Sex Differences in Brown Adipose Tissue Thermogenic Features During Caloric Restriction

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Key Words

UCP1 • Adrenergic receptors • Mitochondria • OXPHOS

Abstract

Caloric restriction (CR) studies have shown that females rats conserve energy more efficiently, showing a higher resistance to weight loss and higher protection of vital organs mass than male rats. Gender-dependent inactivation of thermogenesis in brown adipose tissue (BAT) has been proposed as one of these possible energy conserving mechanisms. To study the changes underlying this inactivation in rats, a three month study with 40% CR was undertaken to unravel the effects on BAT recruitment. Under *ad libitum* conditions female rats had greater BAT recruitment and greater oxygen consumption than their male counterparts. Total and mitochondrial protein, as well as triglyceride and DNA content were more reduced in restricted female rats than in restricted males. Similarly, the levels of key BAT functional proteins (UCP1, LPL, HSL, TFAM) were more reduced in restricted females, whereas no changes were found in mitochondrial DNA levels (mtDNA) and OXPHOS activities in males and females.

Furthermore, α_{2A}/β_3 adrenergic receptor ratio remained constant in male rats whereas in female rats CR increased 60%. In conclusion, our results suggest that female rats, whose BAT thermogenic activity is higher in *ad libitum* conditions, is depressed during CR. This inactivation involves the mitochondrial differentiation process and lipolytic system and could be due, at least in part, to the unfavourable adrenergic receptor balance for thermogenic activation.

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Introduction

Brown adipose tissue (BAT) plays an important role in the regulation of energy balance since it is a major site of both cold- and diet-induced thermogenesis in small mammals. BAT thermogenic activity is dependent on its specific uncoupling protein 1 (UCP1), an inner membrane mitochondrial protein which dissipates energy as heat by uncoupling oxidative phosphorylation [1]. The main

physiological regulator of BAT thermogenesis is norepinephrine, released by sympathetic terminals that densely innervate the tissue. Adrenergic stimulation promotes BAT thermogenic capacity or recruitment state activating UCP1 expression [2], increasing mitochondriogenesis [3], and promoting adipocyte proliferation [4]. Moreover, adrenergic stimulation increases intracellular free fatty acids, released by lipolysis, which directly activates UCP1 [5] and provides metabolic fuel for thermogenesis, suggesting that lipolysis is the flux-generating step controlling BAT activity [6]. Catecholamine effects are mainly mediated by adrenergic receptors (ARs) present on adipocyte membrane [7]. The balance between the cellular content of α_2 -AR (inhibitor) and β_3 -AR (activator) is central to the regulation of thermogenesis as it modulates the net adrenergic signal response in the adipocyte [8].

Caloric restriction (CR) is a condition in which enhanced energy dissipation would be disadvantageous. Periods of short food supply are often repeated in the wild, selecting organisms with more efficient mechanisms for energy metabolism. Some authors have argued that because their relative importance for reproduction and the survival of the species, females have been affected by natural selection pressures to be more resistant to CR than males [9]. It has been hypothesized that these energy conserving mechanisms in the females could be the same as those that give females a higher propensity for obesity under hypercaloric conditions [10, 11]. The mechanisms involved in this gender-dependent adaptation of energy balance remain unclear.

Although the reduced energy expenditure which occurs during CR has been associated partly to a lower BAT activity [12], very few studies have looked for gender differences in BAT during CR. In previous works, we studied the effect of gender in the adaptation of energy metabolism to CR and our results pointed out that BAT may be involved in the gender differences found, with female rats showing a significant drop in UCP1 content [13]. In addition, gender differences in BAT have been also found in cold- and diet-induced thermogenesis [14, 15] suggesting a physiological importance for sex-dependent regulation of BAT uncoupling activity. Taking this into account, the aim of this work was to investigate the gender-dependent changes underlying BAT inactivation during CR, analyzing the effects on important parameters in the activity and control of BAT thermogenesis, i.e. tissue composition, mitochondrial recruitment, enzymes of lipid metabolism and adrenergic receptors.

