Skeletal Muscle and Liver Oxidative Metabolism in Response to a Voluntary Isocaloric Intake of a High Fat Diet in Male and Female Rats

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Key Words

High fat diet • Sexual dimorphism • Isocaloric intake • Muscle oxidative metabolism • Uncoupling proteins • Oxidative stress

Abstract

High fat diets (HFD) usually lead to hyperphagia and body weight gain. However, macronutrient proportions in the diet can modulate energy intake and body fat deposition. The aim of the study was to investigate muscle and liver oxidative metabolism in response to an isocaloric intake of a HFD and to elucidate the possible gender-dependent response. Eight week-old male and female rats were fed either standard chow or HFD for 14 weeks. Energy intake, body weight and whole animal oxygen consumption were determined periodically. Mitochondrial oxygen consumption, hydrogen peroxide production, TBARS levels, Cytochrome-c-oxidase, Citrate synthase and antioxidant enzyme activities were measured in muscle and liver. UCP1 and UCP3 protein levels were analyzed in brown adipose tissue and muscle, respectively. Male rats showed higher energy efficiency, enhanced adiposity, greater hydrogen peroxide production and less effective antioxidant

machinery compared to females. HFD feeding increased energy expenditure but did not modify either tissue oxidative metabolism or oxidative damage in either gender. HFD animals over-expressed uncoupling proteins in order to maintain energy balance (brown adipose tissue UCP1) and to avoid oxidative stress (skeletal muscle UCP3), thus counteracting the alterations induced by the modification of the proportion of macronutrients in the diet.

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Introduction

Diets usually consumed in industrialized countries are rich in animal fat and have been associated with an increased incidence of obesity [1]. The role of different macronutrient combinations in the diet on energy intake, energy metabolism, and adiposity is currently under study. High fat diets lead to increased adiposity, insulin resistance and impaired glucose metabolism in rats [2, 3]. In contrast, high protein diets at the expense of carbohydrates not

only facilitate body weight loss but also improve insulin resistance and lipid metabolism [4, 5].

Oxidative stress is the consequence of an imbalance between pro-oxidants and antioxidants in the favor of more pro-oxidants and has been considered to play a role in diet-induced health impairment [6]. High fat diets increase the amount of substrate available for oxidation and the electron flux through the mitochondrial respiratory chain by enhancing free radical production [7]. Reactive oxygen species (ROS) cause oxidative damage in mitochondrial components in the form of lipid peroxidation, protein modifications, and DNA mutation [8], 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 [9].

The uncoupling proteins (UCPs) have several hypothesized functions including thermogenesis in certain tissues, fatty acid export, mediation of insulin secretion, and protection from ROS. Mitochondrial uncoupling proteins have been shown to dissipate mitochondrial membrane potential, thus reducing free radical production. In fact, muscle UCP3 has been suggested to protect the mitochondria from the toxic effects of fatty acid anions or peroxides by transporting fatty acids out of mitochondria [10, 11].

We have previously reported a gender dimorphism in energy balance in both basal conditions [12, 13] and in response to diet manipulations [14-17], with female rats showing greater total energy expenditure than males. The gender differences in energy efficiency can be linked to changes in the regulation of mechanisms to obtain this energy [13, 18]. In this sense, the hormonal milieu could play an important role in the regulation of the energy metabolism and especially in mitochondrial function.

In the present study we used a high fat diet feeding model with lower carbohydrate content than the control diet, which does not induce either a pro-inflammatory serum lipid profile [19] or hyperphagia. The aim of this study was to investigate muscle and liver oxidative metabolism adaptations to this feeding model and a possible gender-dependent response.

Materials and Methods

Materials

Enzymes, substrates and coenzymes were purchased from Sigma-Aldrich (St Louis, MO, USA). Amplex Red reagent was from Invitrogen (Carlsbad, CA, USA). Antibodies for UCP1 and UCP3 were obtained from Alpha Diagnostic International

(San Antonio, TX, USA). The enhanced chemiluminescence Western blotting analysis system was supplied by Amersham (Little Chalfont, UK). Routine chemicals used were purchased from Sigma-Aldrich (St Louis, MO, USA) and Panreac (Barcelona, Spain). Diets were obtained from Panlab (Barcelona, Spain).

