical production. In addition, an impairment of antioxidant defenses has also been reported in diet-induced obesity [7]. An excess of ROS is harmful to cells but, within a certain level, ROS may be involved in the communication between mitochondria and the nucleus controlling the mitochondrial biogenesis process [8]. Mitochondrial biogenesis is a complex event that includes both mitochondrial proliferation (the increase in the number of mitochondria per cell) and differentiation (the increase in the functional capability of pre-existing mitochondria) [9] and requires the coordinated contribution of both mitochondrial and nuclear genome [10]. Several signaling pathways are involved in the regulation of this process and there is growing evidence to suggest that peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) is the main regulator of mitochondrial biogenesis [11]. In short, PGC-1α can activate different transcription factors such as nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) [12] which regulate the expression of most nuclear genes involved in mitochondrial biogenesis, mitochondrial transcription factor A (TFAM), one of the regulatory factors necessary for proper mtDNA transcription [13], as well as genes encoding subunits of respiratory complexes.

Previous studies in our laboratory performed in several tissues point to rat gender differences in morphology and mitochondrial function [7, 14-19]. Taking this background into account, the aim of the present study was to investigate the gender-related differences in rat liver mitochondrial morphology, function and biogenesis in response to the overweight induced by chronic feeding with a HFD, imitating the dietary habit increasingly found in developing countries.

Materials and Methods

Materials

17β-estradiol and thyroid hormone enzyme immunoassay kits were from Diametra (Milano, Italy) and colorimetric HT Glutathione Assay kit was from Trevigen (Gaithersburg, MD, USA). Amplex Red and Oxyblot™ Protein Oxidation Detection kits were obtained from Invitrogen (Carlsbad, CA, USA) and from Chemicon International (Temecula, CA, USA), respectively. Oligonucleotide primer sequences, Lightcycler-FastStart DNA Master SYBR Green I® for real-time PCR and Tripure® isolation reagent were purchased from Roche Diagnostics (Basel, Switzerland). RT-PCR chemicals were from Applied Biosystems (Lincoln, CA, USA). Rabbit antisera against TFAM was kindly provided by Dr. H. Inagaki [20]. Anti-Pan-Actin Ab5 (Cat. num. MS-1295-PO) was from Neomarkers (Fremont, CA, USA), anti-UCP2 (Cat. num. UCP22-A) was from Alpha Diagnostic International (San Antonio, TX, USA), anti COX II (Cat. num. sc-23984) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-COX IV (Cat. num MS407) was from MitoSciences (Eugene, OR, USA). The enhanced chemiluminescence Western blotting analysis reagents were supplied by Amersham (Little Chaffont, UK). Routine chemicals used were purchased from Sigma-Aldrich (St Louis, MO, USA) and Panreac (Barcelona, Spain). Control pelleted diet (A04) was obtained from Panlab (Barcelona, Spain).

Animals and diets

Animal experiments were performed in accordance with general guidelines approved by our institutional ethics committee and EU regulations (86/609/CEE). Ten week-old Wistar rats of both genders (Charles River, Barcelona, Spain) were kept at 22°C on a 12-hour light-dark cycle with free access to food and water. Both male and female rats were divided into two experimental groups (6 animals per group) with a similar mean body weight (332 ± 4g for male rats and 217 ± 2g for female rats) and were fed a control pelleted diet (3,385Kcal/Kg; 2.9% (w/w) fat) or a high fat diet (HFD) (3,876Kcal/Kg diet; 26% (w/w) fat) for 26 weeks. The HFD components (a homemade diet called cafeteria diet) were cookies, pork liver paté, fresh bacon, chocolate, ensaïmada (a typical Majorcan pastry) and pelleted standard diet. The energy composition of the HFD was 12.7% protein, 32.8% carbohydrate and 54.5% lipid, while the control pelleted diet was made up of 19% protein, 73% carbohydrate and 8% lipid. Animal body weights were assessed monthly; food and energy intake was analyzed the week before sacrifice. The rats were introduced into metabolic cages (Panlab, Barcelona) for 24-hours to measure their food intake (HFD and/or standard diet). All the components of the HFD were presented in several small pieces and in gross excess so as to allow the recovery the following day of at least part of all the components offered. The amount of each component consumed by each animal was calculated from the difference between the amount offered and the amount recovered the next day.

Rats were sacrificed by decapitation after a 12-hour period of fasting. Blood was collected and liver was removed rapidly and weighed. Mitochondrial isolation was performed in fresh samples of liver. The rest of the tissue and serum samples were frozen in liquid N_2 and stored at -80°C until analyzed.

Indirect calorimetry

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured at the end of the dietary

treatment by open-circuit respirometry (flow rate 1L/min). The measurements were performed at 22°C during the second hour of the light phase. The animals were individually placed in calorimetric chambers and were allowed to become acclimated for 2h. Data recordings were made sequentially in each cage for 90-second periods throughout a total 30 min period. Both VO_2 and VCO_2 were adjusted for body surface ($mL \cdot min^{-1} \times kg^{0.75}$) and averaged over the collection period [21]. The respiratory quotient ($RQ = CO_2$ production/O_2 consumption) was calculated from indirect calorimetry data.

Serum 17β-estradiol and thyroid hormone levels

Given the role that the hormonal milieu can play in the regulation of the energy metabolism and especially in mitochondrial function, 17β-estradiol and thyroid hormone levels were measured by enzyme immunoassay kits.

