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Moderate caloric restriction in lactating rats programs their offspring for a better response to HF diet feeding in a sex-dependent manner

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Abstract

We aimed to assess the lasting effects of moderate caloric restriction in lactating rats on the expression of key genes involved in energy balance of their adult offspring (CR) and their adaptations under high-fat (HF) diet. Dams were fed with either ad libitum normal-fat (NF) diet or a 30% caloric restricted diet throughout lactation. After weaning, the offspring were fed with NF diet until the age of 15 weeks and then with an NF or a HF diet until the age of 28 weeks, when they were sacrificed. Body weight and food intake were followed. Blood parameters and the expression of selected genes in hypothalamus and white adipose tissue (WAT) were analysed. CR ate fewer calories and showed lower body weight gain under HF diet than their controls. CR males were also resistant to the increase of insulin and leptin occurring in their controls under HF diet, and HF diet exposed CR females showed lower circulating fasting triglyceride levels than controls. In the hypothalamus, CR males had higher ObRb mRNA levels than controls, and CR females displayed greater InsR mRNA levels than controls and decreased neuropeptide Y mRNA levels when exposed to HF diet. CR males maintained WAT capacity of fat uptake and storage and of fatty-acid oxidation under HF diet, whereas these capacities were impaired in controls; female CR showed higher WAT ObRb mRNA levels than controls. These results suggest that 30% caloric restriction in lactating dams ameliorates diet-induced obesity in their offspring by enhancing their sensitivity to insulin and leptin signaling, but in a gender-dependent manner.

Keywords: Caloric restriction; Lactation; Obesity; Insulin and leptin sensitivity; Hypothalamus; Adipose tissue

1. Introduction

Epidemiological and experimental studies have described that the programming of energy balance already begins in very early development. Indeed, particular conditions in the nutritional environment during the perinatal period may lead to adjustments in the physiology of humans and animals, with lasting effects in adulthood [1–4].

The hypothalamus plays a major role in the regulation of energy balance, producing many orexigenic and anorexigenic peptides that stimulate or inhibit food intake in response to different factors, such as circulating hormones like leptin and insulin [5]. The brain is particularly sensitive to external factors during the early period of life [4]; in this sense, many studies have shown the importance of nutritional factors during this period in the programming of appetite behavior in the adult life [6–8]. Perinatal nutrition leading to changes in the control of body weight and food intake has been associated with developmental programming of structural and functional changes in hypothalamus affecting the expression of key genes involved in food intake and energy balance [6,7].

On the other hand, the adipose tissue is recognized to have different functions that are important in the regulation of energy balance and substrate metabolism [9]. Both fat storage and fat mobilization processes normally occur in the white adipose tissue (WAT) under the habitual food intake/fasting patterns of feeding, and allow the maintenance of energy homeostasis [10–12]; the response to these situations involve hormonal and metabolic adaptations which are accompanied by changes in gene expression [12–14]. Alterations of these processes by different stressor factors such as high-fat (HF) diet feeding promote dysregulation in the overall control of fat deposition affecting energy balance [14].

In mammals, maternal nourishment establishes the first nutritional environment of their offspring; thus, changes in maternal nutrition may program alterations in the metabolism of offspring later on in life [15–17]. In this sense, strong evidence has linked low birth weight with the susceptibility to suffer obesity in adult life. This has allowed to establish the thrifty phenotype hypothesis [2,3,18], which relates malnutrition during the fetal stage and the adaptations for survival experienced by the offspring. On the other hand, early postnatal nutrition may also cause differential programming of energy homeostasis. In particular, malnutrition produced by protein restriction in lactating dams has been associated with reduced body weight in the adult offspring, despite no changes in food intake [19–21]. Other models of energy restriction obtained by severe food

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restriction in lactating dams [22] or by increasing the litter size [23,24] have also been associated with lower body weight and lower food intake in their adult offspring. In this sense, we have also recently described in rats that moderate caloric restriction (30%) in lactating dams protects their offspring against obesity as well as against insulin resistance in adulthood [25]. Most of these studies analyzing the lasting effects of maternal caloric restriction in offspring have mainly focused on the changes in body weight, food intake and/or circulating hormone levels [24,26–28]. However little is known about the changes occurring at gene expression level in key tissues involved in energy balance as a consequence of maternal food restriction during suckling period that may be determinant of the lower propensity to obesity. We hypothesized that early postnatal food restriction may induce developmental programming of hypothalamic and WAT gene expression of key factors involved in the regulation of energy balance. Thus, we aimed to gain further insight into the mechanisms that could underlie the substantial outcome in the male and female offspring of moderate caloric restricted (30%) lactating dams by analyzing the mRNA expression levels of selected genes involved in the regulation of food intake and fat accumulation in the hypothalamus and WAT, respectively, and establishing their relationship with energy homeostasis-related parameters when these animals were exposed in adulthood to HF diet conditions. In addition, considering that the adaptation to fed/fasting conditions may be impaired in obese animals, HF diet exposed animals were studied under both feeding and fasting conditions.

2. Materials and methods

2.1. Experimental animals

The study was performed in male and female rats from 12 different litters, following the protocol below. All rats were housed under controlled temperature (22°C) and a 12 h light–dark cycle (light on from 0800 to 2000), and had unlimited access to tap water and standard chow diet (Panlab, Barcelona, Spain) unless mentioned otherwise. Briefly, twelve virgin female Wistar rats weighing between 200 g and 225 g were mated with male rats (Charles River Laboratories, Barcelona, Spain). After matching, each female was placed in an individual cage. At day 1 after delivery, excess pups in each litter were removed in order to keep 10 pups per dam (five males and five females, when possible) and dams were assigned to either control or caloric restricted group (n=6 in each group). The control group was fed ad libitum with standard diet, while the caloric restricted group was provided daily with a 30% caloric restricted diet throughout lactation, starting on day 1 after delivery and ending at weaning (day 21) as previously described [25]. After weaning, 36 animals from control dams (controls) (18 males and 18 females) and 36 from caloric restricted dams (CR) (18 males and 18 females) were placed two per cage, paired with another animal of the same group, and fed with standard diet until the age of 15 weeks; then they were distributed into two groups; normal fat (NF) group [animals (n=6/group) continued with standard diet (2.9% calories from fat)] and high-fat (HF) group [animals (n=12/group) fed with a chow diet (4.7 kcal/g) with 45% calories from fat (Research Diets, NJ, USA)]. HF diet contained 5.5% calories from soybean oil and 39.5% from lard. Body weight and food intake were followed.