Animals and diets

Experiments were performed in accordance with the general guidelines approved by our institutional ethics committee and EU regulations (2003/65/CE). At the start of the study, 14 male and 14 female Wistar rats (Charles River, Barcelona, Spain) of 8 weeks of age were housed two per cage with free access to food and water in a temperature controlled room (22 °C) under a 12 h light/dark cycle. For each gender, rats were divided into two dietary groups: a *control group*, fed with a pelleted standard diet with 2.9% (w/w) fat (SFA 0.6%, MUFA 0.7% and PUFA 1.5%) and a *high fat group*, fed with a high fat diet (HFD) with 30% (w/w) fat (SFA 13%, MUFA 14% and PUFA 3%). The content of protein, carbohydrate and fat in the standard diet represented 18.7%, 73.3% and 8% respectively of the total energy content of the diet (3385 Kcal/Kg diet). In the HFD, protein, carbohydrate and fat were 13.5%, 31.3% and 55.2% of the total energy content of the diet (5055 Kcal/Kg diet) [19]. Energy intake and body weight were determined weekly and fortnightly respectively throughout the 14 weeks of the experiment. The energy efficiency was calculated as a ratio between body weight gain and total food eaten over the period studied. After a period of 12 h fasting, rats were killed by decapitation and gastrocnemius muscle, liver and brown (interscapular) and white adipose depots (inguinal, gonadal, mesenteric and lumbar) were dissected and weighed. Mitochondrial isolation was performed in fresh samples of muscle and liver. The rest of these tissues and the adipose depots were kept at -80 °C until analyzed.

Indirect Calorimetry

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured monthly (4 animals/group) by open circuit respirometry (flow rate 1 L/min). The measurements were performed during the last 2 h of the dark phase at 22 °C. The animals were individually placed in calorimetric chambers and were allowed to become acclimated for 1 h. Data recordings were made sequentially in each cage for 90-second periods throughout a total 90 min period. VO_2 and VCO_2 were adjusted for body surface ($\text{mL} \cdot \text{min}^{-1} \times \text{kg}^{0.75}$) and averaged over the collection period [20]. Energy expenditure was calculated from indirect calorimetry data:

$$\text{EE (Kcal)} = 3.9 \times \text{L O}_2 \text{ used} + 1.1 \times \text{L CO}_2 \text{ produced,}$$

where L O_2 is O_2 consumption (L/h) and L CO_2 is CO_2 production (L/h) [21].

Tissue composition

Muscle and liver homogenates were used to determine protein [22] and DNA [23]. Tissue triglycerides were extracted from 30 mg of muscle as previously described [24]. Triglycerides in the extract were determined spectrophotometrically with a commercial kit.

	Male		Female		ANOVA
	Control	High fat	Control	High fat	
Body weight (g)	468±8	471±13	267±8	268±5	G
Weight gain (g)	156±6	166±10	62.7±8.5	69.4±6.0	G
Food intake (g/100g body weight)	4.91±0.09	3.58±0.19	5.87±0.46	4.45±0.46	G, D
Energy intake (Kcal/100g body weight)	17.2±0.2	15.8±0.8	20.6±1.6	19.8±1.9	G
Carbohydrate intake (Kcal/100g body weight)	12.3±0.3	3.30±0.06°	15.7±0.5*	4.53±0.32°*	G, D, GxD
Protein intake (Kcal/100g body weight)	3.14±0.07	2.65±0.05	3.99±0.12	3.65±0.26	G
Lipid intake (Kcal/100g body weight)	1.32±0.03	10.6±0.2°	1.70±0.05*	14.7±1.1°*	G, D, GxD
Energy efficiency (weight gain/100g food intake)	6.90±0.24	10.4±0.6°	4.63±0.67*	5.72±0.54*	G, D, GxD
Energy expenditure (Kcal/h kg ^{0.75})	6.43±0.34	7.53±0.50	8.60±0.53	9.05±0.64	NS
White fat depot weight (g)	25.3±3.0	42.8±4.1°	13.7±2.0*	18.9±2.0*	G, D, GxD
White fat depot weight(g/100g body weight)	5.55±0.57	9.02±0.70	5.30±0.74	6.40±0.71	G, D

Table 1. Effect of HFD feeding on food and energy intake in male and female rats. White fat depot weight is the sum of inguinal, gonadal, mesenteric and lumbar fat depot weights. Values are expressed as the mean ± SEM of 7 animals per group. ANOVA ($p < 0.05$): G, gender effect; D, diet effect; GxD, interactive effect; NS, non significant. Student's t -test ($p \leq 0.05$): °HFD vs control, *female vs male.