Mitochondria isolation

Liver mitochondria were isolated as previously described [22]. Fresh liver (2g) was homogenized with a Teflon/glass homogenizer in 15mL of isolation buffer (250mM sucrose, 5mM Tris-HCl and 2mM EGTA, pH 7.4). Briefly, homogenate was centrifuged at 500xg for 10min at 4°C to remove the nuclei and cell debris and the resulting supernatant was centrifuged twice at 8000xg for 10min at 4°C. The mitochondria pellet was resuspended with 1mL of isolation buffer.

Mitochondrial oxygen consumption

Mitochondrial oxygen consumption was measured polarographically as previously described [23], with minor modifications. Isolated mitochondria (2.5mg protein/mL) were incubated in a water-thermostatically regulated chamber with a computed-controlled oxygen Clark-type electrode Oxygraph (Hansatech, Norfolk, UK) in a respiration buffer (145mM KCl, 30mM HEPES, 5mM KH_2PO_4, 3mM $MgCl_2$, 0.1mM EGTA, and 0.1% BSA, pH 7.4 at 37°C). Succinate (10mM) was used as substrate in the absence (state 4) and presence (state 3) of 500μM ADP. Mitochondrial functionality was checked by the respiration control ratio (RCR = state 3 VO_2/state 4 VO_2), rejecting the samples with a RCR lower than 2.50.

Mitochondrial hydrogen peroxide production

Hydrogen peroxide production was assayed in isolated mitochondria by measuring the increase in fluorescence (530nm excitation, 590nm emission) due to the reaction of Amplex Red with hydrogen peroxide in the presence of horseradish peroxidase [24]. Assays were performed at 37°C in a 96-well microplate fluorimeter FLx800 (Bio-tek instruments, Winooski, VT, USA). Mitochondria (0.25mg protein/mL) were added to the respiration buffer without BSA supplemented with horseradish peroxidase 0.1U/mL and 50μM Amplex Red reagent. The assays were performed in presence of succinate (10mM) as substrate and antimycin A (2μM). The rate of hydrogen peroxide production was calculated using a standard curve of H_2O_2 stabilized solution.

Enzymatic activities

In isolated mitochondria, cytochrome *c* oxidase (COX) [25], citrate synthase [26], Mn-superoxide dismutase (Mn-SOD) [27] and glutathione peroxidase (GPx) [28] activities were assayed.

Measurement of glutathione content

Glutathione content was measured in diluted homogenates (1g tissue/20mL isolation buffer) using the HT Glutathione Assay kit according to the manufacturer's protocol. The samples were treated with 4-vinylpyridine to determine the oxidized glutathione concentration. To obtain the levels of reduced glutathione, the oxidized glutathione was subtracted from the total glutathione.

Liver composition

Total protein was determined in homogenates, as previously described [29]. Total DNA and lipids were measured in homogenates using the diaminobenzoic acid method [30] and the Folch method [31], respectively.

Mitochondrial composition

Mitochondrial protein was determined by the method of Bradford [29]. Mitochondrial DNA (mtDNA) extraction and semi-quantification was carried out in isolated mitochondria as previously described [32]. Briefly, 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 (primer sequences shown in Table 1).

Electron microscopic analysis

Mitochondrial pellets were obtained under the same conditions indicated in the mitochondrial isolation section and were placed in ice-cold fixative buffer (4% glutaraldehyde in 200mM trihydrated sodium cacodylate buffer, pH 7.2) for 24h. The specimens were washed three times in 200mM trihydrated sodium cacodylate buffer and postfixed (1% OsO_4) for 2h. They were then dehydrated in graded acetone steps, stained with 2% uranyl acetate overnight, and embedded in Spurr's resin. Ultrathin sections for electron microscopy, ~50 nm thick, were stained with saturated lead citrate solution and examined using a Hitachi H-600 electron microscope at 75 kV. Transmission electron photomicrographs were obtained at x10,000 magnification and analyzed using Scion Image software, randomly measuring 75-150 mitochondria per animal (two animals per group).

Measurement of thiobarbituric acid-reactive substances (TBARS), carbonyl groups and oxidized mtDNA

Levels of TBARS, as an index of lipid peroxides, were measured spectrophotometrically in liver homogenates as previously described [33]. Protein carbonyl groups, as an index of protein oxidation, were measured in liver homogenates by immunoblot detection using the OxyBlot™ Protein Oxidation Detection Kit according to the manufacturer's protocol with

Table 1. Oligonucleotide primer sequences and conditions used in real-time PCR amplification. mtDNA, mitochondrial DNA; PGC-1α, peroxisome proliferator-actived receptor-gamma coactivator-1; NRF-1, nuclear respiratory factor 1; NRF-2α, nuclear respiratory factor 2 DNA-binding α subunit.