Materials and Methods

Materials

Routine chemicals used were supplied by Sigma-Aldrich (St. Louise, USA), Panreac (Barcelona, Spain), Pronadisa (Madrid, Spain) and Amersham Pharmacia Biotech (Little Chalfont, UK). Oligonucleotide primer sequences were from Roche Diagnostics (Basel, Switzerland). Antibodies for α_2 -AR and β_3 -AR were obtained from Santa Cruz Biotechnology (Calif., USA). Antibodies for HSL and TFAM were kindly supplied by Dr. F.B. Kraemer [16] and Dr. H. Inagaki [17], respectively. Antibody for LPL was previously developed in our laboratory [18]. Appropriate secondary antibodies were purchased from Sigma-Aldrich.

Animals and diets

All animals were treated in accordance with the university bioethical committee guidelines for animal care and EU regulations (86/609/EEC). 12 male and 12 female Wistar rats aged 60 days were purchased from Charles River (Barcelona, Spain) and housed individually in wire-bottomed cages at 22°C with 12h light-dark cycle. To study the effects of CR in both genders, males and female rats were randomly divided into two dietary groups: *ad libitum* and caloric restricted. *Ad libitum* animals were fed with standard chow pellets (A04, supplied by Panlab, Barcelona, Spain) and restricted animals were fed for 100 days at the start of the dark cycle with 60% of the mean amount of diet consumed the previous week by the *ad libitum* group of the same gender.

Indirect calorimetry

Whole body oxygen consumption (VO_2) was measured by open-circuit respirometry in metabolic cages ventilated with fresh air at a rate of 1L/min. Oxygen consumption was calculated from the difference in O_2 concentration between inflow and outflow air using a differential O_2 analyzer (Panlab). Animals were placed individually in the cages and acclimated for 1h. Data recordings were obtained during the last 2h of the dark phase. Average VO_2 was calculated from three repeated measures of each animal during the two weeks before sacrifice.

BAT collection and mitochondria isolation

Animals were killed by decapitation at the start of the light phase. The interscapular BAT was excised, weighed and homogenized in isolation buffer (250mM sucrose, 5mM Tris-HCl, 2mM EGTA, pH 7.2, 4°C) in a Teflón/glass homogenizer. The homogenate was filtered through a layer of gauze. Aliquots were used for determination of total protein, DNA and triglycerides content as described elsewhere [19-21] and also cytochrome c activity and western blotting measurements (see below). The rest of the homogenate was used for isolation of mitochondria by differential centrifugation. The nuclei and cell debris were removed by centrifugation at 500xg for 10min. The supernatant was centrifuged at 8000xg to yield the mitochondrial pellet. The pellet was washed once by resuspension, centrifuged (8000xg) and the final pellet was resuspended in the same buffer. The gravitational force used was chosen in

Fig. 1. Effect of CR on body mass, BAT weight and oxygen consumption. A: Body mass. B: BAT weight. C: Oxygen consumption rate adjusted for body mass^{0.75} (VO₂). The data represent the means ± SEM, from six animals per group. ANOVA (p<0,05): R, effect of diet; G, effect of gender; GxR, interaction of gender and diet. Student's t test (p<0,05). °ad libitum vs. CR; * males vs. females.

order to achieve a representative fraction of whole mitochondrial population preserving integrity [22]. Mitochondrial protein was measured by the Bradford method.

OXPHOS activities

The measurement of the specific activities of the oxidative phosphorylation (OXPHOS) complexes (complex I (NADH-ubiquinone oxidoreductase; EC 1.6.99.5) [23], complex III (ubiquinol:cytochrome c reductase; EC 1.10.2.2) [24], complex IV or COX (cytochrome c oxidase; EC 1.9.3.1) [25], complex V or ATPase (F₁F₀-ATP synthase; EC 3.6.1.3) [23], were performed as described adapted to microtiter plate assay with some modifications. The assays were performed in 200µl final volume with 1-5µg mitochondrial proteins. COX activity was also measured in homogenate in order to calculate the mitochondrial recovery.