Mitochondria isolation

Muscle mitochondria were isolated as previously described [25]. Briefly, tissue samples were minced at 4 °C and then incubated in constant stirring for a total of 8 min at 4 °C with buffer A (0.1 M KCl, 0.05 M Tris-HCl, 2 mM EGTA, 1 mM ATP, 5 mM MgCl₂, 0.5% BSA, 0.15 g/L collagenase type VIII, pH 7.4) in a proportion of 10 mL of buffer A per gram of tissue. After the first 4 min of incubation, tissue was homogenized with a Potter/Elvehjem homogenizer and then incubated again until the end of the aforementioned 8 min. To remove the nuclei and cell debris the homogenate was centrifuged at 500 xg for 10 min at 4°C. The resulting supernatant was filtered through a nylon mesh and centrifuged at 10000 xg for 10 min at 4 °C. The pellet was resuspended with buffer B (0.1 M KCl, 0.05 M Tris-HCl, 2 mM EGTA, pH 7.4) and centrifuged at 10000 xg for 10 min at 4 °C. Finally, the resulting pellet was resuspended with buffer B and centrifuged at 4000 xg for 10 min at 4 °C. The mitochondria pellet was resuspended with 1 mL of buffer B.

Liver mitochondria were isolated as previously described [15]. Tissue samples were homogenized in STE buffer (250 mM Sacarose, 5 mM Tris-HCl, 2 mM EGTA, 0.1% BSA, pH 7.4) in a proportion of 7 mL per gram of tissue. Homogenate was centrifuged at 500 xg for 10 min at 4 °C to remove the nuclei and cell debris and the resulting supernatant was centrifuged at 8000 xg for 10 min at 4 °C. The pellet was resuspended with STE buffer and centrifuged at 8000 xg for 10 min at 4 °C. The mitochondria pellet was resuspended with 1 mL of STE buffer.

Mitochondrial protein was measured as previously described [26].

Measurement of mitochondrial respiration rate

Muscle and liver mitochondria oxygen consumption (VO₂) were measured polarographically as previously described [27]. Isolated mitochondria (0.5 and 2 mg protein/ mL for muscle and liver respectively) were incubated in a water-thermo-

statically regulated chamber with a computer-controlled oxygen Clark-type electrode Oxygraph (Hansatech, Norfolk, UK) in a respiratory buffer (145 mM KCl, 30 mM HEPES, 5 mM KH₂PO₄, 3 mM MgCl₂, 0.1 mM EGTA, 0.1% BSA, pH 7.4 at 37 °C). Succinate/rotenone (10 mM/50 µM) was used as substrate in the absence (state 4) and presence (state 3) of 250 µM ADP. Mitochondrial viability was checked by the respiratory control ratio (RCR = state 3 VO₂/state 4 VO₂).

Mitochondrial hydrogen peroxide production

Hydrogen peroxide production was assayed in isolated mitochondria by measuring the increase in fluorescence (530 nm excitation, 590 nm emission) due to the reaction of Amplex Red reagent with hydrogen peroxide in the presence of horseradish peroxidase [28]. Assays were performed at 37 °C for 30 min in a 96-well microplate fluorimeter FLx800 (Bio-tek instruments, Winooski, VT, USA). Mitochondria (0.2 mg protein/mL for muscle and 0.3 mg protein/mL for liver) were added to the respiratory buffer (see composition in the above section) supplemented with 0.1 U/mL horseradish peroxidase and 50 µM Amplex Red reagent. In muscle samples, hydrogen peroxide production was measured in the presence of succinate/rotenone (10 mM/50 µM), and in liver samples, in the presence of pyruvate/malate (10 mM/2.5 mM). The rate of hydrogen peroxide production was calculated using a standard curve of hydrogen peroxide generated by addition of β-D(+)-glucose 14 mM in the presence of glucose oxidase.