Gene	Forward Primer (5'-3') Reverse Primer (5'-3')	Denaturation temp. °C (time in secs)	Annealing temp. °C (time in secs)	Extension temp. °C (time in secs)	Product length	Tm
mtDNA	TAC ACG ATG AGG CAA CCA AA GGT AGG GGG TGT GTT GTG AG	95 (10)	60 (12)	72 (12)	161 pb	80.0
PGC-1α	ATG TGT CGC CTT CTT GCT CT ATC TAC TGC CTG GGG ACC TT	95 (10)	65 (10)	72 (12)	179 pb	83.4
NRF-1	TTA CTC TGC TGT GGC TGA TGG CCT CTG ATG CTT GCG TCG TCT	95 (10)	56 (5)	72 (8)	87 pb	83.2
NRF-2α	CAG AGC AAG TGA CGA GAT GG CCG AAA TGT TGA GTG TGG TG	95 (10)	64 (10)	72 (12)	176 pb	85.3
GAPDH	ATG GGG AAG GTG AAG GTC GGA G TCG CCC CAC TTG ATT TTG GAG G	95 (10)	62 (10)	72 (12)	265 pb	89.0

Table 2. Body weight and energy intake. BW, body weight. White fat depot weight is the sum of inguinal, gonadal, mesenteric and lumbar depot weights. Adiposity index is the white fat depot weight relative to 100g of BW. Values are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect and GxD indicates gender and diet interactive effect. Student's t-test ($p<0.05$): [a]HFD *vs* control, [b]females *vs* males.

	Males		Females		ANOVA
	Control	HFD	Control	HFD	
BW (g)	555 ± 12	671 ± 23	296 ± 6	450 ± 23	G,D
BW gain (g)	225 ± 11	336 ± 22	83.9 ± 5.0	230 ± 21	G,D
White fat depot weight (g)	57.2 ± 5.3	106 ± 8	23.0 ± 1.8	86.2 ± 9.5	G,D
Adiposity index (g/100g BW)	10.1 ± 0.8	15.5 ± 0.6[a]	7.61 ± 0.52[b]	18.6 ± 1.2[a,b]	D,GxD
Energy intake ($kJ/kg^{0.75} \cdot day$)	603 ± 24	1104 ± 101	972 ± 90	1121 ± 129	D

Table 3. Liver weight and composition and liver mitochondrial composition. Levels of mtDNA are expressed as A.U. (arbitrary units). Values are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect, GxD indicates gender and diet interactive effect and NS stands for non significant. Student's t-test ($p<0.05$): [a]HFD *vs* control, [b]females *vs* males. Mitochondrial mean area (μm^2) was obtained by measuring 75-150 mitochondria per animal (two animals per group).

	Males		Females		
	Control	HFD	Control	HFD	ANOVA
Liver weight (g)	15.8 ± 0.5	19.0 ± 1.2	7.52 ± 0.23	9.91 ± 0.59	G,D
(g/100g body weight)	2.86 ± 0.07	2.81 ± 0.11	2.52 ± 0.07	2.20 ± 0.06	G,D
Lipid (mg/g tissue)	58.5 ± 3.4	105 ± 9[a]	51.4 ± 1.1[b]	68.4 ± 4.9[a,b]	G,D,GxD
DNA (mg/g tissue)	8.25 ± 0.47	8.71 ± 0.87	12.5 ± 1.6	10.8 ± 1.2	G
Protein (mg/g tissue)	198 ± 11	169 ± 8	192 ± 11	184 ± 6	NS
mtDNA (A.U./mg DNA)	0.438 ± 0.054	0.722 ± 0.137	0.300 ± 0.043	0.613 ± 0.092	D
mtprotein (mg/mtDNA)	3.61 ± 0.55	2.56 ± 0.21	5.48 ± 0.62	3.70 ± 0.28	G,D
Mitochondrial area (μm^2)	0.373 ± 0.015	0.356 ± 0.013	0.336 ± 0.012	0.381 ± 0.018	NS

minor modifications [34]. The assay used to examine the ROS-induced mtDNA damage was carried out as previously described [34]. This method is based on the amplification of two mtDNA fragments by real-time PCR. The large fragment, which includes a polycytidine stretch, is more sensitive to oxidative damage than the small fragment and, as was observed in previous experiments, its amplification was delayed when compared with short fragment after exposure to DNA-damaging agents in a dose-dependent manner. In this regard, A.U. stands for the ratio between the crossing point of the large fragment and the crossing point of the small fragment, which correlates with mtDNA oxidative damage.

Analysis of mRNA levels by real-time RT-PCR

Total RNA was isolated from 100mg of frozen tissue using TriPure® isolation reagent and quantified using a spectrophotometer set at 260nm.

One µg of the total RNA was reverse transcribed to cDNA at 42°C for 60min with 25U MuLV reverse transcriptase in a 10µl volume of retrotranscription reaction mixture containing 10mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X®-100, 2.5mM $MgCl_2$, 2.5µM random hexamers, 10U RNase inhibitor and 500µM each dNTP. Each cDNA solution was diluted 1/10 and aliquots were frozen (-70°C) until the PCR reactions were carried out.

Real-time PCR was performed for three target genes: peroxisome-proliferator-activated receptor-gamma co-activator 1 alpha (PGC1-α), nuclear respiratory factors 1 and 2α (NRF-1 and NRF-2α), and GAPDH as a housekeeping gene. All oligonucleotide primer sequences were obtained from Primer3 and tested with IDT OligoAnalyser 3.0 (Table 1). 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, Switzeland). Each reaction contained 1µl LightCycler-FastStart DNA Master SYBR™ Green I (containing FastStart Taq polymerase, dNTP mix, reaction buffer, $MgCl_2$ and SYBR™ Green I dye), 0.5µM of the sense and antisense specific primers, 2mM $MgCl_2$ and 3µl of the cDNA dilution in a

final volume of 10µl. The amplification program consisted of a preincubation step for denaturation of the templated cDNA (10min 95°C), 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. A negative control without cDNA template was run in each assay.

