Gender Dimorphism in High-Fat-Diet-Induced Insulin Resistance in Skeletal Muscle of Aged Rats

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Key Words

Obesity • Oxidative stress • Oxidative damage • GLUT4 • UCP3 • Adipokine

Abstract

Muscle resistance to insulin plays a key role in the metabolic dysregulation associated to obesity. A pro-inflammatory and pro-oxidant status has been proposed to be the link between dietary obesity and insulin resistance. Given the gender differences previously found in mitochondrial function and oxidative stress, the aim of the present study was to investigate whether this gender dimorphism leads to differences in the development of high-fat-diet-induced insulin resistance in rat skeletal muscle. Male and female rats of 15 months of age were fed with a high-fat-diet (32% fat) for 14 weeks. Control male rats showed a more marked insulin resistance status compared to females, as indicated by the glucose tolerance curve profile and the serum insulin, resistin and adiponectin levels. High-fat-diet feeding induced an excess of body weight of 16.2% in males and 38.4% in females, an increase in both muscle mitochondrial hydrogen peroxide production and in oxidative damage, together with a decrease in the

Mn-superoxide dismutase activity in both genders. However, high-fat-diet fed female rats showed a less marked insulin resistance profile than males, higher mitochondrial oxygen consumption and cytochrome c oxidase activity, and a better capacity to counteract the oxidative-stress-dependent insulin resistance through an overexpression of both muscle UCP3 and GLUT4 proteins. These results point to a gender dimorphism in the insulin resistance status and in the response of skeletal muscle to high-fat-diet feeding which could be related to a more detrimental effect of age in male rats.

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Introduction

Skeletal muscle constitutes the main insulin-sensitive tissue in the body and is the primary site for insulin-stimulated glucose utilization. Muscle resistance to insulin plays a key role in the metabolic dysregulation associated to obesity contributing to the development of the metabolic syndrome [1].

A pro-inflammatory and pro-oxidant status has been proposed to be the link between obesity associated to

excessive nutrient and energy intake and insulin resistance [2, 3]. High-fat-diet intake increases muscle fatty acid uptake and oxidation leading to increased mitochondrial reactive oxygen species (ROS) production in this tissue [4]. Moreover, an impairment of antioxidant defences has also been reported in diet-induced obesity [5]. The excessive ROS production, not counteracted by the antioxidant defences, leads to an oxidative stress status and to mitochondrial damage. Furthermore, ROS-induced mitochondrial dysfunction can lead to disruptions of the lipid metabolism, by increasing the intracellular lipid content and contributing to lipid-dependent insulin resistance in muscle cells. Insulin resistance has been considered a compensatory mechanism to protect the cells from further insulin-stimulated glucose and fatty acid uptake and therefore from oxidative damage [4, 6].

Several studies in rats have reported gender differences in the development of obesity-associated to high fat diet feeding, in which males showed a greater resistance to increase body weight than females [7, 8]. However, females seem to be more protected from obesity-associated disorders, such as hypertension [7] and sucrose-induced insulin resistance [9], and this protection has been attributed to the sex hormone milieu [7]. Gender differences have also been observed in mitochondrial function and in oxidative stress parameters in different tissues such as muscle [10, 11], liver [12-14] and brown adipose tissue. Sex dimorphism in oxidative capacity, antioxidant enzyme activities and hydrogen peroxide production points to a higher protection against mitochondrial oxidative damage in female rats.

Taking into account the reported role of oxidative stress in the development of obesity-associated insulin sensitivity impairment, the aim of the present study was to investigate whether the gender differences found in mitochondrial function and oxidative stress lead to differences in the development of high-fat-diet-induced insulin resistance in rat skeletal muscle.

Materials and Methods

Animals and diets

Animal experiments were performed in accordance with general guidelines approved by our institutional ethics committee and EU regulations (86/609/EEC). Male and female *Rattus norvegicus* Wistar rats of 15 months of age (Charles River, Barcelona, Spain) were housed two per cage with free access to food and water and were kept at 22°C under a 12-hour light-dark cycle. Both male and female rats were divided into two groups (n=6) with a similar mean body weight (568 ±