Western blotting

As described elsewhere [26], 15µg mitochondrial protein for UCP1 and 20µg homogenate protein for ARs, LPL, and HSL were fractioned by SDS-PAGE [27] (12% polyacrylamide for UCP1 and 10% for the rest of analysed proteins) and electrotransferred onto nitrocellulose filters. Ponceu S staining was used to provide visual evidence of correct loading and electrophoretic transfer of proteins to nitrocellulose filter. Membranes were incubated overnight at 4°C in a blocking solution of 5% non-fat powdered milk in T-PBS (phosphate-buffered saline, pH 7.5, containing 0.1% Tween 20). Developments of the immunoblots were performed using an enhanced chemiluminescence Western blotting analysis system (Amersham). Bands in films were analysed by scanner photodensitometry and quantified using Kodak 1D Image Analysis Software.

Extraction and quantification of mitochondrial DNA

Mitochondrial DNA (mtDNA) was extracted by digestion with proteinase K (100µg/µl) in a buffer containing 50mM KCl, 10mM Tris-HCl, 2.5mM MgCl₂ and 0.5% Tween 20. Homogenate samples were incubated overnight at 37°C and then boiled for 5min in order to inactivate the enzyme. Mitochondrial DNA was linearised by digestion with *Bcl I* restriction enzyme for 3h at 50°C and boiled for 5min. Samples were centrifuged at 7000xg for 5min and the resulting supernatant was used for amplification. A quantitative PCR assay was adapted to the LightCycler technology from Koekemoer et al [28]. PCR was performed to amplify a 162-nts fragment of the mitochondrial NADH dehydrogenase subunit 4 gene. The primer sequences were 5'-TACACGATGAGGCCAACAAA-3' and 5'-GGTAGG GGG TGT GTT GTG AG-3'. The concentration of the purified

template was determined spectrophotometrically. Increasing amounts of template were amplified in parallel reactions to obtain a standard curve. Amplification was carried out in a LightCycler rapid thermal cycler system (Roche, Switzerland) using a total volume of 10µl containing 0.375µM of each primer, 3mM MgCl₂, 1µl LightCycler-FastStart DNA Master SYBR™ Green I (Roche, Switzerland) and 2.5µl of sample prepared as described above. The PCR reactions were cycled 35 times after initial denaturation (95°C, 10min), with the following parameters: denaturation at 95°C for 10s, annealing at 60°C for 12s, extension at 72°C for 12s.

Parameter	MALES		FEMALES		ANOVA
	Ad libitum	40% CR	Ad libitum	40% CR	
DNA					
mg DNA/g BAT	1.88 ± 0.20	3.28 ± 0.35	2.53 ± 0.10*	4.00 ± 0.35°	R
mg/depot	1.04 ± 0.05	1.01 ± 0.15	1.07 ± 0.05	0.791 ± 0.07	NS
Triglyceride					
mg/g BAT	246 ± 69	298 ± 56	533 ± 71	323 ± 76	G
mg/mg DNA	131 ± 33	91.2 ± 14.8	213 ± 30	76.9 ± 13.6°	R
mg/depot	140 ± 42	88.1 ± 12.7	233 ± 42	63.9 ± 15.2	R
Protein					
mg/g BAT	41.9 ± 2.5	68.0 ± 7.3°	66.7 ± 5.6*	80.8 ± 9.5	G,R
mg/mg DNA	22.9 ± 1.7	21.3 ± 2.2	26.3 ± 2.5	20.1 ± 1.4°	R
mg/depot	23.7 ± 2.0	20.3 ± 1.6	29.4 ± 3.3	15.9 ± 1.9°	R, GxR
Mitochondrial protein					
mg/g BAT	22.6 ± 3.7	54.9 ± 16.0°	45.6 ± 5.7*	57.5 ± 15.6	NS
mg/mg DNA	12.1 ± 1.6	15.9 ± 4.3	18.8 ± 2.6*	14.7 ± 4.3	NS
mg/depot	11.6 ± 2.1	12.7 ± 3.2	18.1 ± 3.0	10.1 ± 2.7°	NS

Table 1. Effect of CR on BAT composition in male and female rats. The data represent the means ± SEM, from six animals per group. ANOVA ($p < 0.05$): R, effect of diet; G, effect of gender; GxR, interaction of gender and diet. Student's t test ($p < 0.05$): °Ad libitum vs. CR; * males vs. females.