The real-time PCR efficiencies (e) were estimated on average of all sample efficiencies, which were calculated by the following formula: $e = (F/F_0)^{1/n-n0}$, where n and n_0 were the crossing point values of F and F_0 respectively, fluorescences that belong to the linear section of the logarithmic quantification curve.

Western blot analysis of COX II, COX IV, UCP2 and TFAM protein levels

15 (COX IV), 50 (COX II and UCP2) and 25 (TFAM) µg of mitochondrial protein that had been frozen with protease and phosphatase inhibitors (10µM leupeptin, 10µM pepstatin, 0.2mM PMSF and 0.2mM ortovanadate) were fractionated on 10% SDS-PAGE gels, and electrotransferred onto a nitrocellulose filter. Rabbit polyclonal antibodies against rat TFAM, mouse monoclonal antibodies against rat UCP2 and COX IV and goat polyclonal antibodies against rat COX II were used as primary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands in films were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). Autoradiograms revealed an apparent molecular mass of 18kDa, 27.5KDa, 25kDa and 33kDa for COX IV, COX II, TFAM and UCP2, respectively.

Statistical analysis

All data are expressed as mean values ± SEM of 6 animals per group. Real-time PCR data normalization was performed using the Genex software v.4.3.8 (MultiDAnalyses). The stability and suitability of GAPDH as a reference gene was validated by NormFinder application of Genex program. Statistical differences between experimental groups were analysed by ANOVA, and Student's t-test, as post-hoc comparison, was performed when an interactive effect of gender and diet was shown. Since mitochondrial area data did not follow a normal distribution, differences between groups were assessed using a nonparametric Mann-Whitney test. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed by using a statistical software package (SPSS 17.0 for Windows, Inc., Chicago, IL, USA).

Results

Energy intake, body weight gain and liver composition

The body weight increased more in HFD female rats (51.8%) than in males (22.8%) compared to their control counterparts (Table 2). The white fat depots weight and the adiposity index were lower in control female rats than in males and increased with the dietary treatment especially in females. In fact, adiposity index increased 2.4 fold in females and only 1.5 fold in males, reaching higher values in HFD females than in males. Control female rats showed higher energy intake than males, although these differences did not reach statistical significance (p=0.059). In response to HFD feeding, energy intake increased significantly in both male and

	Males		Females		
	Control	HFD	Control	HFD	ANOVA
17β-estradiol (ng/L)	45.7 ± 2.3	39.6 ± 1.0	53.1 ± 2.0	40.6 ± 1.4	G,D
T3 (ug/L)	0.846 ± 0.047	0.925 ± 0.046	0.866 ± 0.038	1.04 ± 0.05	D
T4 (ug/L)	39.2 ± 1.6	32.8 ± 2.3[a]	28.7 ± 4.5[b]	35.0 ± 2.5	GxD

Table 4. Serum 17β-estradiol and thyroid hormone levels. Values are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect, GxD indicates gender and diet interactive effect and NS stands for non significant. Student's t-test ($p<0.05$): [a]HFD *vs* control, [b]females *vs* males.

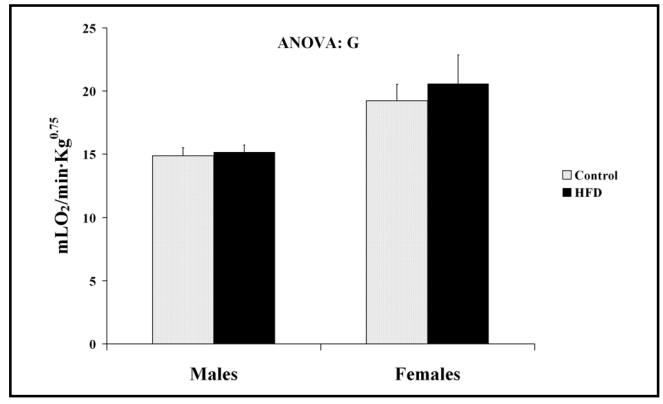

Fig. 1. Whole body oxygen consumption. Data are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect.

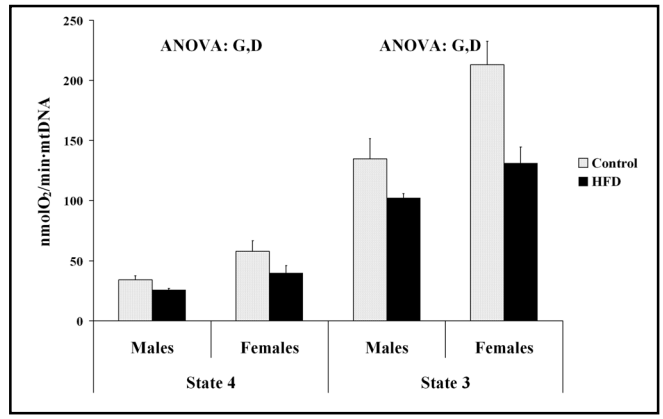

Fig. 2. Liver mitochondrial oxygen consumption. O_2 consumption rates were measured in isolated mitochondria using succinate as substrate in absence (state 4) and presence (state 3) of ADP. Data are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect and D indicates diet effect.