At the age of 28 weeks, NF diet and a half of the HF diet fed rats (n=6/group) were killed under ad libitum feeding conditions, while the second half of HF diet group (n=6/group) was killed after 12-h fasting. All animals were sacrificed by decapitation during the first 2 h of the beginning of the light cycle and on different consecutive days (including animals from each group every day). Blood samples were collected in heparinized containers, then centrifuged at 700 g for 10 min to obtain the plasma, and stored at −20°C until analysis. The hypothalamus and the main WAT depots (retroperitoneal, mesenteric, gonadal and inguinal) were rapidly removed, weighed, frozen in liquid nitrogen and stored at −70°C until ulterior studies. The hypothalamus was harvested by using the following landmarks, i.e., frontal edge of the optical chiasm, lateral sulci, caudal edge of the mammary bodies and a depth of 2 mm. Although different WAT depots were sampled to be weighed, the retroperitoneal depot was selected as representative to be analyzed for gene expression.

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of our University, and guidelines for the use and care of laboratory animals of the University were followed.

2.2. Measurement of circulating parameters under fed/fasting conditions and calculation of the homeostatic model assessment for insulin resistance

Blood glucose concentration was measured by Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). Plasma insulin concentration was determined using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden) following standard procedures. Plasma leptin concentration was measured using a mouse leptin ELISA kit (R&D Systems, Minneapolis, MN, USA). Circulating triglycerides (TGs) were measured by commercial enzymatic colorimetric kit [Triglyceride (INT), Sigma Diagnostics, St. Louis, MO, USA].

The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was used to assess insulin resistance. It was calculated from fasting insulin and glucose concentration using the formula of Matthews et al. [29]: HOMA-IR=fasting glucose (mmol/L)×fasting insulin (mU/L)/22.5.

2.3. RNA extraction

Total RNA was extracted from the hypothalamus and the retroperitoneal WAT depot by Tripure Reagent (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies, Wilmington, DE, USA) and its integrity confirmed using agarose gel electrophoresis.

2.4. Real-time quantitative polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was used to measure mRNA expression levels of neuropeptide Y (NPY), proopiomelanocortin (POMC), long form leptin receptor (ObRb), insulin receptor (InsR), and suppressor of cytokine signalling 3 (SOCS3) in hypothalamus, peroxisome proliferator activated receptor gamma 2 (PPARγ2), acetyl-coenzyme A carboxylase alpha (ACC1), glycerol-3-phosphate acyltransferase (GPAT), glucose transporter 4 (GLUT4), lipoprotein lipase (LPL), the free fatty acid transporter CD36, muscle carnitine palmitoyltransferase 1a (CPT1m), ObRb and InsR in retroperitoneal WAT. 0.25 μg of total RNA (in a final volume of 5 μl) was denatured at 65°C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20°C for 15 min, 42°C for 30 min, with a final step of 5 min at 95°C in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted cDNA template, forward and reverse primers (1 μM each) and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Primers were obtained from Sigma (Madrid, Spain) and sequences are described in [6,14,30], except for the SOCS3 sequences that were: forward 5′-ACTGAGCCGACCTCTCTCCT-3′ and reverse 5′-CCCC-TCTGACCCTTTCTTTG-3′. Real-time PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied

Biosystems) with the following profile: 10 min at 95°C, followed by a total of 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0), and the relative expression of each mRNA was calculated as a percentage of NF control rats under ad libitum feeding conditions, using the $2^{-\Delta\Delta Ct}$ method; beta-actin was used as reference gene for the hypothalamus analysis and beta-actin and 18S for WAT analysis [31]. All primers were obtained from Sigma Genosys (Sigma Aldrich Química, Madrid, Spain).

2.5. Statistical analysis

Given that the animals studied were from six different litters in each treatment group, the effect of litter was simultaneously factored with all data by repeated measures analysis of variance (ANOVA). No interactions between the litter and treatment were found across all the data, thus, data were expressed as mean±S.E.M. of animals from the six different litters. Multiple comparisons were assessed by two-way ANOVA. Single comparisons between groups were assessed by Student's *t* test. $P<.05$ was the threshold of significance.

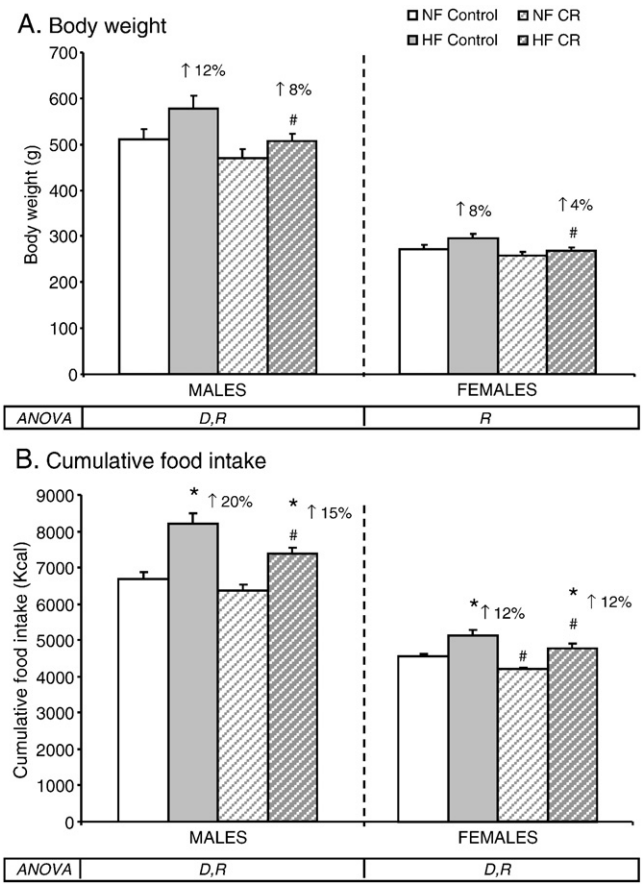

Fig. 1. Body weight (A), and cumulative food intake from day 105 until day 200 (B) of male and female offspring of controls and caloric restricted dams during lactation (CR) that were fed after day 105 with a NF or a HF diet until the age of 28 weeks. Results are expressed as the mean±S.E.M. of 6 to 12 animals per group. Statistics: D, effect of the type of diet; *R*, effect of caloric restriction during lactation; and *DxR*, interaction between caloric restriction and diet (two-way ANOVA). *NF vs. HF diet; #Control vs. CR (Student's *t* test). The percentages in brackets represent the increase as a consequence of the HF diet.