27 g for male rats and 355 ± 10 g for female rats) and were fed a control pelleted diet (3,385 Kcal/Kg diet; 2.9% fat by weight) or a high fat diet (HFD) (4,516 Kcal/Kg diet; 32% of fat by weight) for 14 weeks. The HFD (namely cafeteria diet) components were cookies, pork liver pâté, fresh bacon, fairy cakes, chocolate, carrots and ensaïmada (a typical Majorcan pastry). The energy composition of the HFD was 8% protein, 34% carbohydrate and 64% lipid, and of the control pelleted diet (A04, Panlab, Barcelona) 19% protein, 73% carbohydrate and 8% lipid. Animal body weights were assessed weekly and food intake fortnightly throughout the dietary treatment. 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-hours period of fasting. Serum was collected and gastrocnemius skeletal muscle was rapidly dissected and weighed. Serum samples and a piece of the muscle were frozen in liquid N₂ and stored at -70 °C until analysed, the rest of the tissue was immediately processed.

Materials

Acutrend® GCT-meter and glucose test strips were supplied by Roche Diagnostics (Basel, Switzerland). Rat insulin enzyme immunoassay kit was purchased from Mercodia (Uppsala, Sweden) and enzyme immunoassays for measurement of rat adiponectin, leptin and resistin were from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). The kit for measurement of triglycerides was acquired from Linear Chemicals SL (Barcelona, Spain). Amplex Red kit was obtained from Invitrogen (Carlsbad, CA, USA). Oxyblot™ Protein Oxidation Detection Kit was purchased from Chemicon International (Temecula, CA, USA). Rabbit polyclonal antibodies to rat UCP3 (Cat. Num. AB4036) and GLUT4 (Cat. Num. sc-7938) were obtained from Chemicon International (Temecula, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Routine chemicals used were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain) and Sigma-Aldrich (St. Louis, MO, USA).

Oral Glucose Tolerance Test

The test was performed on the week before the sacrifice. Rats were fasted for 12 hours and then glucose (2 g/Kg body weight) was given orally. Blood was collected from the saphena vein before glucose administration and after 15, 30, 60, 90 and 120 min, and glucose concentrations were measured using the Accutrend® system. The analysis of the area under the curve (AUC) of the blood glucose response was calculated as described previously [17].

Serum glucose, insulin and adipokine levels

Serum glucose levels were measured by using the Accutrend® system and serum insulin, adiponectin, leptin and resistin levels were measured by enzyme immunoassay kits.

	Male		Female		ANOVA
	Control	HFD	Control	HFD	
Food intake (g/Kg day)	30.8±1.1	112±12	47.9±3.0	130±13	D
Energy intake (Kcal/Kg day)	92.5±10.5	404±67	143±16	458±73	D
Body weight gain (g)	12.8±8.5	129±17	19.7±10.2	169±18	D
Energetic efficiency (g weight gain/Kg body weight 100Kcal)	0.325±0.203	0.676±0.091	0.978±0.497	1.45±0.22	G
Muscle weight (g)	4.98±0.14	5.40±0.41	3.48±0.07	3.94±0.08	G, D
Relative muscle weight (g/100g)	0.817±0.047	0.809±0.028	0.895±0.044	0.797±0.053	NS
Triglyceride (mg/g tissue)	1.83±0.05	1.85±0.01	1.22±0.06 [†]	1.84±0.02*	G, D, GxD
Protein (mg/g tissue)	102±6	107±8	102±6	99.0±7.6	NS
DNA (mg/g tissue)	1.37±0.06	1.46±0.22	1.17±0.05	1.53±0.07	D

Table 1. Energy intake, body weight gain and muscle composition. Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): G indicates gender effect, D indicates diet effect, GxD indicates gender and HFD interactive effect and NS stands for non significant. Student's t-test (p<0.05): * diet vs control, [†] females vs males.

Homeostasis Model Assesment HOMA-β and HOMA-IR were used to estimate insulin secretion and insulin resistance, respectively [18]. HOMA-β was calculated as [20 x fasting insulin (μU/mL)]/[fasting glucose (mM) - 3.5] and HOMA-IR as [fasting glucose (mM) x fasting insulin (μU/mL)]/22.5.

Muscle triglyceride content

Tissue triglycerides were extracted from 15 mg of muscle in 2 mL of 2:1 chloroform-methanol [19]. Triglycerides were determined spectrophotometrically with a commercial kit.