	Males		Females		
	Control	HFD	Control	HFD	ANOVA
COX					
(A.U./mtDNA)	14.0 ± 0.9	9.50 ± 0.67	18.8 ± 3.5	13.0 ± 1.1	G,D
(A.U./mg mtProtein)	3.50 ± 0.28	3.45 ± 0.27	3.71 ± 0.27	4.05 ± 0.54	NS
CS					
(I.U./mtDNA)	0.277 ±0.018	0.236 ±0.012[a]	0.595 ± 0.079[b]	0.288 ± 0.014[a,b]	G,D,GxD
(mI.U./mg mtProtein)	68.5 ± 5.6	94.7 ± 4.1	91.5 ± 10.1	95.3 ± 5.6	NS

Table 5. Cytochrome C oxidase and citrate synthase activities. COX, Cytochrome c oxidase, and CS, Citrate synthase, activities were measured in isolated mitochondria and are expressed as A.U. (arbitrary units) and I.U. (µmol/min determined at 37°C) respectively. Values are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect, GxD indicates gender and diet interactive effect and NS stands for non significant. Student's t-test ($p<0.05$): [a]HFD *vs* control, [b]females *vs* males.

	Males		Females		
	Control	HFD	Control	HFD	ANOVA
GPx (I.U./mtDNA)	1.29 ± 0.21	1.26 ± 0.20	4.18 ± 0.42[b]	1.71 ± 0.21[a]	G,D,GxD
Mn-SOD (I.U/mtDNA)	26.0 ± 3.4	23.9 ± 1.8	51.9 ± 2.6[b]	29.8 ± 1.5[a,b]	G,D,GxD
GSH + GSSG (µmol/g tissue)	1.67 ± 0.12	1.13 ± 0.12	1.37 ± 0.20	1.05 ± 0.08	D
GSH (µmol/g tissue)	1.12 ± 0.20	0.879 ±0.095	1.17 ± 0.08	0.802 ±0.088	D

Table 6. Glutathione peroxidase and superoxide dismutase activities and glutathione levels. GPx, glutathione peroxidase and Mn-SOD, superoxide dismutase, activities were measured in isolated mitochondria and are expressed as I.U. (µmol/min determined at 37°C). Glutathione levels were measured in tissue homogenate. Values are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect and GxD indicates gender and diet interactive effect. Student's t-test ($p<0.05$): [a]HFD *vs* control, [b]females *vs* males.

	Males		Females		
	Control	HFD	Control	HFD	ANOVA
H_2O_2 production (pmol/min·mg protein)	78.9 ± 5.9	111 ± 8	135 ± 7	153 ± 11	G,D
(pmol/min·mtDNA)	284 ± 21	283 ± 20	740 ± 41[b]	562 ± 41[a,b]	G,D,GxD
TBARS (nmol/g tissue)	250 ± 7	277 ± 7	288 ± 16	337 ± 51	NS
Protein carbonyl groups (%)	100 ± 21	91.2 ± 13.5	85.7 ± 11.5	115 ± 9	NS
mtDNA oxidized (%)	100 ± 18	67.3 ± 6.6	151 ± 14	97.5 ± 8.5	G,D

Table 7. Mitochondrial hydrogen peroxide production, lipid peroxide levels, protein carbonyl groups and mtDNA oxidized. The value of protein carbonyl groups of control male rats was set as 100%. mtDNA oxidized is expressed in A.U. (arbitrary units) of mtDNA oxidized per mtDNA and the value of control male rats was set as 100%. Values are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect, GxD indicates gender and diet interactive effect and NS stands for non significant. Student's t-test ($p<0.05$): [a]HFD *vs* control, [b]females *vs* males.

female rats. The consumption of the HFD increased liver weight in both genders (Table 3). Liver lipid content was higher in male rats compared to females and increased in both genders in response to the HFD feeding. This increase was higher in males (79.5%) compared to females (33.1%). Female rats showed greater total DNA content and this parameter was not affected by HFD feeding. No significant gender or diet effects were found in total protein levels. The mtDNA level per total DNA (i. e. per cell) was similar in both genders and rose in HFD animals. Mitochondrial protein levels expressed per mtDNA (i. e. per mitochondrion) were higher in female

	Males		Females		
	Control	HFD	Control	HFD	ANOVA
PGC-1α	2.62 ± 0,64	3.83 ± 0.76	6.63 ± 0.98	7.13 ± 0.68	G
NRF-1	2.06 ± 0.11	3.20 ± 0.32	1.47 ± 0.16	2.09 ± 0.19	G,D
NRF-2α	1.45 ± 0.10	1.60 ± 0.20	1.49 ± 0.09	1.94 ± 0.16	NS

Table 8. PGC-1α, NRF-1 and NRF-2α mRNA levels. PGC-1α, peroxisome proliferator-actived receptor-gamma coactivator-1α; NRF-1, nuclear respiratory factor 1; NRF-2α, nuclear respiratory factor 2 DNA-binding α subunit. Real-time PCR data normalization was performed using the Genex software v.4.3.8 (MultiDAnalyses). Values are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect and NS stands for non significant.

