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Impaired insulin and leptin sensitivity in the offspring of moderate caloric-restricted dams during gestation is early programmed☆,☆☆,★

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Abstract

We aimed to assess the mechanisms responsible for hyperphagia and metabolic alterations caused by maternal moderate caloric restriction during gestation. Male and female offspring of control and 20% caloric-restricted rats (CR) were studied. They were fed a normal-fat diet until 4 months of age and then moved to a high-fat diet until 6 months of age. Blood parameters and expression of selected genes in hypothalamus, retroperitoneal white adipose tissue (rWAT) and liver were analyzed at 25 days and 6 months of age. Plasma leptin was measured during suckling. Levels of proteins involved in insulin and leptin signaling were determined at 6 months of age. CR ate more calories than controls, but only males gained more weight. A peak in plasma leptin was found in 9-day-old controls, but was absent in CR. Twenty-five-day-old CR showed lower insulin receptor mRNA levels in hypothalamus, rWAT and liver, and long-form leptin receptor (ObRb) in hypothalamus. At the age of 6 months, homeostatic model assessment for insulin resistance index was higher in CR than controls, and CR males also displayed hyperleptinemia. Adult CR also showed lower ObRb mRNA levels in the hypothalamus (only females, but both showed altered neuropeptide Y/proopiomelanocortin mRNA ratio), rWAT and liver (males), and a decrease of protein kinase C zeta levels in rWAT (females) and liver (males) and of phosphorylated signal transducer and activator of transcription 3 in liver (females). These results suggest that CR animals are programmed for insulin and central leptin resistance, which may explain the dysregulation of appetite and other metabolic alterations, favoring obesity development, although only manifested in males. These early programming effects could be associated with the absence of leptin surge during lactation.

Keywords: Insulin and leptin sensitivity; Caloric restriction; Gestation; Early programming; Leptin surge

1. Introduction

It is becoming increasingly clear that environmental conditions during critical periods of development may lead to differential programming of the mechanisms involved in the control of energy balance [1]. Gestation and lactation are considered critical periods for development, and food restriction during these periods has been described to induce permanent adaptive changes that may have lasting effects on the metabolic regulatory mechanisms of the offspring, leading to different outcomes in the propensity to suffer obesity in adult life [2,3]. While moderate caloric restriction during lactation has been associated with certain protection against later obesity in rats [4,5], caloric restriction during pregnancy has been reported to be a risk factor increasing the vulnerability to later obesogenic environmental stimuli [1,6,7], in both cases with different outputs depending on the severity or type of restriction and also on the gender of animals [4,5,7]. In this sense, we have previously described that 20% caloric restriction in rats during the first half of gestation results in higher food intake in their offspring in adulthood and that this concludes in higher body weight in males but not in females [7].

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Central resistance to insulin and/or leptin has been proposed as an important mechanism responsible for the dysregulation of energy homeostasis, which may lead to obesity [7–9]. In fact, the offspring of 20% caloric-restricted animals during gestation, both males and females, display hyperinsulinemia, which is already present at a juvenile age and previous to any apparent effect on body weight [7]. In addition, these animals, but only males, also display hyperleptinemia in adulthood when exposed to a high-fat (HF) diet [7]. These results suggest that mechanisms involved in insulin and/or leptin sensibility could have been affected as a consequence of this prenatal condition, with these later consequences on body weight control capacity. However, the concrete mechanisms involved and why programming mechanisms had different outcomes in male and female were not determined.

The hypothalamus is the main organ responsible for the central control of energy balance and appetite behavior by the production of many neuropeptides and the establishment of sympathetic connections responding to different stimuli, such as the circulating hormones insulin and leptin [10,11]; the brain is particularly sensitive during the perinatal period, and it has been described to undergo alterations in response to particular nutritional conditions during fetal development and neonatal life [3]. Leptin has been shown to play an important role during the perinatal period [12]. In fact, supplementation to neonate rats with physiological doses of leptin during lactation has been described to improve body weight control and leptin and insulin sensitivity in adult life [13,14], while a lack of leptin during this period occurring in leptin-deficient mice disrupts the normal postnatal developmental pattern of neural projection in the hypothalamus [15]. Previous studies have shown that maternal 20% caloric restriction during the first half of pregnancy resulted in lower cellularity and neuropeptide Y (NPY) and α-melanocyte-stimulating hormone (αMSH) neurons in the arcuate nucleus (ARC) in the offspring [16]. Delahaye et al. [17] also described that maternal severe (50%) caloric restriction during both gestation and lactation reduced fiber projections from ARC neurons to other hypothalamic structures and decreased proopiomelanocortin (POMC) and αMSH mRNA expression levels in neonate rats. In addition, adult offspring from 30% caloric-restricted pregnant dams have been shown to have the hypothalamic gene expression of POMC, NPY, Agouti-related protein and long-form leptin receptor (ObRb) altered [18]. In addition, without neglecting the important role of the hypothalamus in regulating energy homeostasis, both white adipose tissue (WAT) and liver are also key organs in the regulation of energy balance and substrate metabolism and targets of the peripheral actions of insulin and leptin [19]. In fact, the response of these tissues to feeding conditions may be another major factor determining the higher susceptibility to developing obesity and related metabolic alterations [19,20].

Thus, the aim of the present study was to determine the effects of moderate (20%) maternal caloric restriction during the first 12 days of gestation on determinants of later leptin and insulin resistance by exploring the expression of selected genes involved in insulin and leptin signaling in key tissues such as the hypothalamus, retroperitoneal WAT (rWAT) and the liver, both at a juvenile age (25 days) and in adulthood (6 months, after a 2-month period of HF diet exposure), to analyze whether this dietary stressor is able to step up early programmed disorders. Ultimately, considering the important role of leptin during a critical window of development, it was also the aim of this study to ascertain whether the effects of caloric restriction during gestation on later leptin and insulin homeostasis could be related, in part, with an alteration or a deficiency in leptin during the suckling period.

2. Materials and methods

2.1. Animals and experimental design

The study was performed in male and female rats from 32 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 8:00 a.m. to 8:00 p.m.) and had unlimited access to tap water and standard chow diet (3 kcal/g, with 2.9% calories from fat; Panlab, Barcelona, Spain) unless mentioned otherwise. Briefly, virgin female Wistar rats weighing between 200 g and 225 g were mated with male rats (Charles River Laboratories, Barcelona, Spain). Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm, and then female rats were single caged. Pregnant rats were divided into two groups: one had free access to standard chow diet, and the other one underwent 20% restriction of caloric intake from day 1 to day 12 of pregnancy. Caloric restriction was performed by offering each dam a daily amount of food corresponding to 80% of the calories that should be eaten according to body weight. This amount was calculated considering the calories daily consumed by their control animals under *ad libitum* feeding conditions. After the caloric restriction period, rats were allowed to eat *ad libitum*, and food intake was measured. At day 1 after delivery, excess pups of each sex in each litter were removed to keep 10 pups per dam (five males and five females, when possible). Weaning was conducted at 21 days of life.

One set of animals from 10 control dams and from 9 caloric-restricted dams was used to obtain blood samples at different stages of lactation (days 5, 9 and 15 of lactation) under *ad libitum* feeding conditions (n=5–10 animals/group). Blood was collected in heparinized containers to obtain plasma for leptin determination.