Table 1
Weights of inguinal, retroperitoneal, mesenteric and gonadal WAT of male and female offspring of control and caloric restricted dams during lactation (CR), at the age of 28 weeks, under NF and HF diet feeding and under ad libitum feeding conditions

		Males		Females	
		NF diet	HF diet	NF diet	HF diet
iWAT (g)	Control	11.5±1.4	19.9±2.6[a]	3.12±0.19	5.28±0.50[a]
	CR	8.34±0.94[b]	12.7±0.9[a,b]	2.87±0.39	3.76±0.31[b]
	ANOVA	D, R		D, R	
rWAT (g)	Control	14.5±2.5	26.6±3.9[a]	2.78±0.28	4.90±0.81[a]
	CR	10.9±1.5	18.5±3.0[a]	2.90±0.32	4.42±0.61[a]
	ANOVA	D		D	
mWAT (g)	Control	6.63±1.09	11.4±2.4[a]	2.82±0.36	4.36±0.78
	CR	4.64±0.76	6.06±0.52[b]	2.03±0.18[b]	2.78±0.30[a,b]
	ANOVA	D, R		D, R	
gWAT (g)	Control	15.1±2.4	22.9±4.9	8.77±0.95	14.4±2.2[a]
	CR	12.2±1.7	14.4±2.1	7.09±0.93	9.48±1.27[b]
	ANOVA			D, R	
Adiposity index (AI)	Control	9.08±1.07	13.5±1.2[a]	6.27±0.39	9.62±1.09[a]
	CR	7.52±0.84	10.3±1.2[a,b]	5.73±0.48	7.57±0.65[a]
	ANOVA	D, R		D	

Weights are expressed in grams. Data are means±S.E.M. of 6 animals per group. Statistics: *D*, effect of the type of diet; and *R*, effect of caloric restriction during lactation (two-way ANOVA).
[a] NF vs. HF diet.
[b] Control vs. CR (Student's *t* test).

3. Results

3.1. Weight-related parameters and food intake

As shown in Fig. 1A, moderate caloric restriction in dams during lactation resulted in lower body weight of their offspring in adulthood, both males and females (two-way ANOVA). This effect was more pronounced under HF diet feeding; in fact, by individual comparison, there were no statistical differences between control and CR rats under NF diet (Student's *t* test). This lower body weight can be explained, at least in part, by lower food intake. Both male and female CR animals ate fewer calories than their controls, both under NF and HF diet (Fig. 1B).

Differences in body weight between control and CR animals can be attributed to the size of fat depots (Table 1). The inguinal and mesenteric adipose tissue weights in both CR male and female animals and the weight of the gonadal depot in CR females were lower than in control animals (two-way ANOVA). Differences were generally found both under NF and HF diet conditions, but were more marked under HF diet. Moreover, CR males presented lower adiposity index than their controls both under NF and HF diet (two-way ANOVA), and this effect was also more pronounced under HF (Student's *t* test).

3.2. Circulating parameters under fed and fasting conditions

Table 2 shows circulating glucose, insulin, leptin, and TG levels of male and female control and CR animals exposed to NF and HF diet, under feeding conditions, as well as after 12-h fasting conditions in HF diet exposed animals. No significant differences were found in glucose levels between control and CR rats as an effect of the caloric restriction or HF diet feeding (two-way ANOVA). Fasted rats presented lower glucose levels than fed animals (two-way ANOVA). Control male animals, but not CR males, showed increased insulin concentration under feeding conditions when exposed to HF diet (two-way ANOVA); in fact, under HF diet conditions, CR males had lower insulin levels than their controls both under feeding and fasting conditions (two-way ANOVA). In females, no changes were found as an effect of caloric restriction or HF diet feeding (two-way ANOVA).

Table 2
Plasma glucose, insulin, leptin and TG concentration under NF and HF diet under feeding conditions, and also for the latter under fasting conditions of male and female offspring of controls and caloric restricted dams during lactation (CR) at the age of 28 weeks

		Males			Females		
		Feeding		Fasting	Feeding		Fasting
		NF diet	HF diet	HF diet	NF diet	HF diet	HF diet
Glucose (mg/dl)	Control	104±6	111±2	94±5[c]	106±3	104±5	89±4[c]
	CR	108±5	104±3	95±4[c]	113±2	114±7	90±5[c]
	ANOVA						
Insulin (μg/l)	Control	2.87±0.72	6.01±0.32[a]	1.71±0.27[c]	1.52±0.29	1.34±0.310	0.55±0.04[c]
	CR	2.15±0.36	1.71±0.24[b]	0.97±0.20[c]	1.14±0.23	1.11±0.21	0.55±0.10[c]
	ANOVA	DxR					
Leptin (μg/L)	Control	7.68±1.38	17.9±1.4[a]	8.98±1.70[c]	2.33±0.21	4.03±0.65[a]	2.80±0.64
	CR	6.21±1.39	9.48±1.46[b]	5.31±1.17[c]	2.13±0.44	2.23±0.38[b]	1.00±0.16[b,c]
	ANOVA	DxR			R		
Triglycerides (mg/ml)	Control	2.81±0.54	2.76±0.40	1.46±0.35[c]	2.87±0.66	1.91±0.39	1.00±0.16
	CR	2.76±0.50	2.47±0.51	0.902±0.207[c]	2.77±0.50	1.18±0.16[a]	0.500±0.115[b,c]
	ANOVA				D		

Results are expressed as the mean± S.E.M. of 6 animals per group. Statistics: *D*, effect of the type of diet; *R*, effect of caloric restriction during lactation; *DxR*, interaction between caloric restriction and diet (two-way ANOVA).

[a] NF vs. HF diet.
[b] Control vs. CR.
[c] Ad libitum vs. fasting (Student's *t* test).

Both males and females showed a decrease in insulin levels after 12-h fasting (Student's *t* test). Interestingly, under HF diet, control males showed a tendency to higher insulin resistance index (HOMA-IR) versus CR rats ($P = .062$, Student's *t* test) (Fig. 2).

Both male and female control rats, but not CR rats, increased their plasma leptin levels as an effect of HF diet (two-way ANOVA). In addition, CR females showed lower leptin concentration under both NF and HF diet than their controls (two-way ANOVA). Male animals and CR females, but not control females, decreased their circulating leptin levels after 12-h fasting (Student's *t* test); in fact, under HF diet conditions, CR females had lower leptin levels than their controls both under feeding and fasting conditions (Student's *t* test).

Regarding TG, no differences were observed between control and CR rats or after HF diet feeding in males (two-way ANOVA). A decrease in TG levels was found in both control and CR male rats exposed to HF diet as an effect of fasting. However, unlike males, control and CR females displayed lower TG concentration under HF diet compared with NF diet conditions (two-way ANOVA), but differences were more marked and significant by Student's t test only in CR animals. Under HF diet conditions, TG levels decreased in CR females, but not control females, as an effect of fasting, and levels in fasted CR were lower than in fasted control animals (Student's *t* test).

3.3. Hypothalamic mRNA levels of selected genes involved in energy balance in control and CR male and female rats

Fig. 3 shows mRNA expression levels of selected genes in the hypothalamus of male rats, under NF and HF diet under feeding conditions, and also for the latter under fasting conditions. No significant differences were found concerning NPY and POMC mRNA levels as an effect of caloric restriction or HF diet exposure, either in the ratio NPY/POMC (two-way ANOVA) (Fig. 3A). Neither were any differences found concerning InsR mRNA levels. However, CR animals showed higher ObRb mRNA levels than their controls (two-way ANOVA) (although these levels were lower by Student's *t* test under HF diet compared with NF diet), and a tendency to lower SOCS3 expression levels ($P = .07$, two-way ANOVA).