Enzymatic analysis

Catalase activity was measured in tissue homogenates [29]. Glutathione reductase (Gsr), Glutathione peroxidase (Gpx), Superoxide dismutase (SOD) and Cytochrome C oxidase (COX) and Citrate synthase (CS) activities were measured in isolated mitochondria as previously described [30-34].

Fig. 1. Effect of HFD feeding on O_2 consumption in male and female rats. At the beginning of the experiment, animals were 8 weeks old. Values are expressed as the mean $\pm$ SEM of 4 animals per group. ANOVA ($p < 0.05$): G, gender effect; D, diet effect.

Fig. 2. Effect of HFD feeding on brown adipose tissue UCP1 protein levels in male and female rats. Values are expressed as the mean $\pm$ SEM of 7 animals per group. Mean values of control male rats were set as 100%. ANOVA ($p < 0.05$): D, diet effect.

Measurement of thiobarbituric acid-reactive substances (TBARS)

Levels of TBARS, as an index of lipid peroxides, were measured spectrophotometrically in muscle and liver homogenates as previously described [35].

Western blot analysis of brown adipose tissue UCP1 and muscle UCP3

Either 5 (UCP1) or 30 (UCP3) μ g of protein were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% polyacrylamide), and electrotransferred onto a nitrocellulose filter as described elsewhere [36]. Ponceau S staining was used to provide visual evidence for correct loading and electrophoretic transfer of proteins onto the nitrocellulose filter. Blocking and development of the immunoblots were performed using an enhanced chemiluminescence Western blotting analysis system. Rabbit polyclonal antibodies against UCP1 and UCP3 were used as primary antibodies. Bands in films were quantified by scanner photodensitometric analyses (Kodak 1D Image Analysis Software). Autoradiograms revealed an apparent molecular mass of 32 kDa and 34 kDa for UCP1 and UCP3, respectively.

Statistical analysis

All data are presented as mean values $\pm$ SEM of 7 animals per group. Statistical analyses were performed using a statistical software package (SPSS version 13 for Windows, Inc., Chicago, IL, USA). Statistical differences between experimental groups was analysed by two-way analysis of variance (ANOVA). Student's t-test, as post-hoc comparison, was performed when an interactive effect of gender and diet was shown. A p-value of less than 0.05 was considered statistically significant.

Results

Food and energy intake

Consumption of the HFD (Table 1) did not induce an increase in body weight in either gender, but did induce a significant increase in adiposity in male rats. The food intake of the HFD animals was lower than that of the controls, but due to the high caloric content of the HFD, the energy intake was at the same levels as control rats.

Table 2. Effect of HFD feeding on muscle and liver composition in male and female rats. Values are expressed as the mean $\pm$ SEM of 7 animals per group. ANOVA ($p < 0.05$): G, gender effect; D, diet effect; GxD, interactive effect; NS, non significant. Student's t -test ($p \leq 0.05$): °HFD vs control, *female vs male.

	Male		Female		ANOVA
	Control	High fat	Control	High fat	
Muscle					
Weight (g)	5.70±0.14	5.33±0.14	3.40±0.11	3.45±0.09	G
Weight (g/100g body weight)	1.26±0.02	1.13±0.02	1.32±0.04	1.31±0.02	G, D
DNA (mg/g tissue)	1.39±0.16	1.66±0.08	1.47±0.12	1.27±0.12	NS
Protein (mg/g tissue)	125±14	128±6	132±6	127±5	NS
Triglycerides (mg/g tissue)	4.0±0.4	8.4±0.3 ^o	6.4±0.4*	8.8±0.7 ^o	G, D, GxD
Liver					
Weight (g)	11.1±0.3	11.1±0.3	6.22±0.44	6.20±0.18	G
Weight (g/100g body weight)	2.45±0.08	2.37±0.04	2.41±0.13	2.36±0.07	NS
DNA (mg/g tissue)	7.35±0.73	6.85±0.46	7.73±0.88	7.05±0.48	NS
Protein (mg/g tissue)	135±13	159±11	169±7	182±11	G

Relative food and energy intake of HFD female rats was higher than males, thus resulting in a greater intake of energy as carbohydrates, proteins and fat. Energy efficiency was lower in HFD female rats than their male counterparts. No significant differences between groups were observed in energy expenditure.