On day 25 of life, another set of control and CR animals (n=5–7 animals/group) (from six and eight dams, respectively) was killed by decapitation under fed conditions during the first 2 h at the beginning of the light cycle. 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, the rWAT depot and the liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at −80°C until ulterior studies.

At weaning, a third set of animals from the same dams as those killed on day 25 of life, including 24 controls (12 males and 12 females) and 28 CR animals (12 males and 16 females), was kept alive. They were placed two per cage, paired with another animal of the same group and fed with standard diet until the age of 4 months; then they were exposed to an HF diet (4.7 kcal/g, with 45% calories from fat; Research Diets, Inc., NJ, USA) until the age of 6 months. HF diet

Table 1
Nucleotide sequences of primers used for PCR amplification

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplicon size (pb)
β-Actin	GAAGCTGTGCTATGTTGCCC	GGATTCCATACCCAGGAAGG	184
GDI1	CCGCACAAGGCAAATACATC	GACTCTCTGAACCGTCATCAA	210
InsR	GTCCGGCGTTCATCAGAG	CTCCTGGGATTCATGCTGTT	242
ObRb	AGCCAAACAAAAGCACCATT	TCCTGAGCCATCCAGTCTCT	174
Leptin	TTCACACACGCAGTCGGTAT	AGGTCTCGCAGGTTCTCCAG	186
NPY	TGGACTGACCCTCGCTCTAT	GTGTCTCAGGGCTGGATCTC	188
POMC	CCTGTGAAGGTGTACCCCAATGTC	CACGTTCTTGATGATGGCGTTC	266
TNFα	CCGATTTGCCATTTCATACC	TCGCTTCACAGAGCAATGAC	230
ATGL	TGTGGCCTCATTCCTCCTAC	AGCCCTGTTTGCACATCTCT	271
CPT1	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG	180
SREBP1c	AGCCATGGATTGCACATTTG	GGTACATCTTTACAGCAGTG	260
ACC1	TGCAGGTATCCCCACTCTTC	TTCTGATTCCCTTCCCTCCT	212
GPAT	CAGCGTGATTGCTACCTGAA	CTCTCCGTCCTGGTGAGAAG	194

contained 5.5% calories from soybean oil and 39.5% from lard. Body weight and food intake of the offspring were followed.

At the age of 6 months, both control and CR rats were killed under two feeding conditions: *ad libitum* feeding conditions (fed group, n=6–8/group) and 12-h fasting conditions (fasted group, n=6–8/group). All animals were sacrificed by decapitation during the first 2 h of the beginning at 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, WAT depots (retroperitoneal, mesenteric, gonadal and inguinal) and liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at −80°C until ulterior studies.

Although different WAT depots were sampled to be weighed, the retroperitoneal depot was selected as representative to be analyzed for gene expression based on literature showing that this depot seems to be more sensitive to nutritional status compared with other depots [21].

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 (HOMA-IR) (under fasting conditions)

Blood glucose concentration was measured using 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). Circulating triglycerides (TG) were measured by commercial enzymatic colorimetric kit [Triglyceride (INT), Sigma Diagnostics, St. Louis, MO, USA].

The HOMA-IR was used to assess insulin resistance. It was calculated from fasting insulin and glucose concentration using the formula of Matthews et al. [22]: HOMA-IR=fasting glucose (mmol/L)×fasting insulin (mU/L)/22.5.

2.3. RNA extraction

Total RNA was extracted from the hypothalamus, rWAT and the liver by Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) according to the

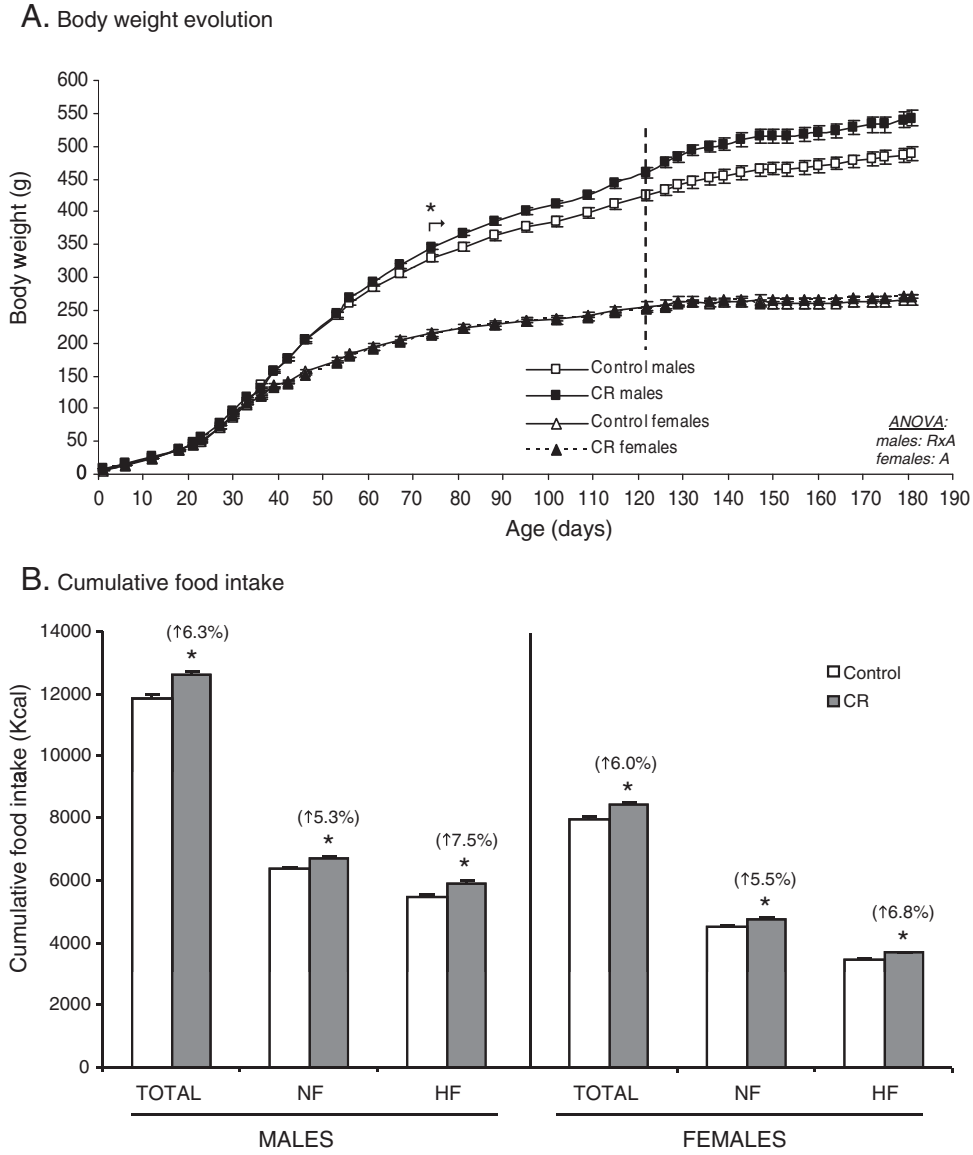

Fig. 1. (A) Body weight over time until the age of 6 months of male and female offspring of controls and caloric-restricted dams during gestation (CR). Animals were fed with standard normal-fat (NF) diet until the age of 4 months and then exposed to a high-fat (HF) diet (the dotted line indicates the time point of change from NF to HF diet). (B) Cumulative caloric intake from weaning at the age of 21 days until 6 months of age (TOTAL), as well as during NF diet feeding (from 21 days to 4 months old) (NF) and when animals were exposed to HF diet (from 4 to 6 months old) (HF), of male and female control and CR rats. The percentage increase in food intake of CR compared with controls is indicated in brackets. Data are expressed as the mean±S.E.M. of 12–16 animals per group. Statistics: *A*, effect of age; *R*×*A*, interaction between caloric restriction during gestation and age (P<.05, ANOVA repeated measures). *Different from their respective control group (P<.05, Student's t test). The arrow indicates the starting point of significant effects on body weight in male animals.

manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc., Wilmington, DE, USA), and its integrity was confirmed using agarose gel electrophoresis.