HF diet exposed male animals showed no significant changes in the expression levels of the analysed genes as an effect of fasting, with the exception of NPY, whose expression levels increased in control and CR animals (two-way ANOVA) (Fig. 3B), and of InsR, whose expression levels decreased only in control animals. Interestingly, the resulting NPY/POMC mRNA ratio increased in CR male rats after 12-h fasting, compared with fed conditions (Student's *t* test).

Results on gene expression in the hypothalamus of females are shown in Fig. 4. Control females increased NPY expression levels under HF diet, while CR rats showed a decreased expression (interaction between caloric restriction and the type of diet, two-way ANOVA) (Fig. 4A); no significant differences appeared in the expression of POMC as an effect of caloric restriction or HF diet feeding (two-way ANOVA). The resulting NPY/POMC mRNA ratio was lower in CR female animals under HF diet compared with levels under NF diet, without significant changes in control animals. In addition, CR female rats showed higher expression levels of InsR than their controls (two-way ANOVA), and a tendency to higher ObRb mRNA levels ($P = .07$; two-way ANOVA). SOCS3 mRNA levels were higher in control and CR animals under HF diet compared with levels under NF diet, without differences between control and CR animals (two-way ANOVA).

Interestingly, under fasting conditions, HF diet exposed CR female animals increased NPY mRNA levels and the NPY/POMC mRNA ratio; however, the expression levels of this gene and the NPY/POMC mRNA ratio did not change in control animals as an effect of fasting (Student's *t* test) (Fig. 4B). Furthermore, SOCS3 mRNA levels decreased in control animals as an effect of fasting, but remained unaltered in CR female animals (Student's *t* test).

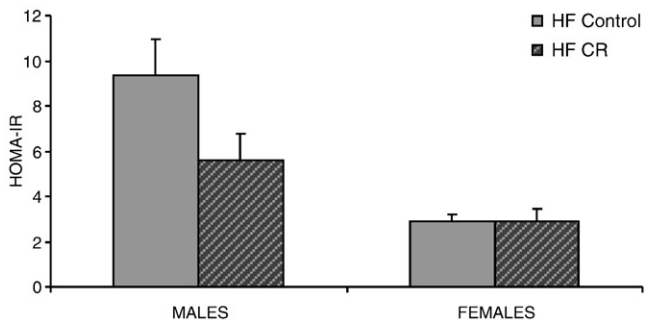

Fig. 2. HOMA-IR index at the age of 28 weeks of male and female offspring of controls and caloric restricted dams during lactation (CR), which where fed with HF diet. Results are expressed as the mean±S.E.M. of 6 animals per group.

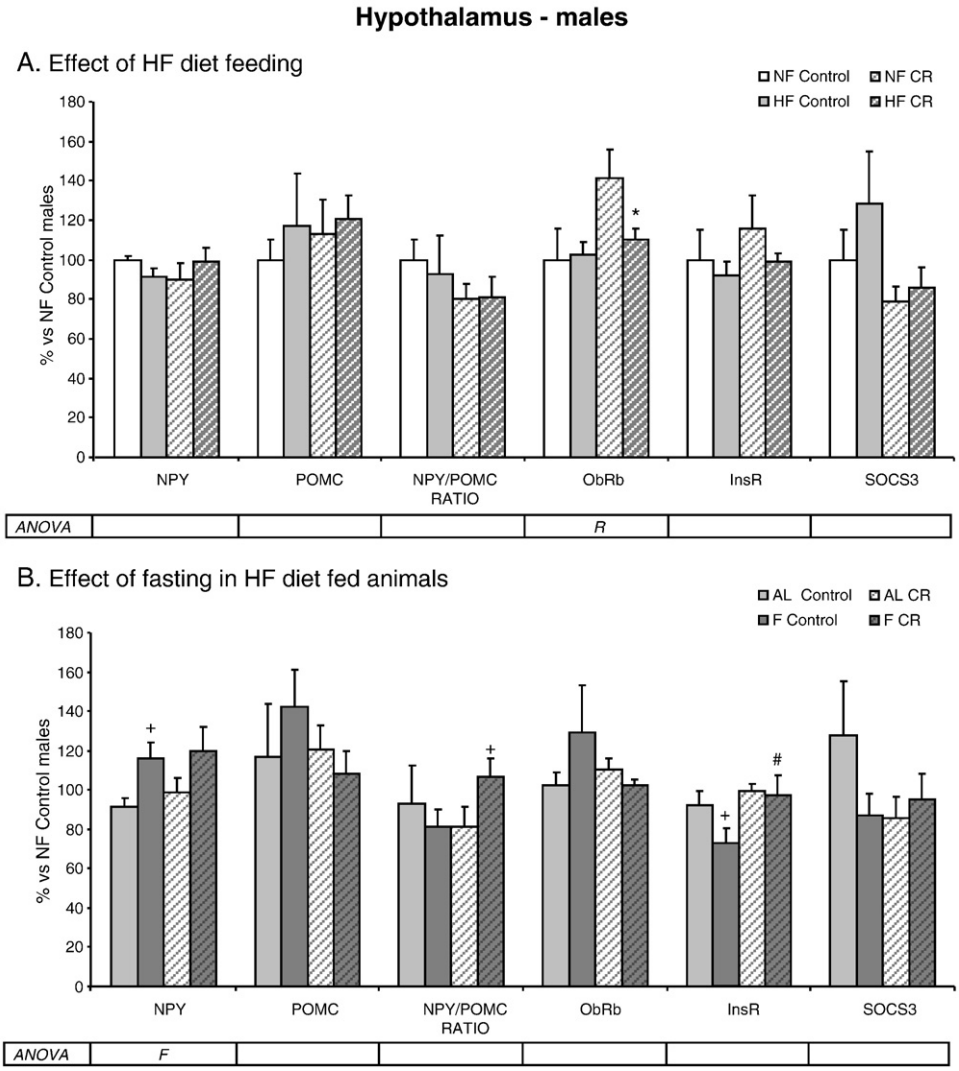

Fig. 3. mRNA expression levels of NPY, POMC, ObRb, InsR, and SOCS3 and the NPY/POMC ratio in the hypothalamus of male offspring of controls and maternal caloric restricted dams during lactation (CR), under NF and HF diet under feeding conditions (A), and also, in HF diet fed animals, under both ad libitum feeding (AL) and fasting (F) conditions (B). mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of NF diet fed control males under ad libitum feeding conditions. Data are means±S.E.M. (n=6). STATISTICS: *R*, effect of caloric restriction during lactation; *F*, effect of fasting (two-way ANOVA). *NF vs. HF diet; #Control vs. CR +Ad libitum vs. fasting (Student's *t* test).