Whole animal respirometry

Whole animal O_2 consumption data are compiled in Figure 1. Female rats showed greater O_2 consumption than males over the period studied, reaching statistical significance on day 90 (the rise was 136% in control and 119% in HFD females compared to their male counterparts). HFD feeding induced an increase in O_2 consumption in both male and female rats, although significant differences were found only on day 30 (HFD males showed 122% and HFD females 120% increase compared to their control counterparts). No significant differences were found in the respiratory quotient values (data not shown).

Muscle and liver weight and composition

The relative contribution of gastrocnemius muscle to body weight was greater in female rats (Table 2) and decreased in response to HFD. Triglyceride content was higher in control female rats than in males and increased in response to HFD in both genders, although more markedly in male rats. No significant differences were found either in DNA levels or protein content.

Liver protein content was higher in female rats than in males. No significant differences were seen in liver DNA content. HFD did not induce changes in liver composition.

Brown adipose tissue UCP1 protein levels

UCP1 protein levels are shown in Figure 2. Female rats had higher UCP1 protein levels than male rats, although differences did not reach statistical significance. In both genders, HFD feeding increased UCP1 protein levels.

Mitochondrial oxygen consumption and oxidative activities

The effect of HFD on mitochondrial O_2 consumption in muscle and liver is shown in Figure 3. In muscle, O_2 consumption was higher in female rats but did not rise in response to the HFD. In liver, neither gender-related nor diet-related differences were found.

COX and CS activities (Table 3) were slightly higher in female than in male rats, although differences were not statistically significant. There were no diet related differences.

Muscle and liver mitochondrial peroxide production and TBARS levels

Muscle mitochondria from female rats generated less hydrogen peroxide than males, and no effects of HFD were found (Table 4). Neither were any changes between groups observed in muscle TBARS levels.

In liver, hydrogen peroxide and TBARS levels showed similar values in both genders. No differences related to diet either in hydrogen peroxide generation or in TBARS levels were observed.

Antioxidant enzyme activities

Antioxidant enzyme activities are shown in Table 5. In muscle, Gpx, Gsr and SOD activities were higher in

Fig. 3. Effect of HFD feeding on mitochondrial O₂ consumption by muscle and liver mitochondria from male and female rats. O₂ consumption rates were measured in isolated mitochondria using succinate/rotenone in presence (phosphorylating, state 3) and absence (resting, state 4) of ADP. Values are expressed as the mean $\pm$ SEM of 7 animals per group. ANOVA ($p < 0.05$): G, gender effect.

Table 3. Effect of HFD feeding on muscle and liver Cytochrome c oxidase and Citrate synthase in male and female rats. COX, Cytochrome c oxidase; CS, Citrate synthase. All enzymatic activities were measured in isolated mitochondria and are expressed in mIU/mg of protein. Values are expressed as the mean $\pm$ SEM of 7 animals per group. ANOVA ($p < 0.05$): NS, non significant.

	Male		Female		ANOVA
	Control	High fat	Control	High fat	
Muscle					
COX	602 $\pm$ 62	676 $\pm$ 38	714 $\pm$ 88	669 $\pm$ 83	NS
CS	421 $\pm$ 48	494 $\pm$ 62	549 $\pm$ 62	579 $\pm$ 86	NS
Liver					
COX	271 $\pm$ 18	272 $\pm$ 25	316 $\pm$ 49	262 $\pm$ 38	NS
CS	25.2 $\pm$ 3.3	29.2 $\pm$ 6.8	33.4 $\pm$ 5.2	30.1 $\pm$ 3.2	NS

	Male		Female		ANOVA
	Control	High fat	Control	High fat	
Muscle					
Mitochondrial H ₂ O ₂ production (pmolH ₂ O ₂ /min mg prot)	181 $\pm$ 34	201 $\pm$ 42	95.3 $\pm$ 19.3	75.3 $\pm$ 17.9	G
TBARS (nmol TBARS/g tissue)	180 $\pm$ 25	209 $\pm$ 26	151 $\pm$ 10	171 $\pm$ 9	NS
Liver					
Mitochondrial H ₂ O ₂ production (pmolH ₂ O ₂ /min mg prot)	94.3 $\pm$ 23.6	52.5 $\pm$ 17.4	55.6 $\pm$ 9.2	73.2 $\pm$ 11.5	NS
TBARS (nmol TBARS/g tissue)	240 $\pm$ 15	240 $\pm$ 14	235 $\pm$ 28	233 $\pm$ 14	NS