Statistics

The Statistical Program for the Social Sciences software for Windows (SPSS, Version 12.0) was employed for all statistical analyses. Data are presented as mean ± SEM. Differences between groups were assessed by two-way analysis of variance (ANOVA) and Student's t-test. Statistical significance was set at $p < 0.05$.

Results

Body mass, BAT weight and respirometry

Figure 1A shows body mass of the four groups studied. CR decreased body mass in both genders. No significant differences were found in body mass loss between genders (26.5% in males and 24.2% in females).

BAT weight is shown in Figure 1B. Although BAT absolute weight was higher in males, ad libitum female rats had a greater BAT weight adjusted per body mass (163mg/g) than males (114mg/g). BAT weight was reduced 54% in females and 43% in males by CR.

Oxygen consumption (VO_2) was higher in ad libitum fed female rats than in males (Fig 1C). VO_2 was decreased by CR in the females, and neared those levels in males. Similar findings were obtained in previous works [13, 21].

BAT composition

To test whether the decrease in energy expenditure was related to impaired BAT thermogenesis, we decided to study the composition and recruitment features of BAT.

Table 1 shows the values of DNA, triglyceride and protein content in BAT. These parameters (per gram of tissue) have also been normalized to mg of DNA, to avoid the concentration effects of CR when data is normalized per gram, and per whole depot.

Ad libitum female rats showed higher triglyceride and protein contents than males, supporting the higher lipid multilocular arrangement and thermogenesis reported by other authors [21]. The higher loss of BAT weight produced by CR in the female in comparison to male rats was accompanied by a higher loss of triglyceride (-63% vs. -30%), protein (-23% vs. -7%) and total DNA (-26% vs. -3%, respectively), this latter indicating a significant decrease in cell number. These changes suggest that females, whose BAT seems to be active in *ad libitum* conditions, deactivate thermogenesis in a greater extent than males in response to CR. In agreement with this, whole mitochondrial protein (per depot) was higher in female than in male rats and it was decreased by CR in a gender-dependent way. This fact indicates that caloric restricted female rats displayed a lower mitochondrial protein content, which could be due to a lower mitochondria number and/or lower differentiation state of these mitochondria.

Mitochondrial DNA and TFAM levels

To test whether the lower mitochondrial protein content in restricted females was due to a decrease in mitochondrial number, we studied the mitochondrial DNA content (mtDNA). The levels of mtDNA could be a good

Parameter	MALES		FEMALES		ANOVA
	Ad libitum	40% CR	Ad libitum	40% CR	
mtDNA					
ng/g BAT	44.1 ± 16.1	76.4 ± 36.5	35.2 ± 3.4	69.4 ± 19.1	NS
ng/mg tDNA	21.3 ± 6.1	22.1 ± 9.5	13.8 ± 1.9	17.2 ± 3.9	NS
ng/mg mt protein	6.04 ± 2.48	5.12 ± 2.23	2.62 ± 0.23	5.01 ± 1.90 ^o	NS
TFAM					
a.u./mg mt protein	100 ± 14	91.4 ± 14.6	92.2 ± 21.8	46.5 ± 9.5 ^{o*}	NS

Table 2. Effect of CR on mtDNA and TFAM specific protein levels in BAT mitochondria of male and female rats. TFAM levels are expressed relative to levels found in AL male rats, which were set at 100%. The data represent the means ± SEM, from six animals per group. ANOVA (p<0,05): R, effect of restriction; G, effect of gender; GxR, interaction of gender and diet. Post hoc analysis Student's t test (p<0,05): ^oAd libitum vs. CR; * males vs. females.

indicative parameter of mitochondrial number as there is a direct relationship between the number of mtDNA copies and the number of mitochondria [29-31]. As shown in table 2, restricted rats displayed higher mtDNA content adjusted per gram of tissue as a result of the concentrative effect of the fat loss induced by CR. This effect is avoided when data is adjusted per cellular DNA, showing restricted rats similar levels than *ad libitum* rats. Considering mtDNA indicative of mitochondria number, the number of mitochondria would not be affected by CR, thus the lower mitochondrial protein would be due to the reduced protein content per mitochondria.