3.4. Retroperitoneal WAT mRNA levels of selected genes involved in energy homeostasis in control and CR male and female rats

Fig. 5 shows mRNA expression levels of selected genes in WAT of male rats, under NF and HF diet under feeding conditions, and also for the latter under fasting conditions. Gene expression analyses were performed in the retroperitoneal depot, based in literature showing that this depot seems to be more sensitive to nutritional status, compared with other depots [12]. Control male rats showed lower mRNA expression levels of PPARγ2, ACC1, GPAT, LDL, CPT1m and InsR under HF diet, compared with those under NF diet (Student's *t* test), whereas this decrease was not found (for PPARγ2, CPT1m and InsR) or was not significant (for ACC1 and GPAT) in CR animals (Student's *t* test) (Fig. 5A). Moreover, GPAT mRNA levels were also significantly lower in CR animals compared with their controls (two-way ANOVA), but were significant by Student's *t* test only under NF diet. GLUT4 expression levels decreased under HF diet in both control and CR male rats, although the decrease was more marked and significant by Student's *t* test in CR animals. Notably, when HF diet fed animals were deprived of food (Fig. 5B), GLUT4 expression also decreased under fasting conditions (two-way ANOVA), but the decrease was significant by Student's *t* test only in CR animals. However, GPAT mRNA levels decreased by Student's *t* test only in control rats, and remained unchanged in CR animals. No response to fasting was found for the other genes studied, either in control or in CR animals exposed to HF diet.

Results on gene expression in WAT of females are shown in Fig. 6. No significant differences were found in females as an effect of HF diet or caloric restriction in the expression of PPARgamma2, ACC1, GLUT4, LPL and InsR in WAT (two-way ANOVA) (Fig. 6A). CR females showed higher ObRb mRNA levels than their controls (two-way ANOVA), greater CPT1m mRNA levels but only under NF diet (Student's *t* test), and lower mRNA levels on GPAT but only under HF diet (Student's *t* test).

CR female animals under HF diet, besides expressing greater ObRb mRNA levels than their controls under this diet, they showed a different response to fasting conditions compared with their controls (interaction between caloric restriction and fasting, two-way ANOVA): ObRb mRNA levels tended to decrease in CR animals, but to increase in controls (Fig. 6B). In turn, GPAT mRNA levels decreased after fasting in control rats without showing significant changes in CR (interaction between caloric restriction and fasting, two-way ANOVA). Both control and CR females exposed to HF diet

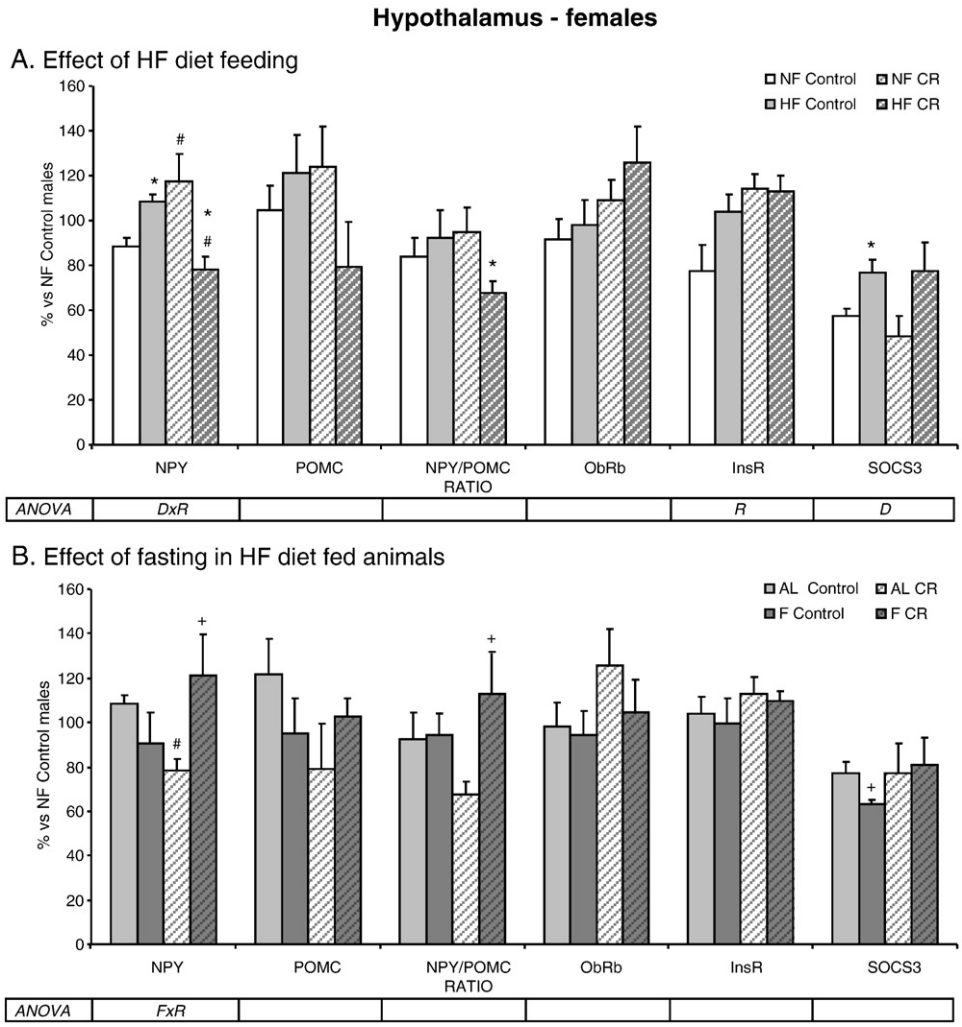

Fig. 4. mRNA expression levels of NPY, POMC, ObRb, InsR, and SOCS3 and the NPY/POMC ratio in the hypothalamus of female offspring of controls and maternal calorie restricted dams during lactation (CR), under NF and HF diet under feeding conditions (A), and also, in HF diet fed animals, under both ad libitum feeding (AL) and fasting (F) conditions (B). mRNA levels were measured by Real-time PCR and expressed as a percentage of the mean value of NF diet fed control males under ad libitum feeding conditions. Data are means± S.E.M. (n=6). STATISTICS: *D*, effect of the type of diet; *R*, effect of caloric restriction during lactation; *DxR*, interaction between caloric restriction and diet; and *FxR*, interaction between caloric restriction and feeding conditions (two-way ANOVA). *NF vs. HF diet; #Control vs. CR +Ad libitum vs. fasting (Student's *t* test).

decreased their GLUT4 mRNA levels with fasting (two-way ANOVA), while mRNA levels of ACC1, LPL, CPT1m and InsR did not change under fasting conditions in either control or CR females (two-way ANOVA).

4. Discussion

It is well known that maternal nutrition during perinatal periods may program the development of their offspring towards different consequences [1–4,32]. We previously described that a moderate caloric restriction (30%) in lactating dams confers certain protection against obesity development and from related metabolic alterations in adult life, particularly insulin resistance and hyperleptinemia [25]. However, little is known about the mechanisms that underlie these alterations and whether they are associated with changes in the expression of key genes involved in energy homeostasis. Here, we show that programming of gene expression related with central and peripheral leptin and insulin sensibility may be involved in the beneficial effects of moderate caloric restriction during lactation.