	Males		Females		
	Control	HFD	Control	HFD	ANOVA
UCP2/mg protein (%)	100 ± 19	95.4 ± 8.2	112 ± 19	90.2 ± 12.1	NS
UCP2/mtDNA (%)	100 ± 19	67.6 ± 5.8	170 ± 29	92.5 ± 12.4	G,D
COX II/mg protein (%)	100 ± 16	159 ± 7[a]	131 ± 17	115 ± 10[b]	GxD
COX II/mtDNA (%)	100 ± 16	113 ± 5	199 ± 26[b]	117 ± 11[a]	G,GxD
COX IV/mg protein (%)	100 ± 18	106 ± 6	91.0 ± 13.9	98.8 ± 6.3	NS
COX IV/mtDNA (%)	100 ± 14	75.0 ± 10.7	138 ± 47	101 ± 16	G,D

Table 9. UCP2, COX II and COX IV protein levels. UCP2, uncoupling protein 2; COX II and COX IV, cytochrome c oxidase subunit II and IV, respectively. Values are means ± SEM of 6 animals per group. The value of control male rats was set as 100%. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect, GxD indicates gender and diet interactive effect and NS stands for non significant. Student's t-test ($p<0.05$): [a]HFD *vs* control, [b]females *vs* males.

rats than in males and decreased by the diet effect. No significant differences between groups were observed in mitochondrial area.

Serum hormone levels

Serum 17β-estradiol levels were higher in control female rats than in males and decreased in response to HFD feeding in both genders (Table 4). The consumption of the HFD increased T3 levels in both genders and reduced T4 levels in male rats.

Indirect calorimetry

Female rats showed greater O_2 consumption than males, which was not modified by the HFD feeding (Fig. 1). In both genders, the respiratory quotient decreased significantly in response to the dietary treatment (from 0.861 ± 0.046 to 0.796 ± 0.045 in males, and from 0.923 ± 0.068 to 0.755 ± 0.030 in females, $p=0.028$), suggesting a greater lipid oxidation in the HFD groups.

Mitochondrial oxygen consumption and oxidative enzyme activities

Liver mitochondrial oxygen consumption was higher in female rats than in males and decreased in both genders in response to HFD feeding in both mitochondrial respiratory states 3 and 4 (Fig. 2). RCR did not show significant differences between groups (Control males 3.74 ± 0.11; HFD males 4.02 ± 0.15; Control females 3.68 ± 0.23; HFD females 3.32 ± 0.25). The results of oxygen consumption are in agreement with COX and CS activity data (Table 5) which were also greater in female rats than in males and decreased by the diet effect in both genders, although remaining higher in females. No significant differences between groups were found in COX or CS activities when these parameters are expressed per mtProtein (mitochondrial protein). Neither was any significant difference between groups observed in oxygen consumption expressed per mtProtein (data not shown).

Antioxidant enzyme activities and glutathione levels

GPx and Mn-SOD activities were higher in females than in males (Table 6). In both genders, the consumption of the HFD induced a decrease in both GPx and Mn-SOD activities, but levels in females remained increased compared to males. In addition, HFD feeding decreased both total and reduced glutathione levels in both genders.

Mitochondrial H_2O_2 production and oxidative damage

Hydrogen peroxide production was higher in female rats than in males and increased in both genders in response to HFD feeding, when referred to mitochondrial protein (Table 7). In contrast, hydrogen peroxide production per mitochondrion decreased in female rats with the dietary treatment. Oxidized mtDNA decreased in both genders with HFD feeding. There were no significant differences between groups either in lipid peroxide levels or in protein carbonyl groups.

Relative mRNA levels of PGC-1α, NRF-1 and NRF-2α

The mRNA levels of PGC-1α were higher in female rats compared to males but the expression of NRF-1 showed the opposite profile. In response to HFD feeding NRF-1 mRNA levels increased in both genders (Table 8). There were no gender or diet differences in NRF-2α mRNA levels.

Liver UCP2, COX II, COX IV and TFAM protein content

The levels of COX subunit II increased in response to HFD feeding in male rats, whereas the levels of UCP2 and COX subunit IV referred to protein showed neither gender nor diet effect. The levels of UCP2 and COX subunits II and IV per mitochondrion were higher in control female rats than in their male counterparts (Table 9). Consumption of the HFD decreased both UCP2 and COX IV levels in both genders, but gender differences were maintained. COX II protein levels decreased in response to HFD feeding only in female rats. TFAM protein levels (Fig. 3) were higher in female rats than in males and these gender-related differences were more evident in control rats when expressed per mtDNA. In both genders, HFD feeding decreased TFAM protein levels per mitochondrion but did not modify total TFAM levels referred to mitochondrial protein content. Representative immunoblots are shown in Fig. 4.