In order to evaluate the differentiation state of these mitochondrias, we examined the mtDNA/mtprotein ratio and the mitochondrial transcription factor A (TFAM), a transcription factor involved in the expression of the proteins encoded by the mitochondrial genome [32]. The *ad libitum* fed female rats showed the lowest ratio mtDNA/mtprotein, indicating a tendency for a higher degree of maturation, which was in agreement with previous reports [21, 33]. On the contrary, restricted female rats showed similar mtDNA/mtprotein ratio than males (which levels were not affected by CR).

As regards to TFAM levels, no differences were found between genders in *ad libitum* feeding conditions (all representative western blots are given in figure 2). However, the specific levels of this differentiation marker were dramatically decreased in restricted female rats. On the whole, these results support the notion of a lower differentiation state in mitochondria of restricted female rats rather than a lower mitochondrial pool.

UCP1 content and mitochondrial OXPHOS activities

BAT thermogenic function relies on its characteristic mitochondrial protein UCP1, which uncouples oxidative phosphorylation [34]. In order to assess the qualitative

Fig. 2. Representative western blot of TFAM, UCP1, HSL, LPL, α_{2A} -AR, β_3 -AR and PKA. 15µg of total protein was loaded for UCP1 and 20µg for TFAM, HSL, LPL, PKA. Developed western blot nitrocellulose membranes were exposed to Hyperfilm ECL (Amersham Biosciences), and representative bands in films are shown.

changes in mitochondrial thermogenic machinery we analyzed in detail the main components involved in the generation of the electro-chemical gradient and its dissipation as heat: the OXPHOS activities and the UCP1 content, respectively. As shown in Table 3, no differences were found in the specific activity of the four OXPHOS complex studied (I, III, IV and V). However, the specific UCP1 content showed gender differences both in *ad libitum* and CR conditions. The *ad libitum* fed female

Parameter	MALES		FEMALES		ANOVA
	Ad libitum	40% CR	Ad libitum	40% CR	
Complex I activity IU/mg mitochondrial protein	1.06 ± 0.12	0.77 ± 0.11	0.87 ± 0.16	1.03 ± 0.20	NS
Complex III activity IU/mg mitochondrial protein	11.6 ± 0.9	14.2 ± 2.0	11.8 ± 1.8	13.4 ± 2.9	NS
Complex IV activity a.u./mg mitochondrial protein	100 ± 17	65.5 ± 7.5	56.9 ± 13.2	81 ± 17.4	NS
Complex V activity mIU/mg mitochondrial protein	1.37 ± 0.17	0.96 ± 0.05	1.14 ± 0.13	1.09 ± 0.11	NS
UCP1 % a.u./mg mitochondrial protein	100 ± 8	120 ± 15	130 ± 11 *	97.6 ± 12.1 °	GxR

Table 3. Effect of CR on mitochondrial OXPHOS activities and UCP1 specific protein level in BAT of male and female rats. Complex IV (COX) activity is expressed relative to activity found in AL male rats, which were set at 100% The data represent the means ± SEM, from six animals per group. ANOVA (p<0,05): R, effect of diet; G, effect of gender; GxR, interaction of gender and diet. Student's t test (p<0,05): °Ad libitum vs. CR; * males vs. females.

rats showed higher UCP1 levels than males. CR produced a significant decrease (-30%) in UCP1 levels of female rats, whereas the changes in restricted male rats (a 20% increase) were not statistically significant.

Since BAT implies an important component of energy expenditure, we decided to correlate the changes in the VO_2 with the variation in the levels of UCP1 in whole BAT [34]. VO_2 and UCP1 per depot showed a higher correlation coefficient in females ($r^2=0.72$) than in males ($r^2=0.03$). The close relationship between UCP1 and energy expenditure suggest that thermogenesis might be activated in female rats under usual housekeeping conditions and is switched off when the females are faced with low caloric intake.