In accordance with our previous results in the same cohort of animals at younger ages [25], we also show here that the offspring of caloric restricted dams during the suckling period display lower body weight and lower fat content in adulthood, compared with their controls, the difference being more patent when animals are exposed to the challenge of HF diet feeding. The lower body weight can be explained, at least in part, by lower food intake. Comparison of accumulated calories eaten in adulthood during the 13-week period of exposure to NF or HF diet showed that food intake was lower in CR animals compared with their controls, both under NF and HF diet. The increased intake of calories associated with HF diet exposure was lower in CR male animals compared with their controls (increase of 15% and 20%, respectively), but was similar in CR female rats compared with their controls (increase of 12% an 12%, respectively). Remmers et al. [33] also showed reduced body weight, fat content and food intake in male and female caloric restricted rats during lactation obtained by large litter size; in contrast, malnutrition produced by protein restriction to the dams during lactation has been associated in the adult offspring with reduced body weight, despite no changes in food intake [19–21].

Central resistance to insulin and/or leptin have been proposed as important mechanisms responsible for the dysregulation of energy homeostasis, which may lead to obesity [34–36]. Regarding insulin, male rats are known to have a higher tendency for hyperinsulinemia induced by hyperlipidic diets than females [14]. In this sense, we have

previously described that male offspring of caloric restricted rats during lactation are protected against diet-induced hyperinsulinemia and insulin resistance when animals are exposed for 5 weeks in adulthood to HF diet [25]; in agreement, here we also found lower insulin levels under feeding conditions and a tendency to lower HOMA-IR in CR males exposed for a longer period (13 weeks) to HF diet, suggesting a better resistance of CR male rats against the detrimental effects of an obesogenic environment on circulating insulin profile. Concerning the leptin system, CR animals showed lower circulating leptin levels, particularly under HF diet feeding, compared with controls. This is in accordance with their lower body weight and agrees with the results obtained when these animals were younger (20 weeks old) [25]. Interestingly, lower leptin levels have been associated with a better sensitivity to leptin [8] but, in contrast, higher circulating leptin levels may contribute to the energy imbalance induced by HF diet feeding or age and are involved with the impairment of the fasting-induced suppression of leptin production [7,8,37]. On the other hand, gender differences have been described concerning the leptin system; female rats seem to be relatively more sensitive to leptin system regulation, whereas male rats are more sensitive to insulin regulation [14,38,39]. Nevertheless, HF diet fed control male animals showed decreased plasma leptin levels under fasting conditions, whereas the decrease was not significant in female control animals. However, both HF diet fed male and female CR animals responded to fasting conditions by lowering their plasma leptin levels.

Leptin and insulin are thought to regulate feeding behavior through their abilities to modulate the transcription of several neuropeptide genes. Thus, to ascertain whether changes in food intake between control and CR animals could be related with central effects of these hormones, the expression of hypothalamic genes related with leptin and insulin action were determined. NPY is considered one of the main peptides regulating food intake with orexigenic activity [40]. Increased NPY protein and/or mRNA levels have been described in the hypothalamus of different animal models of obesity [40]; for instance, higher NPY mRNA levels have been found in female rats chronically fed with HF diet [41]. We also found here that NPY mRNA levels increased in control female animals when exposed to HF diet, but, interestingly, they decreased in CR females exposed to this diet; moreover HF diet exposed CR females, but not their controls, displayed higher NPY mRNA levels after fasting conditions; this suggests not only a better adaptation to this

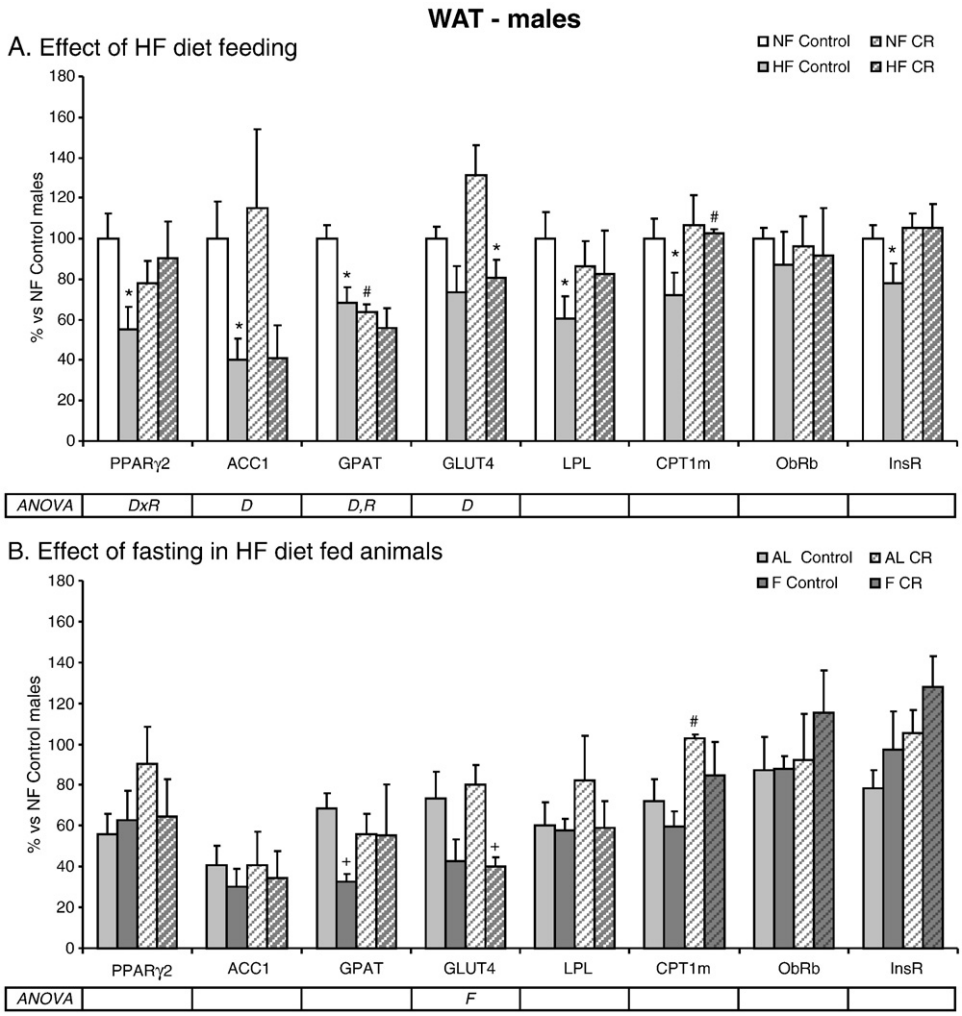

Fig. 5. mRNA expression levels of selected genes in the WAT of offspring of controls and maternal caloric restricted dams during lactation (CR), under NF and HF diet under feeding conditions (A), and also, in HF diet fed animals, under both ad libitum feeding (AL) and fasting (F) conditions (B). mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of NF diet fed control males under ad libitum feeding conditions. Data are means±S.E.M. (n=6). Genes determined were: PPARγ2, ACC1, GPAT, GLUT4, LPL, CPT1m, ObRb and the InsR. STATISTICS: *D*, effect of the type of diet; *R*, effect of caloric restriction during lactation; *F*, effect of feeding conditions; and *DxR*, interaction between caloric restriction and diet (two-way ANOVA). *NF vs. HF diet; #Control vs. CR +Ad libitum vs. fasting (Student's *t* test).