Muscle mitochondria isolation

Mitochondria were isolated from muscle as previously described [20]. Briefly, tissue samples were minced at 4°C and then incubated in constant stirring for a total of 8 minutes 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% free fatty acid BSA, 0.15 g/L type VIII collagenase, pH 7.4) in a proportion of 10 mL per gram of tissue. After the first 4 minutes of incubation, tissue was homogenized with a Potter-Elvehjem homogenizer and then incubated again until the end of the aforementioned 8 minutes. To remove nuclei and cell debris the homogenate was centrifuged at 500xg for 10 minutes at 4°C. The resulting supernatant was filtered through a nylon mesh and centrifuged at 10,000xg for 10 minutes 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 again centrifuged at 10,000xg for 10 minutes at 4°C. Finally, the resulting pellet was resuspended with buffer B and centrifuged at 4,000xg for 10 minutes at 4°C. The mitochondria pellet was resuspended with 1 mL of buffer B.

Muscle mitochondrial oxygen consumption and cytochrome c oxidase activity

Mitochondrial oxygen consumption was measured polarographically as described previously [21], with minor modifications. Isolated mitochondria (0.5 mg protein/mL) were incubated in a water-thermostatically regulated chamber with a computer-controlled oxygen Clark-type electrode Oxygraph (Hansatech, Norfolk, UK) in 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). Mitochondrial respiratory states 4 and 3 were assayed with pyruvate/malate (5 mM/5 mM) in the absence (state 4) and in the presence (state 3) of 500 μM ADP. Mitochondrial cytochrome c oxidase (COX) activity was determined as previously described [22].

Muscle 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 [23]. Assays were performed at 37°C for 20 min in a 96-well microplate fluorimeter FLx800 (Bio-tek instruments, Winooski, VT, USA). Mitochondria (0.2 mg protein/mL) were added to the same medium used for respiration supplemented with 0.1 U/mL horseradish peroxidase and 50 μM Amplex Red reagent. Maximal hydrogen peroxide generation rates of complex III were measured as previously described [24], using succinate (5 mM), antimycin A (5 μM) and rotenone (2 μM). 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.

Measurements of muscle thiobarbituric acid-reactive substances (TBARS) and protein carbonyl groups

TBARS levels were measured as previously described [25] and used as index of serum lipid peroxidation. Protein carbonyl groups were determined as index of protein oxidation by immunoblot detection by using the OxyBlot™ Protein Oxidation Detection Kit according to the manufacturer's protocol.

Muscle antioxidant enzyme activity measurements

Catalase activity was determined in homogenate [26] and activities of glutathione peroxidase, glutathione reductase and Mn-superoxide dismutase were measured in isolated mitochondria as previously described [27-29].

Fig. 1. Oral Glucose Tolerance Curves. Values are expressed as the mean $\pm$ S.E.M of 6 animals per group. ANOVA ($p < 0.05$): D indicates diet effect. Values of area under the blood glucose curve were 3,889 in control males, 5,383 in HFD males, 2,558 in control females and 3,315 in HFD females rats.

Western blot analysis of muscle UCP3 and GLUT4 protein levels

30 μ g (UCP3) or 50 μ g (GLUT4) of muscle protein were fractionated on 15% SDS-PAGE gels and electrotransferred onto a nitrocellulose filter. Rabbit polyclonal antibodies against rat UCP3 and GLUT4 were used as primary antibodies. Anti-rabbit IgG-alkaline phosphatase antibodies were used as secondary 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 34 kDa and 45 kDa for UCP3 and GLUT4, respectively.

Statistical analysis

All data are expressed as mean values $\pm$ SEM of 6 animals per group. Statistical analyses were performed by using a statistical software package (SPSS 13.0 for Windows, Inc., Chicago, IL, USA). Statistical differences between experimental groups were 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

Energy intake, body weight gain and muscle composition

Both food and energy intake of the HFD animals was about three times higher than those of the control rats, and the body weight gain was almost ten times higher (Table 1). No significant differences between genders were found either in food and energy intake or in body weight gain. Energetic efficiency was higher in female rats than in males and increased due to the consumption

Fig. 2. Muscle mitochondrial oxygen consumption (A) and COX activity (B). Mitochondrial respiratory states 4 and 3 were assayed with pyruvate/malate (5 mM/5 mM). Values are expressed as the mean $\pm$ S.E.M of 6 animals per group. ANOVA ($p < 0.05$): D indicates diet, GxD indicates gender and diet interactive effect and NS stands for non significant. Student's t-test ($p < 0.05$): *diet vs control.

Table 2. Serum glucose, insulin and adipokine levels. Values are expressed as the mean $\pm$ S.E.M 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 ($p < 0.05$): * diet vs control, $\dagger$ females vs males.