Lipoprotein lipase and hormone-sensitive lipase

Since fatty acids are modulators and metabolic fuel for thermogenesis, we studied the levels of the main enzymes involved in BAT fatty acid metabolism: lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL). These enzymes play a key role in the control of lipolytic capacity in brown adipocytes, catalyzing the limiting steps in fatty acid uptake and use, respectively. As shown in table 4, the LPL levels were decreased by CR in both genders. This drop was greater in female rats (-36%) than in males (-20%), indicating that CR decreases to a larger extent, the capacity for fatty acid captation in the BAT of female rats.

For HSL no significant differences were found, although *ad libitum* female rats showed higher HSL content than *ad libitum* males. CR also produced a significant drop in the HSL content per mg of DNA in

female rats (-15%) whereas this effect was negligible in males. These gender related changes are greater when the loss in whole tissue is taken into account. Thus, female rats decreased 62% LPL and 57% HSL content per depot versus respective 42% and 31% in males, which might have systemic implications in the fatty acid clearance. Taking this all together, these results suggest that in female rats CR decreases in a greater extent the capacity of BAT to uptake and oxidize fatty acids, the main thermogenic fuel.

Adrenergic receptor levels

In view of the changes found in lipolytic and thermogenic capacity, we decided to examine the levels of the main adrenergic receptors, α_{2A} and β_3 . The α_{2A}/β_3 balance is key in the regulation of thermogenesis and lipolysis by modulating the net adrenergic signal response in the adipocyte. It has been established that a higher α_{2A}/β_3 would lead to a lower lipolytic and thermogenic activity and vice versa [15, 35, 36]. *Ad libitum* female rats showed lower values in both adrenoceptor levels as well as lower α_{2A}/β_3 ratio, although these differences did not reach statistical significance (p=0.057). CR was found to induce a statistically significant reduction in the levels of the stimulatory β_3 -AR in both genders. This drop in β_3 -AR was higher in female rats, reaching a 50% decrease vs. 38% in males when normalized per mg of DNA. On the contrary, α_{2A} -AR was found to be reduced only in males, as restricted females had the same levels of their *ad libitum* counterparts. As a result, female rats went from the lowest α_{2A}/β_3 ratio under *ad libitum* feeding conditions to the highest ratio under CR,

Parameter	MALES		FEMALES		ANOVA
	Ad libitum	40% CR	Ad libitum	40% CR	
LPL					
% a.u./mg protein	100 ± 8	79.9 ± 8.0	106 ± 5	68.1 ± 2.5°	R
% a.u./mg DNA	100 ± 12	75.2 ± 12.6	116 ± 12	58.0 ± 5.2°	R
% a.u./depot	100 ± 11	57.8 ± 8.4°	118 ± 14	44.1 ± 6.4°	R
HSL					
% a.u./mg protein	100 ± 18	97.6 ± 15.3	146 ± 23	123 ± 4	NS
% a.u./mg DNA	100 ± 21	92.1 ± 19.7	161 ± 28	90.1 ± 15.5°	R
% a.u./depot	100 ± 22	69.0 ± 13.2	160 ± 27	68.1 ± 14.0°	R
ratio LPL/HSL	1.00 ± 0.19	0.79 ± 0.13	0.75 ± 0.14	0.50 ± 0.03 *	NS

Table 4. Effect of CR on LPL and HSL protein levels in BAT of male and female rats. Levels are expressed relative to AL male rat levels, which were set at 100%. The data represent the means ± SEM, from six animals per group. ANOVA (p<0,05): R, effect of diet; G, effect of gender; GxR, interaction of gender and diet. Student's t test (p<0,05): °Ad libitum vs. CR; * males vs. females.

Parameter	MALES		FEMALES		ANOVA
	Ad libitum	40% CR	Ad libitum	40% CR	
β_3 -AR					
% a.u./mg protein	100 ± 9	63.2 ± 8.6	78.9 ± 10.0	49.3 ± 11.5	R
% a.u./mg DNA	100 ± 5	61.8 ± 14.4	89.6 ± 13.7	43.8 ± 15.2	R
α_{2A} -AR					
% a.u./mg protein	100 ± 10	64.5 ± 16.2	67.7 ± 10.5	68.8 ± 16.0	NS
% a.u./mg DNA	100 ± 9	60.2 ± 19.7	77.6 ± 14.3	61.1 ± 21.2	NS
ratio α_{2A}/β_3 -AR	1.00 ± 0.09	0.92 ± 0.13	0.87 ± 0.11	1.39 ± 0.03°*	GxR
PKA					
% a.u./mg protein	100 ± 6	63.0 ± 3.4°	137 ± 8 *	133 ± 16 *	G
% a.u./mg DNA	100 ± 11	57.9 ± 4.8	151 ± 18	111 ± 19	G,R