Table 4. Effect of HFD feeding on muscle and liver hydrogen peroxide production and TBARS levels in male and female rats. TBARS levels were measured in tissue homogenate and hydrogen peroxide production in isolated mitochondria by addition of succinate and rotenone in muscle and pyruvate and malate in liver. Values are expressed as the mean $\pm$ SEM of 7 animals per group. ANOVA ($p < 0.05$): G, gender effect; NS, non significant.

females compared to males while catalase activity was lower. The consumption of the HFD induced a decrease in Gpx activity only in female rats.

In liver, Gpx activity was higher in female rats than in males, while catalase activity was lower. Gpx activity showed a diet effect, which was lower in HFD females compared to controls. Gsr and SOD

activities did not show either gender or diet-related differences.

Muscle UCP3 protein levels

UCP3 protein levels are shown in Figure 4. In both genders, HFD increased UCP3 protein levels. No significant gender-related differences were observed.

Table 5. Effect of HFD feeding on muscle and liver antioxidant enzyme activities in male and female rats. Gpx, Glutathione peroxidase; Gsr, Glutathione reductase; SOD, Superoxide dismutase. All enzymatic activities were measured in isolated mitochondria except catalase activity which was measured in tissue homogenate. All activities are expressed in mIU/mg of protein except catalase activity that is expressed in IU/mg of protein. Values are expressed as the mean $\pm$ SEM of 7 animals per group. ANOVA ($p < 0.05$): G, gender effect; D, diet effect; GxD, interactive effect; NS, non significant. Student's t -test ($p \leq 0.05$): HFD vs control, *female vs male.

	Male		Female		ANOVA
	Control	High fat	Control	High fat	
Muscle					
Catalase	10.5±1.6	11.8±0.79	8.18±0.67	6.08±0.63	G
Gpx	91.6±6.7	97.3±11.4	178±14*	119±10°	G, D, GxD
Gsr	22.4±1.2	31.1±1.9	40.3±4.6	35.6±4.2	G
SOD	124±18	152±28	188±45	260±65	G
Liver					
Catalase	708±85	538±44	460±47	432±21	G
Gpx	917±60	826±55	1659±113*	1083±74*°	G, D, GxD
Gsr	36.4±1.5	31.1±2.4	30.6±2.0	27.8±3.0	NS
SOD	31.5±2.9	29.8±3.4	25.4±2.7	27.1±3.0	NS

Fig. 4. Effect of HFD feeding on muscle UCP3 protein levels from male and female rats. Values are expressed as the mean $\pm$ SEM of 7 animals per group. Mean values of control males were set as 100%. ANOVA ($p < 0.05$): D, diet effect.

Discussion

In response to HFD feeding both male and female rats reduced their food intake and maintained their energy intake, thus avoiding body weight gain. In fact, the reduced levels of the appetite-stimulating peptide ghrelin shown by the HFD rats [19] point to a satiating effect of this diet. Although many fat-rich diets lead to hyperphagia and body weight gain [37-39], it has recently been reported, in both rodents and humans, that the nutrient proportion in the diet can modulate energy intake and body weight changes [40]. The proportion of macronutrients in the diets used in our study - carbohydrate/fat ratio was 9.2 for the control diet and 0.57 for the HFD while the protein/carbohydrate ratio was 0.25 and 0.43 respectively - could explain the lack of both hyperphagia and body weight gain since high fat diets have been reported to cause hyperphagia only in combination with a high carbohydrate intake [40]. This is in accordance with some studies in humans showing that the weight-reducing

effect of low-carbohydrate/high-protein diets is mainly due to decreased energy intake [41].

Body weight gain and fat accumulation in the tissues result from an imbalance between energy intake and energy expenditure. In rodents, an important component of energy expenditure is adaptive thermogenesis in brown adipose tissue [42], measured as UCP1 protein levels, which is enhanced in both genders by HFD feeding, thus contributing to the maintenance of body weight, which is in agreement with previous results [36, 43, 44].