2.4. Real-time quantitative polymerase chain reaction (PCR) analysis

Real-time PCR was used to measure mRNA expression levels of ObRb and insulin receptor (InsR) in hypothalamus, rWAT and liver of 25-day-old and 6-month-old rats, and NPY and POMC in hypothalamus; tumor necrosis factor alpha (TNFα), adipose triglyceride lipase (ATGL) and carnitine palmitoyltransferase 1 (CPT1) in rWAT; and sterol response element binding protein 1c (SREBP1c), acetyl-coenzyme A carboxylase alpha (ACC1) and glycerol-3-phosphate acyltransferase (GPAT) in liver in rats 6 months of age. A total of 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 and 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 Table 1. 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 normal-fat (NF) control rats under *ad libitum* feeding conditions using the $2^{-\Delta\Delta Ct}$ method [23]. Beta-actin and guanosine diphosphate dissociation inhibitor 1 (GDI1) were used as reference genes depending on the tissue and the age and sex of the animals, according to their better suitability.

2.5. Western blot analysis

The amounts of total insulin receptor substrate 1 (IRS1), phosphorylated IRS1 on Try612 (pIRS1), protein kinase C zeta (PKCζ), signal transducer and activator of transcription 3 (STAT3) and tyrosine 705 phosphorylated STAT3 (pSTAT3) in rWAT and liver of control and CR rats at the age of 6 months were determined by Western blot. Tissue was homogenized at 4°C in 1:3 (w:v) or 1:20 (w:v), for rWAT and liver, respectively, of lysis buffer as previously described in Ref. [24]. The homogenate was centrifuged at 500g for 10 min at 4°C, and the supernatant was used for protein analysis. Total protein content was measured by the method of Bradford [25]. For analysis, 300 μg (for rWAT analysis) or 30 μg (for liver analysis) of total protein was solubilized and boiled for 3 min in Laemmli sample buffer containing 5% 2-beta-mercaptoethanol. Then, total protein was fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5% polyacrylamide) and electrotransferred onto a nitrocellulose membrane (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, the membrane was incubated with the primary rabbit polyclonal anti-IRS1, anti-pIRS1(Tyr632), anti-PKCζ, anti-STAT3 or anti-pSTAT3(Tyr705) antibody (Santa Cruz Biotechnology, Inc., CA, USA), and then with the infrared (IR)-dyed secondary anti-IgG antibody (LI-COR Biociences, Nebraska, USA) diluted 1:10,000. For IR detection, membranes were scanned in Odyssey Infrared Imaging System (LI-COR Biociences, NE, USA), and the bands were quantified using the analysis software provided.

2.6. Statistical analysis

Data were expressed as mean±S.E.M. Multiple comparisons were assessed by one-, two- and three-way analysis of variance (ANOVA) to determine the effects of different factors (sex, caloric restriction during pregnancy, feeding conditions and/or the day of lactation). Single comparisons between groups were assessed by Student's *t* test. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). $P<.05$ was the threshold of significance.

3. Results

3.1. Body weight gain and cumulative food intake of control and CR animals until the age of 6 months

Moderate caloric restriction during the first 12 days of gestation resulted in higher body weight in the male offspring from day 74 of life onwards (Student's *t* test) compared with their controls (Fig. 1A). When animals were 4 months old (just before changing to HF diet), CR male animals weighed 6.9% more than their controls, and the difference was even higher (12.5%) when animals were 6 months old and were under HF diet. Unlike males, no significant changes concerning body weight were found between control and CR female animals either under NF diet or HF diet.

As shown in Fig. 1B, cumulative food intake from weaning until the age of 6 months was significantly higher in both male and female CR compared with their controls. These differences were found during the feeding period with the NF diet as well as, and even higher, when they were under HF diet.

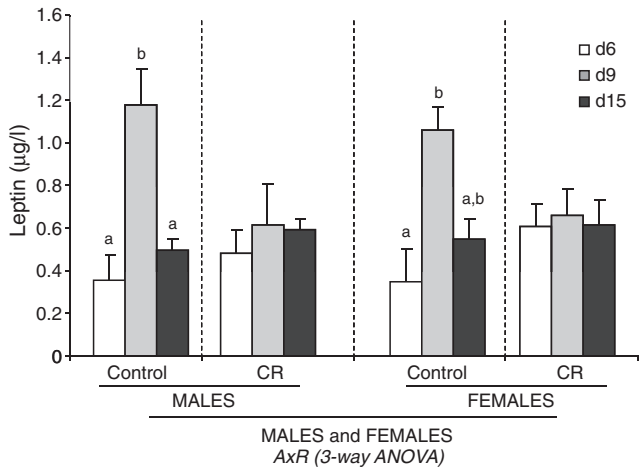

Fig. 2. Plasma leptin levels (μg/L) of male and female offspring of controls and caloric restricted dams during gestation (CR) at different stages of lactation: 6 days (d6), 9 days (d9) and 15 days (d15) of life. Data are means±S.E.M. (n=5–10). Statistics: $R\times A$, interaction between caloric restriction (R) and the day of lactation (A) in male and female pups ($P<.05$, three-way ANOVA). Within each graph, bars not sharing a common letter (a, b) are significantly different ($P<.05$, one-way ANOVA and Bonferroni post hoc test).

3.2. Circulating leptin levels in control and CR pups during lactation

No significant differences were found in body weight between control and CR animals at birth or during lactation (data not shown).

Circulating leptin levels were studied at different stages of lactation (5, 9 and 15 days of life) (Fig. 2). No significant differences were found between control and CR animals concerning leptin levels on days 5, 9 and 15 of lactation. However, interestingly, male and female control animals showed a surge of circulating leptin concentration at the age of 9 days (one-way ANOVA), in contrast with CR rats which maintained similar levels in the 3 days of lactation analyzed (interaction between

Table 2
Weight-related parameter and blood parameters at the age of 25 days

	Males		Females	
	Control	CR	Control	CR
Body weight (g)	61.4±2.8	59.3±2.0	58.6±2.3	55.7±2.8
Liver weight (mg)	2.86±0.11	2.60±0.11	2.79±0.07	2.69±0.12
rWAT weight (mg)	113±11	95.7±7.8	95.4±16.5	92.2±18.4
Glucose (mg/dl)	130±6	137±3	124±4	131±6
Insulin (ng/L)	170±38	122±36	154±41	167±35
Leptin (ng/L)	959±119	608±51[a]	984±164	841±40

Body weigh, liver and rWAT weight, and circulating glucose, insulin and leptin levels at 25 days of life (n=6–8) of male and female offspring from controls and caloric restricted dams during gestation (CR) under *ad libitum* feeding conditions. Data are mean±S.E.M. Statistics: No significant differences were found by two-way ANOVA.