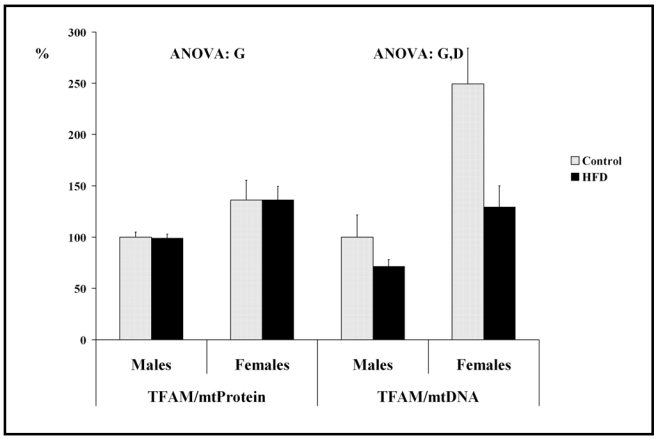

Fig. 3. TFAM protein levels. TFAM, mitochondrial transcription factor A. TFAM/mtDNA was calculated as [TFAM/mg mtprotein]/[mtDNA/mg mtprotein]. Data are means ± SEM of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect and D indicates diet effect. Levels of control male rats were set as 100%.

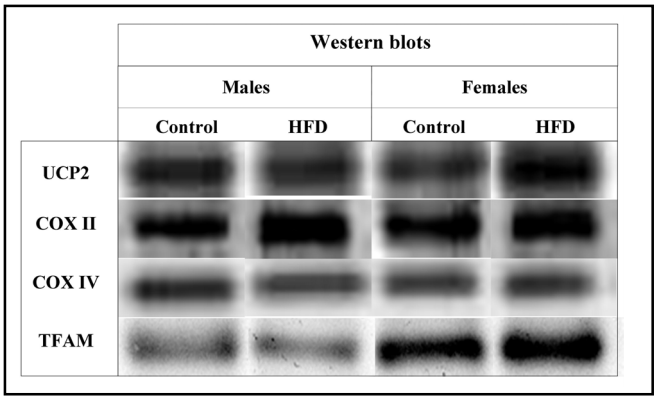

Fig. 4. Representative immunoblots. UCP2, uncoupling protein 2; COX II and COX IV, cytochrome c oxidase subunit II and IV, respectively; TFAM, mitochondrial transcription factor A.

Discussion

We studied the gender-related differences in rat liver mitochondrial function and biogenesis in response to chronic HFD feeding. Compared to male rats, females show a higher mitochondrial oxidative capacity associated to enhanced mitochondrial differentiation. HFD feeding enhances mitochondrial proliferation and decreases mitochondrion differentiation. The sexual dimorphism observed in control animals is maintained under HFD feeding since females continue exhibiting more differentiated mitochondria than males.

Gender effect

Gender-related dimorphism in rat liver mitochondrial oxidative capacity was found, which is indicated by the increased levels of mitochondrial protein and of both COX and CS activities, as well as by the higher whole body and mitochondrial oxygen consumption shown by female rats compared to males, in agreement with previous studies in younger rats [14, 35] and in other tissues [15, 16, 18, 19, 36]. The higher mitochondrial hydrogen peroxide production shown by females is in accordance with their superior oxygen consumption, actually, a relationship between both parameters has been previously reported

[37]. However, the greater hydrogen peroxide production in females compared to males did not lead to higher levels of oxidative damage, which could be due to the enhanced antioxidant capacity found in mitochondria from female rats as it has also been suggested previously [35]. Taking into account that UCP2 uncouples substrate oxidation from ATP synthesis by mediating proton leak across the inner mitochondrial membrane [38] and that an increased ROS production has been related with high levels of UCP2 [39], it can be suggested that the higher ROS production shown by females could activate UCP2 protection against oxidative damage, with this protein playing an essential role in antioxidant defense.

Mitochondrial differentiation is aimed towards building up a more efficient respiratory chain [14]. In this sense, gender differences in oxidative capacity could be the result of more highly differentiated mitochondria with greater machinery per mitochondrion in females compared to males, although this sexual dimorphism is not accompanied by increased mitochondrial size. The lack of differences between genders in mitochondrial morphology in our study is in agreement with previous studies showing unclear differences between genders in this parameter [14]. Moreover, mtDNA levels, a marker of mitochondria content [14, 40], was not different between genders, hence the increase in the number of mitochondria (mitochondrial proliferation) as the cause of the female higher oxidative capacity could be ruled out.

The enhanced differentiation proposed for female rat mitochondria is reinforced by their higher levels of TFAM, a key transcription factor in mtDNA expression. TFAM protein content showed a similar profile to those of PGC-1α mRNA. PGC-1α is the main regulator of mitochondrial biogenesis, since it activates the expression of the subunits of the respiratory chain and of TFAM itself through the induction of the expression of the NRFs [11, 12]. Therefore, the high levels of COX subunits II and IV protein expression per mitochondrion encoded by mitochondrial and nuclear genome, respectively, would be the consequence of the elevated PGC-1α and TFAM levels. Although NRFs mRNA expression did not show the same profile as PGC-1α, the higher levels of PGC-1α found in females could enhance NRF-1 coactivation function, increasing the expression of mitochondrial genes [12]. Thus, the low NRF-1 mRNA expression found in females could be compensated by this higher hypothetical activation, since NRF-1 serine phosphorylation has been associated with enhancement of both its DNA-biding activity [41] and *trans*-activation functions [42].