	Male		Female		ANOVA
	Control	HFD	Control	HFD	
Glucose (mM)	8.01 $\pm$ 0.20	7.87 $\pm$ 0.65	7.76 $\pm$ 0.28	7.99 $\pm$ 0.14	NS
Insulin (μ U/mL)	68.3 $\pm$ 5.8	261 $\pm$ 6.8*	28.6 $\pm$ 3.4	88.7 $\pm$ 5.4* $\dagger$	G, D, GxD
HOMA-IR	24.4 $\pm$ 4.7	93.5 $\pm$ 5.9*	9.86 $\pm$ 1.89 $\dagger$	31.3 $\pm$ 4.8* $\dagger$	G, D, GxD
HOMA- β	304 $\pm$ 55	1213 $\pm$ 208*	138 $\pm$ 25 $\dagger$	401 $\pm$ 59* $\dagger$	G, D, GxD
Resistin (ng/mL)	52.2 $\pm$ 4.1	51.7 $\pm$ 3.3	26.6 $\pm$ 1.8	32.3 $\pm$ 3.5	G
Adiponectin (μ g/mL)	7.90 $\pm$ 0.90	11.1 $\pm$ 1.9	24.4 $\pm$ 4.3 $\dagger$	13.9 $\pm$ 1.2*	G, GxD
Leptin (ng/mL)	9.10 $\pm$ 2.40	18.7 $\pm$ 0.9	6.80 $\pm$ 1.60	14.2 $\pm$ 1.6	D

	Male		Female		ANOVA
	Control	HFD	Control	HFD	
Hydrogen peroxide production (pmolH ₂ O ₂ /min mg protein)	83.1 $\pm$ 9.3	281 $\pm$ 55	205 $\pm$ 56	357 $\pm$ 23	G, D
TBARS (nmol/mg protein)	1.52 $\pm$ 0.11	1.35 $\pm$ 0.24	1.20 $\pm$ 0.07 $\dagger$	1.75 $\pm$ 0.17*	GxD
Protein carbonyl groups (%)	100 $\pm$ 34	204 $\pm$ 58	130 $\pm$ 35	265 $\pm$ 77	D

Table 3. Muscle mitochondrial hydrogen peroxide production, lipid peroxide levels and protein carbonyl groups. Values are expressed as the mean $\pm$ S.E.M of 6 animals per group. ANOVA ($p < 0.05$): G indicates gender effect, D indicates diet effect and NS stands for non significant. Student's t-test ($p < 0.05$): * diet vs control, $\dagger$ females vs males. Hydrogen peroxide production was determined in isolated mitochondria, lipid peroxide levels (TBARS) and protein carbonyl groups were determined in homogenate. Protein carbonyl groups were assessed by immunoblot detection and value of control male rats was set as 100%.

of the HFD, although it did not reach statistical significance. Relative muscle weight was not affected by gender or HFD feeding. Muscle triglyceride content was higher in control male rats compared to females and increased with HFD feeding only in female rats. Muscle DNA levels increased with the HFD but did not change with gender. No significant gender or diet effects were found in protein levels.

Oral Glucose Tolerance Test

HFD feeding increased blood glucose levels at 15 and 30 minutes after glucose load (Fig. 1). For both control and HFD female rats, the glucose level peaked at 15 minutes, and dropped thereafter, while for both groups of male rats the glucose level peaked at 30 minutes.

Serum glucose, insulin and adipokine levels

Serum insulin levels, HOMA-IR and HOMA- β ratios were higher in control male rats than in females (Table 2) and were significantly increased in both genders with HFD feeding (four times in male rats and three times in females). Resistin levels were higher in male rats in comparison to females and no differences were found with HFD feeding. Adiponectin levels, which were 3-fold higher in female control rats compared to males,

strongly decreased (43%) in HFD female rats reaching the same value as males. In both genders leptin levels significantly increased in response to HFD feeding. No significant gender or diet effects were found in serum glucose levels.

Mitochondrial oxygen consumption and cytochrome c oxidase activity

The intake of the HFD increased muscle mitochondrial oxygen consumption in state 4 (Fig. 2A). This increase was 58.2% in female rats and only 22.6% in males, although no significant gender effect was found. This is in agreement with the higher COX activity shown by HFD female rats (Fig. 2B).