[a] Different from their respective control group ($P<.05$, Student's *t* test).

the effect of caloric restriction and the day of lactation, three-way ANOVA).

3.3. Results in 25-day-old control and CR rats

3.3.1. Body weight, tissue weights and blood parameters

As previously described in the same cohort of animals, no significant differences were found in body weight between control and CR animals at the age of 25 days (two-way ANOVA) [16] (Table 2). No significant differences were found in the weight of the rWAT and liver either (Table 2).

Concerning blood parameters, blood glucose and plasma insulin levels were not significantly different in CR animals compared with their controls (two-way ANOVA). Circulating leptin concentration was lower in CR male rats, but not in females, with respect to their controls (Student's *t* test) (Table 2).

3.3.2. Gene expression levels in hypothalamus, rWAT and liver

Fig. 3 shows mRNA expression levels of InsR and ObRb in the hypothalamus, rWAT and liver of control and CR animals at the age of 25 days. Interestingly, both male and female CR animals displayed lower InsR and ObRb mRNA expression levels in the hypothalamus compared with their controls (two-way ANOVA). In rWAT and liver, InsR mRNA levels were also significantly lower in CR animals compared with their controls (two-way ANOVA), but no significant differences were found concerning ObRb mRNA levels.

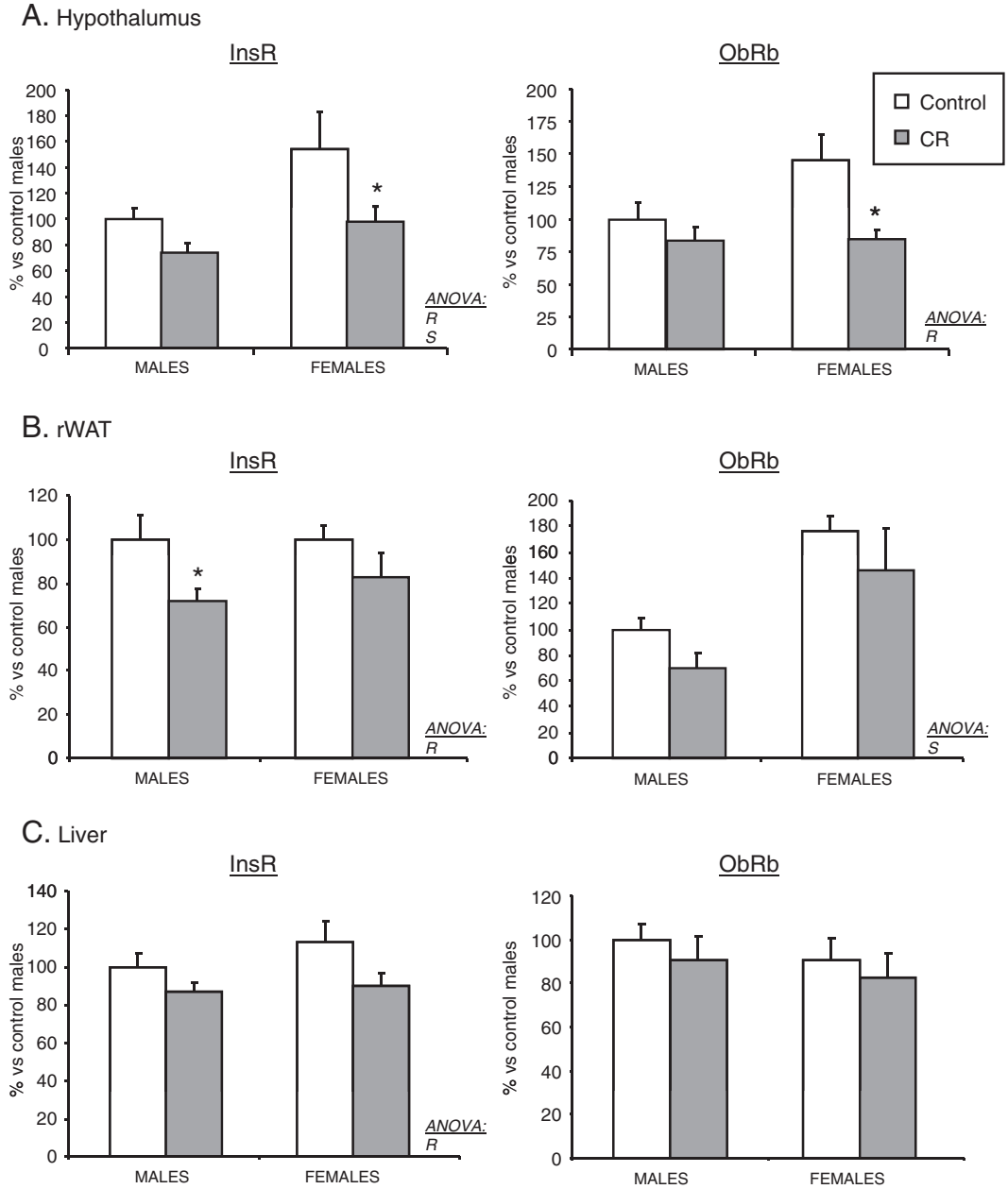

Fig. 3. InsR and ObRb mRNA expression levels in hypothalamus (A), rWAT (B) and liver (C) of 25-day-old male and female offspring of controls and caloric restricted dams during gestation (CR). mRNA levels were measured by real-time PCR and expressed as a percentage of the value of control male rats. Data are mean±S.E.M. (n=6–8). Statistics: *R*, effect of caloric restriction; *S*, effect of sex (P<.05, two-way ANOVA). *Different from their respective control group (P<.05, Student's *t* test).

3.4. Results in 6-month-old control and CR rats

3.4.1. Body weight, tissue weights and blood parameters under fed and fasting conditions

As described above, moderate maternal caloric restriction during gestation resulted in higher body weight in adulthood of male offspring but not of females (two-way ANOVA). Twelve-hour fasting induced a significant decrease of body weight in all animals (ANOVA repeated measures) (Table 3).

The differences in body weight between control and CR male rats can be attributed to the size of fat depots (Table 3). The adiposity index was higher in CR males with respect to their controls (two-way ANOVA). In fact, CR male animals showed greater fat pad weights in the four depots weighed in comparison to controls (two-way ANOVA). Although no significant differences were found between control and CR female animals, a tendency to higher adiposity was found in the latter (P=.066, two-way ANOVA) especially due to the significantly greater gonadal fat depot (two-way ANOVA). In both males and females, no significant differences were observed in the effect of 12-h fasting in the weights of the fat depots studied, and an increase in the size of rWAT was even observed in the group of female control animals after 12-h fasting. This difference does not seem to be a direct effect of fasting, but may probably be attributed to differences in the initial weight of fat depots between animals, which could also be masking other significant effects due to the fasting state. On the other hand, gestational caloric restriction resulted in higher liver weight in CR male animals but not in females (two-way ANOVA), especially under fasting conditions (Student's t test). As expected, fasted rats from the different groups showed lower liver weight than fed rats (two-way ANOVA).