Diet-induced obesity effects

In response to 26 week-HFD feeding both male and female rats increased their body weight, although male rats showed a higher resistance to becoming obese than females (the excess of body weight was 51.8% for females and only 22.8% for males compared to their control counterparts). This is in agreement with studies using short dietary treatments and similar diets, where the excess of body weight was found to be gender-dependent [43]. However, in spite of this greater body weight, HFD female rats showed a less marked lipid accumulation in liver, which could be related to higher adipose tissue expandability, such as is pointed out by the adiposity index. When the storage capacity of the white adipose tissue is saturated, the excess of fat is accumulated in non-adipose tissues such as liver, muscle or pancreas compromising their normal functionality, a phenomenon known as lipotoxicity [44, 45]. In fact, in response to HFD feeding, adiposity index increased especially in female rats, in which relative adipose tissue increase reached higher levels than in HFD males, without leading to higher liver steatosis. These gender-related differences in white adipose tissue expandability could be associated with females' reproductive function. During evolution, females have been subjected to more severe selection pressure than males, and have developed mechanisms to exploit their energy resources more efficiently to facilitate their own and their progeny's survival [46].

HFD feeding decreased liver antioxidant defenses, though females rats still maintained higher levels than males. Estrogens could be among the liver non-enzymatic antioxidant defenses, since they play an important role by up-regulating the expression of antioxidant, longevity-related genes [47]. Previous studies have shown that liver superoxide dismutase and catalase activities increase in old male rats after hormonal replacement therapy with 17β-estradiol [48], and that liver GPx activity decreases in ovariectomized rats compared to intact females, but increases significantly when the animals are treated with estrogens [49]. Thus, in our study the decreased antioxidant defenses shown by HFD animals could be related to their lower serum levels of estrogens. Although in other tissues estrogens only modulate Mn-SOD expression [50], in liver, estrogens seem to regulate the expression of the main enzymatic antioxidant defenses (SOD, GPx and catalase) and these effects could be mediated by the MAPK pathway, as described in *in vitro* studies [51]. Furthermore, UCP2 protein levels per mitochondrion also decreased in response to HFD feeding,

in accordance with previous studies that associate the absence of UCP2 with a collectively decreased antioxidant defense [52], in spite of others that report that UCP2 is up-regulated in the liver during pathological conditions associated with steatosis [53]. In this sense, UCP2 over-expression could be understood as a short-term mechanism that would not be useful for responding to chronic stimuli such as the one used in this study.

In obese animals, liver mitochondrial proliferation increases in both genders as a compensatory mechanism to counteract the greater amount of substrate available for oxidation, protecting mtDNA and other biomolecules from oxidative damage. This response to the HFD feeding could be mediated by the PPARα, since its expression increases in the liver in obese animals [54] and its activation has been related to an increased mtDNA copy number [55]. In spite of the enhanced mitochondrial proliferation, mitochondrial morphology and synthesis of mitochondrial machinery remained unaltered and, as a consequence, respiratory chain proteins were distributed among the greater number of mitochondria. The result is a mitochondrial population made up of an increased number of less differentiated mitochondria, given their lower oxidative capacity (COX and CS activities, as well as oxygen consumption per mitochondrion, decreased with the dietary treatment). The impaired oxidative capacity of each mitochondrion is compensated by the increased number. In this way, the oxidative capacity of the mitochondrial pool is not altered, since oxidative enzymes activities and oxygen consumption expressed per mtProtein did not show diet effects. In spite of these effects of the HFD feeding in both genders, oxidative capacity and antioxidant defenses continued being higher in female rats than in males.

The lower TFAM/mtDNA ratio shown by HFD animals compared to control rats would result from the distribution of the TFAM protein in a more abundant mitochondrial population, and also suggests that the HFD feeding decreases mitochondrial differentiation, since expression of mtDNA and mitochondrial differentiation is activated only at high concentrations of TFAM [56-58]. These results are also in agreement with the lower COX II and COX IV protein content per mitochondrion exhibited by these animals and could be related to the decrease in estrogen levels and the increase in T3 levels. In fact, ROS inhibit steroidogenesis [48] and estradiol stimulates mitochondrial biogenesis mediated by TFAM [59]. Moreover, the increased levels of T3 in obese rats could also imply variations in mitochondrial biogenesis patterns. Actually, thyroid hormones are one of the main effectors in mitochondrial biogenesis [60] and the hyperthyroid state is associated with increases in both oxidative capacity and oxidative stress [61].

There is some disparity in results concerning the effect of diet-induced oxidative stress on mitochondrial function. Some studies have shown that HFD feeding improves mitochondrial oxidative capacity [62], whereas others have revealed opposite results [63, 64]. The reason for the discrepancy is unclear, although both the length of diet exposure and diet composition could be relevant. In our study, the effects on mitochondrial function found in response to obesity-induced by HFD feeding could be explained by the chronicity of the dietary stimulus that could have the ability to induce adaptive mechanisms to avoid the deleterious consequences of the treatment, this is in agreement with previous studies that relate severe reductions in mitochondrial function to shorter treatments [65].

In summary, differences in mitochondrial function exist between genders that can be due to a different regulation of mitochondrial biogenesis. In comparison to male rats, females show higher oxidative capacity as a consequence of their greater mitochondrial differentiation under both control and obese status. In response to HFD feeding, the oxidative capacity of the whole mitochondrial population is maintained in both genders. This is obtained by means of an enhancement of mitochondrial proliferation, which counteracts the diet-induced impairment of the function of each mitochondrion. Nevertheless, further investigations into the signaling pathways involved in the regulation of the mitochondrial biogenesis process would give more clues as to this gender dimorphism.

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