Muscle mitochondrial hydrogen peroxide production, lipid peroxide levels and protein carbonyl groups

Mitochondrial hydrogen peroxide production was higher in control female rats than in males (Table 3). The lipid peroxide levels (TBARS) were lower in female rats compared to males and no significant gender differences were found in protein carbonyl groups. In both genders, HFD feeding increased the mitochondria hydrogen peroxide production, to a higher degree in males (238%)

Table 4. Muscle antioxidant enzyme activities. Values are expressed as the mean $\pm$ S.E.M of 6 animals per group. ANOVA ($p < 0.05$): G indicates gender effect, D indicates diet effect and NS stands for non significant.

	Male		Female		ANOVA
	Control	HFD	Control	HFD	
Catalase (nKat/mg protein)	308 $\pm$ 37	414 $\pm$ 81	377 $\pm$ 61	375 $\pm$ 75	NS
Glutathione peroxidase (nKat/mg protein)	1.06 $\pm$ 0.24	0.689 $\pm$ 0.120	1.09 $\pm$ 0.27	0.988 $\pm$ 0.130	NS
Glutathione reductase (nKat/mg protein)	0.329 $\pm$ 0.036	0.363 $\pm$ 0.031	0.422 $\pm$ 0.042	0.522 $\pm$ 0.058	G
Mn-Superoxide dismutase (mIU/mg protein)	185 $\pm$ 51	95.5 $\pm$ 16.4	234 $\pm$ 76	82.3 $\pm$ 10.8	D

than in females (74%), and the protein carbonyl groups. The TBARS levels increased with HFD feeding only in female rats.

Muscle antioxidant enzymes activities

Glutathione reductase activity was higher in female rats than in males and no significant differences between genders were found in catalase, glutathione peroxidase and Mn-superoxide dismutase (Table 4). In both genders, HFD feeding significantly decreased the activity of Mn-superoxide dismutase.

Muscle UCP3 and GLUT4 protein levels

The consumption of the HFD strongly increased both UCP3 and GLUT4 protein levels in female rats (Fig. 3). In control rats, no significant differences between genders were found in these parameters.

Discussion

In this study, we found a gender dimorphism in the insulin resistance profile of old rats in both the control situation and in response to high-fat-diet feeding, with female rats showing a lower impairment of insulin sensitivity profile as well as a higher muscle oxidative function and ability to counteract the oxidative stress dependent insulin resistance.

Control male rats of 18 months of age showed a marked insulin resistance state compared to females, as suggested by insulin resistance markers - serum insulin levels, HOMA-IR and HOMA- β indexes and glucose tolerance curve - in accordance with that previously reported in both rodents [30] and humans [19]. Moreover, when the present results are compared with those obtained in a previous study using younger rats [31], we observe an age related increase of both serum insulin levels and HOMA-IR index in both genders that is two-fold higher for male rats than females. This difference in the

Fig. 3. Muscle UCP3 (A) and GLUT4 (B) protein levels. Values are expressed as the mean $\pm$ S.E.M of 6 animals per group. ANOVA ($p < 0.05$): D indicates diet effect and GxD indicates gender and diet interactive effect. Student's t-test ($p < 0.05$): *diet vs control, † females vs males. Levels of control male rats were set as 100%.

magnitude of the increase from similar values in male and female young rats points to the aging process as the main cause for the impairment of insulin sensitivity showed by control male rats.

The gender differences observed in both serum resistin and adiponectin levels also reflect the insulin resistance profile shown by control male rats. The elevated serum adiponectin levels in female rats have

been reported to be consistent with their improved insulin sensitivity compared with their male counterparts [19, 30] which agrees with the lower resistin levels shown by female rats. In this way, the intramuscular accumulation of lipids observed in male rats together with its reported deleterious effect on insulin action [32-34] further supports this idea. This sex dimorphism could point towards a higher tendency of male rats to undergo metabolic syndrome manifestations associated to age-related pro-oxidant state.

Although female rat mitochondria produced more hydrogen peroxide than those from males, the muscle oxidative damage was not enhanced which could be explained by a slight increase in the muscle antioxidant protection and by the lower levels of triglycerides accumulated in this tissue. All in all, 18-month-old male rats showed a more marked insulin resistance profile compared with their female counterparts which could be attributable in part to the different hormonal milieu to which they have been exposed during their fertile period [35-37].