Table 3
Weight-related parameters at the age of 6 months

	Control		CR		ANOVA
	Ad libitum	12-h fasting	*Ad libitum*	12-h fasting	
Males					
Body weight before fasting (g)		495±20		546±15	
Body weight at sacrifice (g)	487±9	486±20	548±20 [a]	536±15	*R*
Liver (g)	14.2±0.4	10.2±0.6 [b]	15.4±0.4	11.9±0.5 [a, b]	*R, F*
rWAT (g)	15.4±1.1	13.6±1.7	19.0±1.7 [a]	17.6±1.7	*R*
mWAT(g)	6.53±0.73	5.79±0.99	9.56±1.06 [a]	8.04±1.04	*R*
iWAT (g)	11.7±1.1	11.3±2.0	16.3±1.6 [a]	15.1±0.8	*R*
gWAT (g)	16.0±1.3	15.1±1.7	22.9±1.7 [a]	20.8±1.5 [a]	*R*
Adiposity index (%)	10.1±0.6	9.32±0.81	12.3±0.6	11.4±0.6	*R*
Females					
Body weight before fasting (g)		269±7		269±4	
Body weight at sacrifice (g)	263±14	261±7	274±5	262±5	
Liver (g)	7.5±0.4	6.1±0.3 [b]	7.8±0.3	6.1±0.2 [b]	*F*
rWAT (g)	2.96±0.34	4.13±0.39 [b]	4.43±0.61	3.89±0.42	
mWAT(g)	2.45±0.34	2.10±0.17	2.83±0.27	2.53±0.39	
iWAT (g)	3.54±0.49	3.10±0.29	3.83±0.38	3.84±0.35	
gWAT (g)	7.64±1.21	8.62±0.73	9.79±1.01	10.8±1.0	*R*
Adiposity index (%)	6.18±0.59	6.88±0.81	7.59±0.52	7.94±0.64	

Body weight, weights of retroperitoneal, mesenteric, inguinal and gonadal WAT (rWAT, mWAT, iWAT and gWAT, respectively), and the adiposity index at the age of 6 months from male and female offspring of controls and caloric restricted dams during gestation (CR) under *ad libitum* feeding conditions and after 12-h fasting conditions. Data are mean±S.E.M. (n=6–8). Statistics: *R*, effect of caloric restriction; *F*, effect of fasting (two-way ANOVA).

[a] Different from their respective control group.

[b] Different from fed conditions (Student's t test). Twelve-hour fasting induced a significant decrease of body weight in all animals (P<.05, effect of fasting and sex, ANOVA repeated measures).

Table 4
Circulating parameters at the age of 6 months

	Control		CR		ANOVA
	Ad libitum	12-h fasting	*Ad libitum*	12-h fasting	
Males					
Glucose (mg/dl)	110±2	89.3±5.9 [b]	113±5	95.8±2.3 [b]	*F*
Insulin (μg/L)	2.52±0.42	0.510±0.069 [b]	2.11±0.35	1.43±0.33 [a]	*F*
Leptin (μg/L)	11.6±1.7	3.48±0.48 [b]	14.8±2.3	7.05±0.89 [a,b]	*R, F*
Triglycerides (g/L)	3.93±0.18	2.44± 0.29 [b]	4.38±0.85	5.32±1.89	
NEFA (mmol/L)	0.959±0.053	0.727± 0.042 [b]	1.08±0.12	0.751±0.060 [b]	*F*
Females					
Glucose (mg/dl)	117±7	90.1±3.0 [b]	111±3	88.3±3.6 [b]	*F*
Insulin (μg/L)	0.871±0.087	0.321±0.023 [b]	0.840±0.101	0.396±0.061 [b]	*F*
Leptin (μg/L)	4.05±0.63	1.71±0.23 [b]	4.27±0.60	1.72±0.26 [b]	*F*
Triglycerides (g/L)	1.83±0.24	1.85±0.05	1.91±0.22	1.67±0.32	
NEFA (mmol/L)	0.694±0.089	0.888±0.114	0.751±0.097	0.957±0.049	*F*

Circulating glucose, insulin, leptin, triglycerides and nonesterified fatty acid (NEFA) in male and female offspring of controls and caloric restricted dams during gestation (CR), at the age of 6 months, under *ad libitum* feeding conditions and after 12 h fasting conditions. Data are mean±S.E.M. (n=6–8). Statistics: *R*, effect of caloric restriction; *F*, effect of fasting (two-way ANOVA).

[a] Different from their respective control group (Student's t test).

[b] Different from fed conditions (Student's t test).

Table 4 shows circulating glucose, insulin, leptin and TG levels of male and female control and CR animals under feeding conditions and after 12-h fasting. No significant differences were found in glucose levels between control and CR rats (two-way ANOVA). Fasted rats presented lower glucose levels than fed animals (two-way ANOVA). Insulin levels also decreased after fasting in male and female control and CR animals (two-way ANOVA), although a little nonsignificant response by Student's t test was found in CR male animals after food deprivation. In fact, CR male rats showed higher insulin concentration under fasting conditions in comparison to their controls (Student's t test). In females, no significant differences were observed between control and CR rats concerning circulating insulin levels (Student's t test).

HOMA-IR was calculated to estimate insulin resistance (Fig. 4). Notably, both male and female CR animals showed a higher HOMA-IR index compared with their controls (two-way ANOVA), although the increase was more pronounced and only significant by Student's t test in CR male rats, which showed 177% increase vs. controls, in contrast with the 31% increase found in CR females. It should also be mentioned that HOMA-IR value was significantly lower in females compared with males (two-way ANOVA).

Regarding leptin (Table 4), CR male animals displayed higher circulating leptin levels than controls (two-way ANOVA), especially under fasting conditions (Student's t test). In contrast, no differences were found between control and CR females (two-way ANOVA). As expected, circulating leptin levels decreased after 12-h fasting in male and female control and CR rats (two-way ANOVA).

Concerning TG, no differences were observed as a consequence of the caloric restriction or fasting conditions in both male and female animals (two-way ANOVA). However, it is interesting to highlight that control male rats showed a significant decrease in circulating TG levels after fasting (Student's t test) which was not present in CR males.

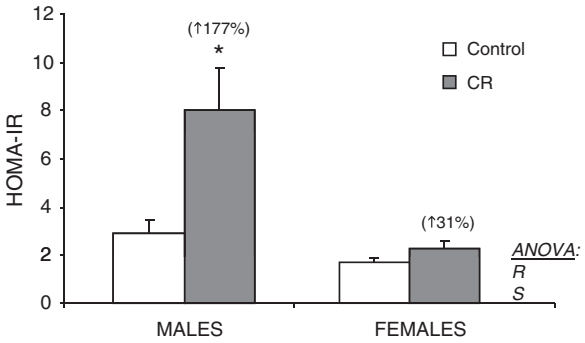

Fig. 4. HOMA-IR index at the age of 6 months of male and female offspring of controls and caloric restricted dams during gestation (CR). Results are expressed as the mean±S.E.M. of six to eight animals per group. The percentage increase in HOMA-IR index of CR compared with controls is indicated in brackets. Statistics: *R*, effect of caloric restriction; *S*, effect of sex ($P<.05$, two-way ANOVA). *Different from their respective control group ($P<.05$, Student's *t* test).