In spite of maintaining a body weight closer to that of the control rats, HFD animals showed a marked increase of fat depots, especially male rats whose body fat rose nearly two-fold. This finding is consistent with both experimental observations in which dietary fat promotes increased adiposity and greater energy efficiency independently of energy intake [45], and with the higher efficiency of fat utilization compared to carbohydrates or proteins [46]. Thus, it has been reported that adult rats fed a HFD could become obese (in terms

of an increase in body fat) compared to rats fed with a low-fat diet, despite minimal changes in body weight [45], as observed in our study, particularly in the male rats. The higher adiposity reached by HFD male rats compared to females occurred despite a lower energy intake, thus conferring on male rats enhanced energy efficiency, which could be due to the lower levels of energy expenditure and whole body oxygen consumption. It has been suggested that one of the possible reasons for energy inefficiency in female rats is the greater proportion of metabolically active organs per unit of body mass [15]. The higher relative muscle weight shown by female rats in our study is in agreement with this idea.

Our results concerning muscle and liver mitochondrial oxidative capacity are in accordance with the whole animal oxygen consumption. In our study, female rats show a greater oxidative capacity in both muscle and liver mitochondria compared to males. In fact, changes in mitochondrial oxidative capacity affect the metabolic rate of specific organs or tissues and subsequently whole body energy expenditure [47]. This increased tissue oxidative activity would further explain that females have higher energy intake but lower body fat deposition compared to males.

The triglyceride accumulation found in skeletal muscle of HFD rats could be the result of a lack of response of the mitochondrial oxidative machinery to compensate the increased fatty acid flux through this tissue [45, 48]. Intramuscular triglyceride deposition has been reported as one of the most important causes leading to insulin resistance [49]. Skeletal muscle is the primary site of insulin action and thus is inherently linked to the development of whole body insulin resistance [49]. In fact, HFD male rats showed both increased insulin plasma levels [19] and a greater increase of muscle triglyceride accumulation compared to controls.

Our results indicate that mitochondria from females produce significantly less hydrogen peroxide and have a more effective antioxidant machinery than males, in accordance with previous results reported in liver [50] and cardiac muscle [16]. In fact, it has been shown that female sexual hormones are involved in the up-regulation of antioxidant enzymes and the subsequent decrease in free radical levels [51]. The age-related increase in muscle mitochondrial hydrogen peroxide production observed in 18 month-old female rats (unpublished data) could be attributed to the decrease of estrogen levels that occurs

with ageing, and further reinforces this idea. In response to HFD feeding, the antioxidant protection of female rats is reduced to the same level of male values, thus suggesting that, although liver and muscle oxidative damage is not increased, a more harmful effect of a prolonged treatment of this diet cannot be discarded.

Although lipid peroxidation seems to be dependent on the amount of lipid substrate present for oxidation [52], HFD feeding did not induce an increase in oxidative damage in spite of the intramuscular triglyceride accumulation found. We suggest that the UCP3 overexpression induced by HFD could be a response to protect against oxidative damage, since UCP3 has been suggested to play an important physiological role in facilitating the outward transport from the mitochondrial matrix of fatty acid anions that cannot be oxidized, thereby protecting against lipid-induced mitochondrial damage by attenuating the production of free radicals. In fact, it has been shown that UCP3 is activated by ROS or lipid peroxidation by-products, which in turn would lead to mild uncoupling and, subsequently, mitigated ROS production [11, 53]. The overexpression of UCP3 that we have found could explain the lack of oxidative damage in spite of the intramuscular triglyceride accumulation found.

In summary, we found a gender dimorphism with male rats showing high energy efficiency and enhanced adiposity, high hydrogen peroxide production and less effective antioxidant machinery. In addition, the consumption of a HFD that does not increase energy intake induced greater energy expenditure but no changes in skeletal muscle oxidative metabolism or oxidative damage. In response to the HFD, animals of both genders overexpressed uncoupling proteins in order to maintain energy balance (brown adipose tissue UCP1) and to avoid oxidative stress (skeletal muscle UCP3), thus counteracting the alterations induced by the modification of the proportion of macronutrients in the diet.

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