HFD feeding generated a pro-oxidant status as reported previous studies [3, 38-40], represented by increases in both muscle mitochondrial hydrogen peroxide production and oxidative damage and by the loss of the SOD activity. The consumption of the HFD also entailed a pro-inflammatory profile with altered adipokine levels and an impairment of insulin sensitivity, as has been previously reported to occur in association with the obese state [41, 42]. In our study, male rats showed a higher resistance to increase body weight in response to HFD feeding than females (the excess of body weight was 38.4% for females and only 16.2% for males). However, in spite of this higher body weight gain, HFD female rats showed a less marked insulin resistance profile than males. The excess of body weight induced in female rats by the dietary treatment caused an insulin resistant state similar to that exhibited by both control and overweight males at 18 months of age. This could be understood as a more deleterious effect of the aging process on males than on females.

The consumption of the HFD - through the increase of the fat supply to muscle - provokes a triglyceride accumulation in female rats that matches male rat levels, in spite of their increased mitochondrial oxidative capacity. Although an impairment of muscle fatty acid oxidation has been proposed to play a key role in the intramuscular accumulation of lipids and their deleterious effects on insulin action [4], Turner and collaborators [43, 44] have recently suggested that the intramuscular lipid

accumulation and insulin resistance in rodents can occur along with an increase in the fatty acid oxidative capacity, such as we have also observed in our study, and can be understood as an homeostatic response to attempt to compensate for the elevated availability of lipids.

Interestingly, HFD female rats show an increase in oxidative damage probably due to the higher pro-oxidant situation they underwent. Nevertheless, in spite of the high excess of body weight, HFD female rats seem to have the ability to induce mechanisms for attenuating the deleterious consequences of the HFD. It can be suggested that the increase in lipid peroxides shown by HFD female rats could be responsible, in part, for the strong increase of muscle UCP3 protein levels found in this gender aimed at counteracting the increased hydrogen peroxide production associated to the obese state. This higher UCP3 protein levels in HFD females does not seem to be attributable to the increase of mitochondrial proteins levels associated to HFD feeding, since the increase occurs in both genders (data not shown). It has been hypothesized that during fatty acid oxidation, UCP3 is specifically activated by free radical species or lipid peroxides, leading to a mild uncoupling that reduces membrane potential and mitigates free radical species production to protect against oxidative damage [45, 46]. In contrast, in male rats HFD feeding did not achieve the UCP3 overexpression, unlike that reported in other studies performed with younger male rats [43, 44, 47]. This apparent controversy could be explained by an age-related decrease in muscle UCP3 expression which has been previously reported [48]. In fact, a similar effect of age decreasing UCP1 levels has also been described in brown adipose tissue [49, 50]. These results reinforce the postulated impaired ability of 18-month-old male rats to counteract the effects of HFD feeding.

Moreover, it could not be discarded an additional role for this UCP3 induction as a mechanism of adiposity control in situations of high fuel availability, as it has been recently reported in human UCP3 transgenic mice [51].

Since UCP3 has been related with an improvement of insulin resistance through an increase in the muscle GLUT4 traslocation [52], gender dimorphism in muscle UCP3 levels could be linked to the gender differences found in the insulin resistance status. In fact, the positive correlation between UCP3 and GLUT4 protein levels ($R=0.786$, $p=0.000$) further reinforces this idea. The reported effects of HFD diets on GLUT4 expression in skeletal muscle are variable and seem to be dependent on the quality of the diet and the age of the animals [53, 54]. However, the enhanced GLUT4 levels did not

prevent the insulin resistance profile in obese female rats, but could attenuate it, thus resulting in a better profile than that of the otherwise less obese HFD males. GLUT4 levels do not necessary reflect changes in GLUT4 traslocation. In fact, a deficient insulin-induced GLUT4 traslocation in the presence of an unaltered GLUT4 expression has been detected in skeletal muscle of different animal models of obesity or type 2 diabetes [55, 56]. This could explain that control male rats showed a higher impaired insulin sensitivity compared to control females, in spite of having similar UCP3 and GLUT4 levels.

In summary, our results point to a gender dimorphism in the response of skeletal muscle to high-fat-diet feeding. Female rats, in spite of showing a higher excess of body weight than males, have a better oxidative and inflammatory profile, and could have a higher ability to

counteract the oxidative-stress-associated insulin resistance, in part through an overexpression of both UCP3 and GLUT4. Moreover, the more marked insulin resistant state of control male rats could be attributed to a more detrimental effect of age in this gender.

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