3.4.2. Gene expression levels in hypothalamus, rWAT and liver under fed and fasting conditions

Hypothalamic mRNA levels of selected genes involved in energy balance in control and CR male and female rats under fed and fasting conditions are shown in Fig. 5. In male animals (Fig. 5A), no significant differences were found concerning mRNA expression levels of InsR, ObRb, NPY or POMC as an effect of caloric restriction or fasting conditions (two-way ANOVA). However, it should be highlighted that the resulting NPY/POMC mRNA ratio increased in control animals after fasting conditions (Student's *t* test), but was unchanged in CR male animals. With regard to females (Fig. 5B), CR animals showed altered gene expression of hypothalamic key genes; in concrete, these animals showed lower ObRb and POMC mRNA levels than their controls, with no changes in InsR and NPY (two-way ANOVA). In addition, CR females showed increased POMC mRNA levels under fasting conditions (Student's *t* test), while no significant changes were found in control animals. Moreover, as occurring in males, the resulting NPY/POMC mRNA ratio increased in control animals after fasting conditions (Student's *t* test), but was unchanged in CR female animals.

Fig. 6 shows mRNA expression levels of selected genes related with energy balance in rWAT of control and CR male and female rats. Although different adipose tissue depots were harvested, gene expression analyses were performed in the retroperitoneal depot based on the literature showing that this depot seems to be more sensitive to nutritional status compared with other depots [21]. CR male animals showed lower InsR and ObRb mRNA levels than their controls (two-way ANOVA) (Fig. 6A). In addition, concerning InsR, their mRNA levels increased in control animals under fasting conditions, but did not change in CR animals as an effect of food deprivation. CR animals also showed higher TNFα mRNA expression levels under fasting conditions in comparison to fasted control rats

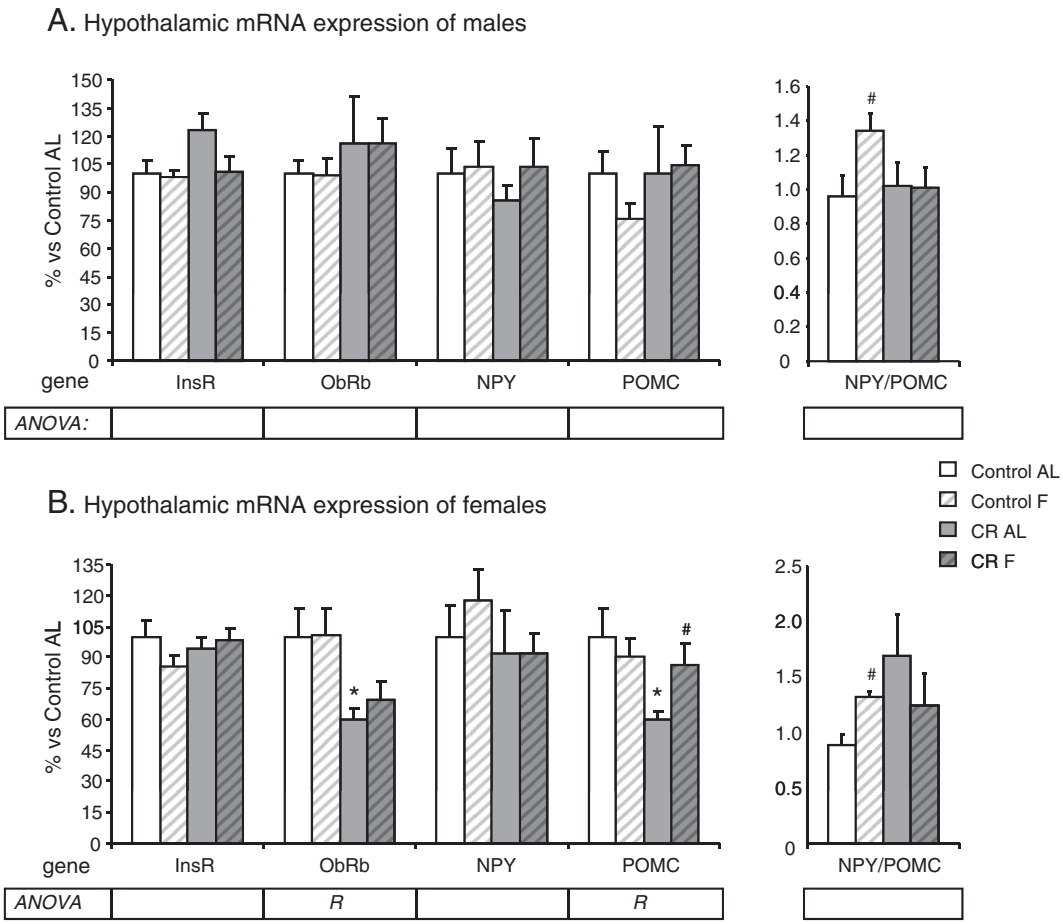

Fig. 5. mRNA expression levels of InsR, ObRb, NPY and POMC, and the NPY/POMC ratio in the hypothalamus of male (A) and female (B) offspring of controls and caloric restricted dams during gestation (CR), at the age of 6 months, under *ad libitum* feeding (AL) and fasting (F) conditions. mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of the control group under *ad libitum* feeding conditions. Data are mean±S.E.M. ($n=6–8$). Statistics: *R*, effect of caloric restriction ($P<.05$, two-way ANOVA). *Control vs. CR; #*ad libitum* vs. fasting ($P<.05$, Student's *t* test).