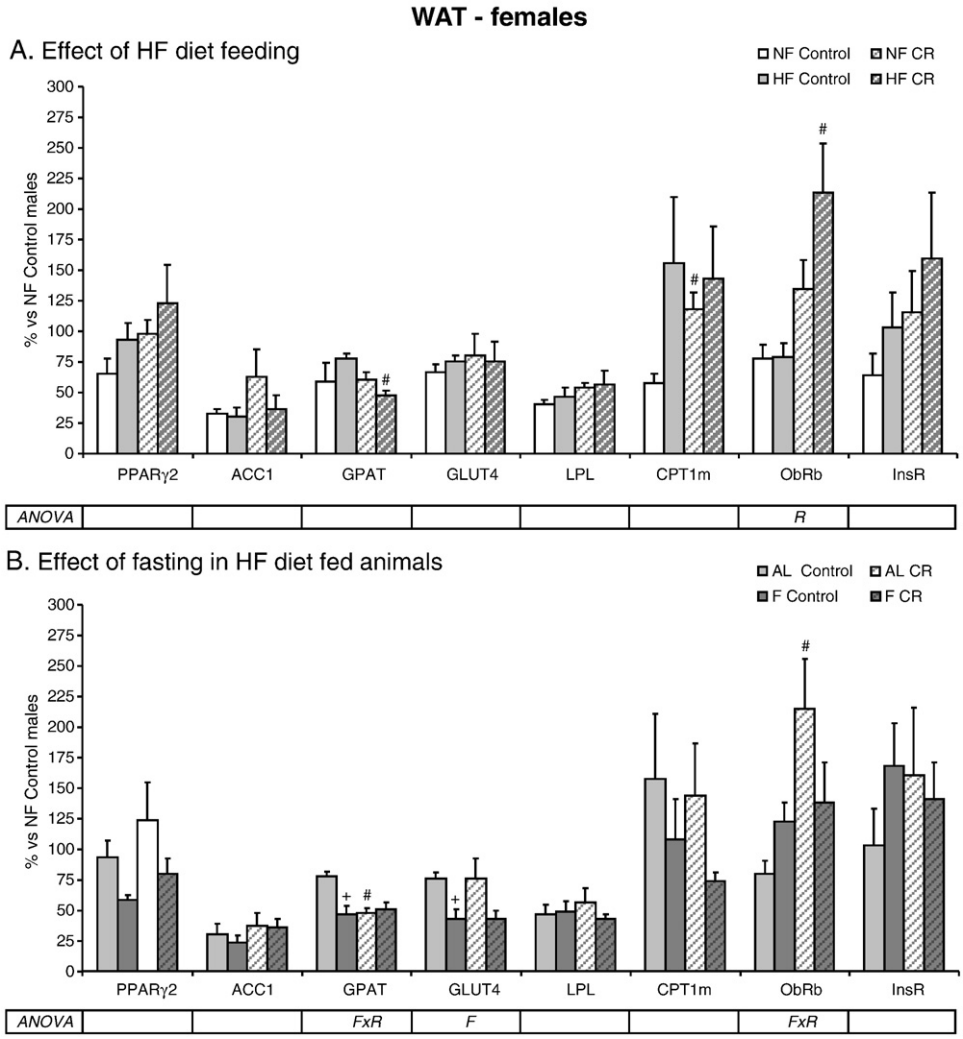

Fig. 6. mRNA expression levels of selected genes in the WAT of female offspring of controls and maternal caloric restricted dams during lactation (CR), under NF and HF diet under feeding conditions (A), and also, in HF diet fed animals, under both ad libitum feeding (AL) and fasting (F) conditions (B). mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of NF diet fed control males under ad libitum feeding conditions. Data are means±S.E.M. (n=6). Genes determined were PPARγ2, ACC1, GPAT, GLUT4, LPL, CPT1m, the ObRb and the InsR. Statistics: *R*, effect of caloric restriction during lactation; *F*, effect of feeding conditions; *FxR*, interaction between caloric restriction and feeding conditions (two-way ANOVA). *NF vs. HF diet; #Control vs. CR $^{+}$Ad libitum vs. fasting (Student's *t* test).

hyperlipidic diet, but also a better response to the feeding/fasting patterns. However, it is worth mentioning that, under NF diet, CR female animals displayed higher NPY mRNA levels than their controls; this fact cannot explain the hypophagia or the lower body weight of these animals compared with their controls. This suggests that caloric restriction may also affect other processes that could counteract the increased expression of NPY in these animals. Unlike females, NPY mRNA expression levels did not change in male animals, either as an effect of diet or caloric restriction.

POMC is the precursor of the anorexigenic peptide alpha-melanocyte stimulating hormone [42], but no significant differences were found between control and CR animals concerning POMC expression. Considering that both NPY and POMC are the main neuropeptides stimulating and inhibiting food intake, respectively, it is interesting to highlight that the NPY/POMC ratio decreased in CR females under HF diet and increased under fasting conditions in both HF diet exposed CR male and female animals, but not in controls, although the difference in males did not reach statistical significance ($P = .06$, Student's t test). These results may also contribute to explain the better control of food intake and body weight in CR rats under HF diet.

This pattern of expression of neuropeptides involved in food intake control and regulated at the central level by leptin and insulin may be indicative of a better sensitivity to these hormones. The responsiveness of the hypothalamus to leptin and insulin action depends not only on the circulating levels of these hormones, but also on different factors determining the sensitivity to these signals, such as the leptin and insulin receptors or the cytokine inhibitory protein SOCS3 [43,44]. The ObRb, the longest isoform of all leptin receptors, is mainly expressed in the hypothalamus and is considered to be the signaling-competent isoform [43]. This form is sensitive to genetic and physiological interventions that change circulating leptin levels, indicating that overexpression of ObRb in the hypothalamus may contribute to increased leptin sensitivity [43]. Here, we measured the expression of ObRb in the hypothalamus to evaluate possible differences in leptin responsiveness between control and CR rats under NF or HF diet feeding. CR male rats showed higher ObRb mRNA levels than controls, particularly under NF diet; a nonsignificant tendency ($P = .073$) was also observed in CR females. This is likely to be an indicator of better leptin sensitivity and a higher resistance to obesity development. On the other hand, SOCS3 is a leptin-inducible inhibitor of leptin signaling, and a potential mediator of leptin