Table 5. Effect of CR on β_3 -AR, α_{2A} -AR and PKA protein levels in BAT of male and female rats. Levels are expressed relative to AL male rat levels, which were set at 100%. The data represent the means ± SEM, from six animals per group. ANOVA (p<0,05): R, effect of diet; G, effect of gender; GxR, interaction of gender and diet. Student's t test (p<0,05): °Ad libitum vs. CR; * males vs. females.

whereas in male rats the ratio was kept constant by decreasing both subtypes of receptors in the same extent. The receptor profile in restricted female rats is aimed at inhibiting the lipolysis and thermogenesis and may be related, at least in part, to the impaired thermogenic features above mentioned.

Discussion

In this work we analyzed the effect of CR on the recruitment features and the levels of key proteins implicated in the control of the thermogenic and lipolytic pathway in BAT of male and female rats.

Ad libitum fed female rats at 22°C had greater thermogenic capacity and activity than males, with a higher UCP1 content, higher total and mitochondrial protein content, and greater BAT size and cell number (both in

relation to body weight). These findings are in agreement with previous works showing greater recruitment in BAT of female rats [21, 33]. In particular, it has been shown that *ad libitum* female rats have larger mitochondria, with longer cristae and higher cristae density [21]. These gender dimorphisms in mitochondria have been related to differences in the distribution pattern of mitochondria subpopulations, with females showing a highly differentiated mitochondrial pool than males [33]. One of the causes for these gender differences in BAT thermogenic capacity may be explained by the action of sex steroid hormones, which have shown to exert effects even at mitochondrial level [37]. Thus, ovariectomy in rats leads to an increase in energy gain, which can be prevented or reversed by estrogens [38, 39], whereas in male rats castration causes a reduction in energy gain [40]. Part of the estrogen effects on energy expenditure are mediated by changes in BAT thermogenesis [41, 42],

although mechanisms based on alterations in food intake and activity cannot be ruled out.

Male and female rats have different response to CR, with females showing a higher loss of recruitment features than male rats. This deactivation was characterized by a considerable decrease in BAT size, cell number, fat and protein content (both mitochondrial and total) when restricted are compared to *ad libitum*. However, in this loss of thermogenic capacity, no decrease in mitochondria number was found as is pointed out by our mtDNA results. Mitochondria from restricted female rats showed lower specific levels of the main thermogenic effector UCP1, whereas no changes were found for the rest of the OXPHOS complexes studied. This profile is indicative of a lesser differentiated mitochondria pool, since differentiation process has been shown to increase UCP1 levels to a greater extent than the electron transport chain complexes in BAT [33]. The ratio of mtDNA/mtprotein suggests that mitochondria in female rats shifted from the highest maturation state in the *ad libitum* conditions to state similar to that of the male rats in CR, which has been shown to have a lower degree of functional differentiation in this tissue [33]. In agreement with this, specific TFAM levels, another mitochondrial differentiation marker used in this study, was found to be lower in restricted female rats. TFAM is necessary for mtDNA transcription and it has a concentration-dependent function, showing higher binding affinity for the mtDNA light strand promoter than for the heavy strand one, which encodes most of the mitochondrial proteins [32, 43]. Thus, TFAM/mtDNA ratio has been considered as a good indicative parameter of mitochondrial differentiation [44, 45]. *Ad libitum* fed female rats showed 2-fold higher ratio than *ad libitum* or restricted males (35.2 vs 16.5 and 17.8 respectively), supporting the notion of higher recruitment/differentiation state, whereas restricted females showed a 70% lesser ratio than *ad libitum* females (9.3). On the whole, the levels of TFAM, UCP1, mtDNA and mitochondrial protein suggest that female rats change their mitochondrial differentiation process under CR, showing a lesser differentiated mitochondria pool than in *ad libitum* conditions, likely without affecting mitochondrial proliferation process.