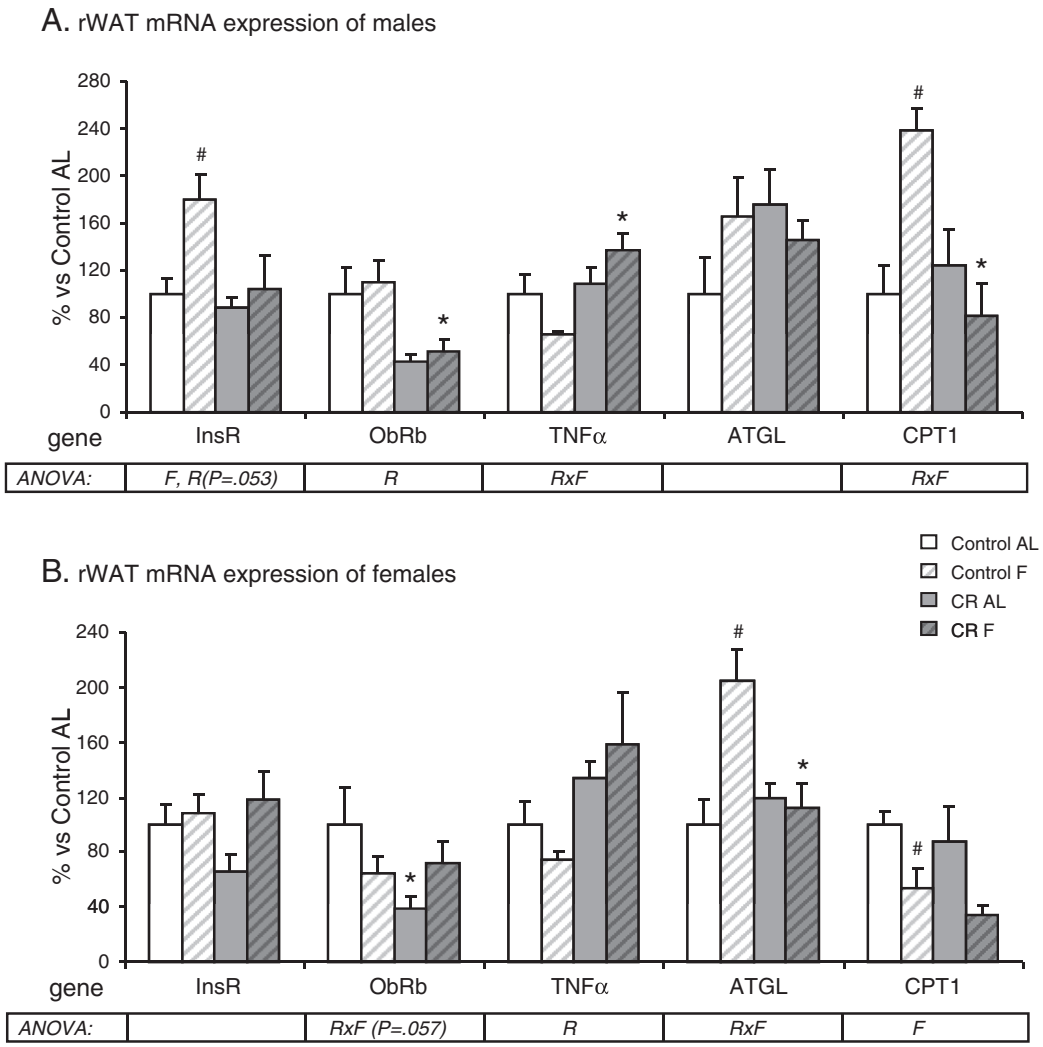

Fig. 6. mRNA expression levels of InsR, ObRb, TNFα, ATGL and CPT1 in rWAT of male (A) and female (B) offspring of controls and caloric restricted dams during gestation (CR), at the age of 6 months, under *ad libitum* feeding (AL) and 12-h fasting (F) conditions. mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of the control group under *ad libitum* feeding conditions. Data are mean±S.E.M. (n=6–8). Statistics: *R*, effect of caloric restriction; *F*, effect of fasting; *R*×*F*, interaction between caloric restriction and feeding conditions (P<.05, two-way ANOVA). *Control vs. CR; $^{\#}$*ad libitum* vs. fasting (P<.05, Student's *t* test).

(Student's *t* test). Moreover, CR male animals showed a different response to fasting conditions concerning CPT1 expression levels: they increased in control animals under fasting conditions, but not in CR animals. In addition, CR male animals showed lower CPT1 mRNA levels under fasting conditions than their controls (Student's *t* test). Fig. 6B shows gene expression in rWAT of female animals. Interestingly, CR female animals, under fed conditions, showed lower ObRb mRNA levels than their controls. CR animals also showed higher TNFα expression levels compared to controls (two-way ANOVA). In addition, there was a different response to fasting conditions between control and CR animals concerning ATGL mRNA expression (interaction between caloric restriction and fasting, two-way ANOVA) since mRNA levels increased in control animals under fasting conditions, but levels did not change in CR animals. In fact, CR female animals under fasting conditions showed lower ATGL mRNA levels than their controls (Student's *t* test). No significant differences were found between control and CR female animals concerning InsR mRNA levels.

Liver mRNA levels of selected genes related with energy balance in control and CR male and female rats are shown in Fig. 7. Interestingly, CR male animals showed lower ObRb mRNA expression levels under *ad libitum* fed conditions than their controls (Fig. 7A). Fasting induced an increase in ObRb mRNA levels in both groups, although this was more pronounced and only significant by Student *t* test in CR rats (interactive effect between caloric restriction during pregnancy and food deprivation, two-way ANOVA). Both SREBP1c and ACC1 mRNA expression levels decreased under fasting conditions in control and CR male animals, but in both cases, the response to starvation was of a greater magnitude in control animals. Concerning GPAT, a significant decrease was found in control animals as an effect of fasting, but no changes were found in CR animals.

The results of hepatic gene expression in females are shown in Fig. 7B. Both control and CR females displayed higher InsR mRNA levels after fasting conditions (two-way ANOVA), but the increase was higher and significant (by Student's *t* test) in controls. ObRb mRNA expression levels also increased after fasting conditions in both groups (two-way ANOVA), but the response was higher in CR animals; therefore, female CR rats showed higher ObRb mRNA levels under fasting conditions compared to controls (two-way ANOVA). SREBP1c, ACC1 and GPAT mRNA levels decreased in both control and CR female animals as an effect of fasting, while no significant differences were found between control and CR female animals concerning the expression of these genes.