resistance in obesity [45]. No significant differences were found between control and CR animals concerning the expression of levels of SOCS3 in the hypothalamus, but notably, CR males presented a tendency ($P = .074$) to lower SOCS3 expression levels both under NF (21% reduction) and HF diet (45% reduction). This reduction could contribute to increase leptin action and attenuate sensitivity to diet-induced obesity. This is supported by the fact that mice, which at are heterozygous for an SOCS3 gene deletion and hence have a 50% reduction of SOCS3 expression, exhibit enhanced ObRb activation induced by leptin [46]. In addition, SOCS3 is also an inhibitor of insulin action [47]. Thus, these changes in the expression levels of SOCS3 in the hypothalamus of CR male animals could also account for a better responsiveness to the central action of insulin. Insulin, like leptin, is secreted in proportion to fat stores, and also enters the central nervous system in proportion to its plasma levels [48]. Insulin receptors are expressed in neurons of brain areas involved in feeding control, and central administration of insulin can reduce food intake and body weight [48,49]. Interestingly, CR female animals showed increased InsR mRNA levels with respect to their controls which may determine a better response to insulin action, improving their feeding behaviour. All in all, these results may illustrate that a better responsiveness to the central action of leptin (in CR male animals) and insulin (in CR female animals) could be the mechanisms underlying the better control of food intake and body weight at the central level.

In addition to the hypothalamus, the adipose tissue is a target of the peripheral action of leptin and insulin. The adaptations of this tissue to an obesogenic environment and also to feeding conditions are another main determinant of the propensity to suffer obesity or other metabolic alterations [9,14]. In this sense, visceral fat accumulation, rather than subcutaneous, has been strongly linked to features of the metabolic syndrome, including leptin resistance, type 2 diabetes, hypertension and dyslipidemia [50–52]. Here, we show that CR rats appear to be more prepared to resist obesity development, the protective effect being more evident in males. This can be associated in male animals with an improvement of adipose tissue responsiveness to insulin. In fact, CR male animals were resistant to the decrease occurring in InsR mRNA levels in the internal (retroperitoneal) WAT depot under HF diet feeding. In addition, CR males had a better response to HF diet feeding, by maintaining fat uptake capacity and its storage by the adipose tissue, as well as the fatty acid oxidation capacity, whereas their controls presented an impairment of these processes. In fact, control male rats showed a decreased expression of lipogenic-related genes such as PPARγ2, LPL, which are regulated by this transcription factor [53], ACC1 and GPAT, and also of the catabolic-related gene CPT1m after HF diet feeding, whereas expression levels were mostly maintained unaltered in CR male animals (with the exception of ACC1, which showed a non significant tendency to decrease). A greater capacity to channel the excess of energy from the diet to the adipose tissue has been related with a better adaptive response to a HF diet [14] and higher sensitivity to insulin [54]. In fact, insulin regulates PPARγ mRNA expression by the adipose tissue [55], and this factor is involved in whole-body insulin sensitivity, probably through its effects on adipocyte metabolism and secretory function [53]. Thus, these results agree with an impairment of insulin sensitivity of control males under HF diet, at least at the gene expression level, whereas insulin sensitivity was not apparently impaired in CR male animals under HF diet. This is also in accordance with the pattern observed in the HOMA-IR in these animals. Increased insulin sensitivity has also been previously described in the offspring of severe protein and caloric restricted rats during lactation [25,56–58]. Other conditions during the early neonatal period, such as the supplement of suckling rats with physiological doses of leptin has also been associated in male animals with improved insulin sensitivity [8] and with better metabolic adaptations to HF diet feeding [59]; in concrete, changes at the gene expression level in the adipose tissue evidenced a better capacity of leptin-treated animals to handle and partition the excess of fuel under HF diet feeding, preventing other metabolic disorders related with HF diet feeding, such as hepatic lipid accumulation [59].

Unlike males, no significant differences were found between control and CR female animals concerning the expression of InsR and of metabolism-related genes in the adipose tissue, although it must be mentioned that female animals appear to be more protected against the detrimental effects of HF diet on the expression of these genes, as previously described [14]. These results also point out the increased capacity of the adipose tissue of female animals to store an excess of fat [14], in accordance with their healthier response to obesogenic environments in comparison with males [14,39,60]. These results are also in accordance with the lack of apparent effects of caloric restriction during lactation on insulin resistance (measured by the HOMA-IR) in CR female animals under HF diet.

It is worth noting, HF diet exposed CR females presented lower circulating fasting TG levels than their controls; thus, although they did not apparently improve their insulin sensitivity, like CR males, a better blood TG profile could be related to an improvement of the capacity to store the excess of fuel in the adipose tissue. Results of Zammit [61] have demonstrated that repeated exposure of the liver to elevated levels of insulin has a potent stimulatory effect on hepatic TG production. Thus, this over-stimulation of hepatic TG production through insulin action may outline a mechanistic basis for the development of leptin resistance, even independently of HF diet feeding [62]. Elevated fasting plasma TG levels have also been described in the offspring of 30% caloric restricted dams throughout pregnancy [62], and this has been found in conjunction with hyperinsulinemia and leptin resistance. In this sense, female CR rats displayed higher ObRb mRNA levels in the adipose tissue than their controls, and, when exposed to HF diet, their expression levels were sensitive to feeding/fasting conditions, which is also in accordance with the circulating leptin patterns observed. Interestingly, CR female animals also showed higher expression levels of CPT1m in the adipose tissue than their controls, although only under NF diet. This suggests that CR female rats may have greater fatty acid oxidation capacity in adipose tissue, which is of great importance for the control of whole body weight and fat reserves, and may be related to the pattern of ObRb expression in this tissue, as previously described [59]. All in all, these results on gene expression may indicate that CR female animals are more sensitive to the peripheral action of leptin, and this agrees with the lower plasma leptin levels and the better response of the circulating hormone to fasting, suggesting that CR females are more resistant to the development of overweight under HF diet conditions than their controls.

In conclusion, we show here that moderate caloric restriction (30%) in lactating dams results in lower body weight, adiposity and food intake in their male and female offspring in adulthood, but the mechanisms underlying these adaptations are gender-dependent. Changes in blood hormone concentration and at the gene expression level suggest that CR male rats seem to be more protected against HF diet induced peripheral insulin resistance, and these results in an improved capacity of the adipose tissue to handle and store the excess of fuel from the diet. In addition, these animals also show improved capacity to respond to leptin at the central level. CR female animals, in turn, appear to be programmed for a better sensitivity to the peripheral actions of leptin on the adipose tissue and to the central action of insulin, based on results at the transcriptional level. Both mechanisms may have similar outcomes in males and females, providing a better adaptation to the challenge of HF diet feeding. These results could help our understanding of the differential regulation of energy homeostasis in both genders, as well as the mechanisms responsible for the beneficial effects of moderate caloric restriction of the dams during lactation.

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