From a physiologic point of view, the lower thermogenic capacity in the restricted female rats is even greater since BAT showed a net diminution of cells in the tissue (total DNA content). Thus in female rats, the total amount of UCP1 protein per BAT depot (per animal), which encompasses both the levels of UCP1 in mitochondria and the effect of cell proliferation or

apoptosis in the tissue [34], was found to be well correlated with the VO_2 , suggesting that the drop in UCP1 could be responsible for a decrease in energy expenditure in this gender.

Furthermore, restricted female rats showed higher depletion than males in the content of fatty acids, the main thermogenic fuel, indicating a lesser substrate availability for thermogenesis. These differences in fat content could be explained, at least in part, by the greater decrease in the levels of LPL found in the restricted female in comparison to the males. The decrease in LPL and HSL suggest a loss in fatty acid metabolic capacity in the BAT, which could be determined by the decreased insulin signalling [46]. Previous studies with CR rats have shown that females preserve lean mass to a greater degree than male rats by utilizing relatively more fat for energy needs [10]. From a systemic point of view, the lesser uptake capacity in BAT of restricted female rats could leave these energy dense fuels available for other tissues, permitting their use for purposes more profitable for survival.

As mentioned previously, the α_{2A}/β_3 -AR balance is key in the regulation of thermogenesis and lipolysis in BAT by modulating the net adrenergic signal response [8]. Taking into account that the inhibitory α_{2A} -AR has the greatest affinity for catecholamines, the higher ratio α_{2A}/β_3 -AR in restricted females could mean a higher catecholamine threshold to trigger the thermogenic and lipolytic response. In addition, it is known that adrenergic stimulation in BAT is decreased during food restriction [47]. Therefore under *in vivo* conditions it is probable that BAT thermogenic activity in restricted female rats would be very low, as it would be depressed at signal and receptor level. Bartness and Wade demonstrated that the BAT and its sympathetic innervation are very important for the estrogen mediated changes in energy expenditure [41]. In agreement with this, several *in vivo* and *in vitro* studies have reported that sex hormones modify the lipolytic and thermogenic signal by changing the responsiveness and/or density of β - and α_{2A} -AR in the adipocyte [42, 48]. α_{2A} -AR has been found to be up-regulated by testosterone in cultured brown adipocytes, whereas female sex hormones (17 β -estradiol and progesterone) up-regulate β_3 -AR and down-regulate α_{2A} -AR, resulting in a lower α_{2A}/β_3 -AR protein ratio [49, 50]. Taking into account these findings, our results suggest the existence of a cross-talk between the CR signals and sex hormones probably at adrenergic receptor level.

Studies of wild animals suggest that stored body fat is regulated under stabilizing selection as part of a dynamic

tradeoff between the risks of starvation, which promote fat storage, and the risks of predation, which promote leanness [51, 52]. In this evolutionary scenario, BAT may have provided a powerful mechanism to regulate energy expenditure [34, 53]. Because of their extended role as caretakers for the young, as well as their obvious role during pregnancy and lactation, females with more favourable mechanisms for coping with food scarcity would be more apt to survive and produce more offspring, promoting the survival of the species [9, 11, 54]. Supporting this notion are the findings reported here, with females showing a higher thermogenesis in *ad libitum* conditions in order to avoid excessive weight gain, and switching off this mechanism in order to economize energy when food is limited. This deactivation takes place at different levels of control of BAT activity, affecting cell number, mitochondrial machinery and fatty acid supplying system. Adrenergic receptor balance appears as candidate point, mediating the cross-talking between sex hormones and adrenergic stimuli in the gender-dependent response to caloric restriction.

Abbreviations

CR (caloric restriction); BAT (interscapular brown adipose tissue); UCP1 (uncoupling protein 1); ARs (adrenergic receptors); OXPHOS (oxidative phosphorylation system); LPL (lipoprotein lipase); HSL (hormone-sensitive lipase); COX (cytochrome c oxidase); VO_2 (oxygen consumption volume); mtDNA (mitochondrial DNA).

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