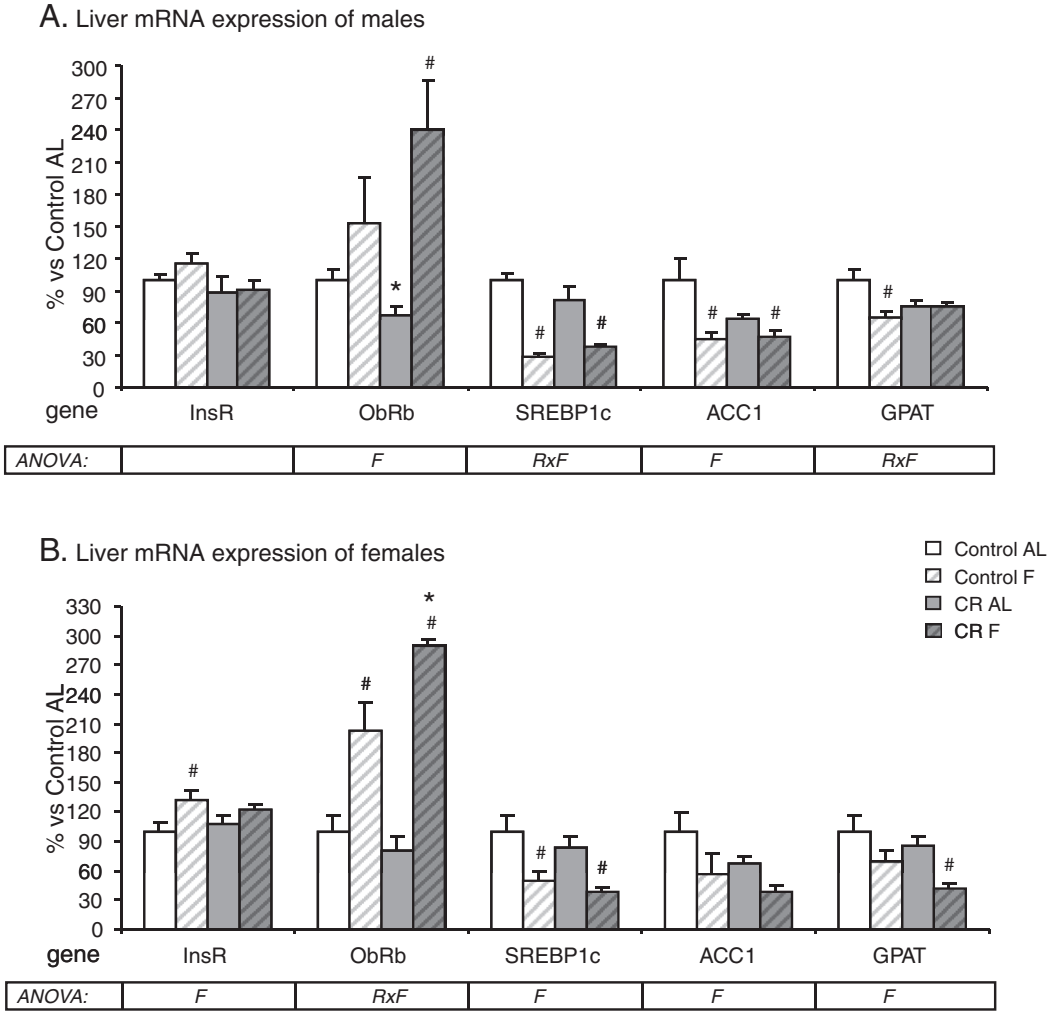

Fig. 7. mRNA expression levels of InsR, ObRb, SREBP1c, ACC1 and GPAT in liver of male (A) and female (B) offspring of controls and caloric restricted dams during gestation (CR), at the age of 6 months, under *ad libitum* feeding (AL) and 12-h fasting (F) conditions. mRNA levels were measured by real-time PCR and expressed as a percentage of the mean value of the control group under *ad libitum* feeding conditions. Data are mean±S.E.M. (n=6–8). Statistics: *F*, effect of fasting; *R*×*F*, interaction between caloric restriction and feeding conditions (P<.05, two-way ANOVA). *Control vs. CR; #*ad libitum* vs. fasting (P<.05, Student's *t* test).

3.4.3. Protein levels of insulin and leptin signaling molecules in rWAT and liver of control and CR male and female rats

Table 5 shows protein levels of IRS1, pIRS1, PKCζ, STAT3 and pSTAT3 in rWAT and liver of control and CR animals under fed and fasting conditions. Compared to controls, CR male animals showed higher levels of total STAT3 in rWAT and lower PKCζ in liver. CR female animals showed lower levels of PKCζ in rWAT and lower STAT3 (only under fed conditions) and pSTAT3 in liver. No changes were found concerning IRS1 and pIRS1 between controls and CR animals in either rWAT or liver. Fasting reduced PKCζ and total STAT3 protein levels in rWAT of both controls and CR male rats (two-way ANOVA), while no changes were found in female animals as an effect of fasting, with the exception of a decrease in PKCζ levels in CR rats (Student's *t* test). In liver, fasting conditions resulted in lower pIRS1 (which was higher and significant by Student's *t* test in controls), PKCζ, STAT3 and pSTAT3 in males, but notably, it resulted in increased levels of IRS1, pIRS1 and of STAT3 in females (two-way ANOVA).

4. Discussion

In agreement with our previous results in the same cohort of animals when younger [7], we show here that moderate maternal caloric restriction of 20% during the first half of pregnancy programs the offspring for higher food intake, which results in higher body weight and higher body fat content in males but not in females. We further show here that the hyperphagia displayed by these animals may be related with early programming of central and peripheral insulin resistance and of central leptin resistance, and it is associated with gender-dependent changes in the expression profile of key genes involved in the control of energy homeostasis in adult rats. In addition, it is suggested that the lack of a circulating leptin surge during the suckling period in the offspring of caloric-restricted animals during gestation may be one of the mechanisms that contribute to the metabolic malprogramming effects on target organs.

Fetal programming of insulin and leptin resistance by nutritional conditions has been proposed as a major mechanism responsible for later energy homeostasis dysregulation [9,26]. In agreement with this, the results obtained here in adult rats exposed to HF diet concerning plasma leptin levels in males, and the HOMA-IR index in both males and females, but particularly in males, also suggest an impairment of insulin and leptin sensitivity in CR rats, which may explain their hyperphagia. It should be mentioned that although changes in leptin levels are found here in male animals under the stressor of HF diet, an increase in HOMA-IR in both males and females was already found in the same cohort of animals when younger and exposed to an NF diet [7].

Table 5
Protein levels of insulin and leptin signaling molecules in the rWAT and liver

	rWAT					Liver				
	Control		CR		ANOVA	Control		CR		ANOVA
	Ad libitum	12-h fasting	*Ad libitum*	12-h fasting		*Ad libitum*	12-h fasting	*Ad libitum*	12-h fasting	
Males										
IRS1	100±18	112±22	154±33	133±32		100±8	94.7±6.1	89.1±4.9	110±11	
pIRS1 (Tyr632)	100±18	116±4	246±95	217±76		100±10	56.1±6.4 [b]	98.4±11.1	70.6±6.3	*F*
PKCζ	100±7	87.1±14.7	96.2±4.0	67.9±11.7	*F*	100±6	82.4±6.1	84.6±4.7	72.0±1.8 [b]	*R, F*
STAT3	100±20	83.9±11.1	141± 12	110±17	*R, F*	100±13	57.2±14.3	102±26	56.4±13.2	*F*
pSTAT3 (Tyr705)	100±29	88.5±10.2	161± 43	145±42		100±14	58.1±11.5 [b]	75.2±11.8	56.1±9.7	*F*
Females										
IRS1	100±22	108±16	81.3±22,5	94.6±19.5		100±5	119±15	113±9	151±13 [b]	*F*
pIRS1 (Tyr632)	100±24	101±17	106±11	93.0±14.1		100±5	157±13 [b]	106±17	157±7 [b]	*F*
PKCζ	100±9	123±16	93.0±10.7	58.4±10.0 [a,b]	*R*	100±10	90.3±10.4	90.9±8.5	89.1±7.3	
STAT3	100±13	87.5±25.8	163± 33	128±28		100±11	144±14 [b]	61.1±10.6 [a,b]	215±61	*F*
pSTAT3 (Tyr705)	100±18	98.1±35.3	71.6±8.9	111±23		100±25	118±9	59.4±16.2	76.2±12.1 [a]	*R*

Protein levels in the rWAT and the liver of male and female offspring of controls and caloric restricted dams during gestation (CR), at the age of 6 months, under *ad libitum* feeding conditions and after 12-h fasting conditions. Data are mean±S.E.M. (n=4–6). Statistics: *R*, effect of caloric restriction; *F*, effect of fasting (two-way ANOVA).
[a] Different from their respective control group (Student's *t* test).
[b] Different from fed conditions (Student's *t* test).

The effects of caloric restriction during gestation in male animals resulted in higher body weight and adiposity in adulthood compared to their controls. In addition, CR male adult animals also displayed an altered circulating TG response to feeding conditions since these animals did not exhibit a decrease in their circulating TG levels after 12-h fasting, while it occurred in control animals. Repeated exposure of the liver to elevated insulin levels has been described to induce hepatic TG production [27]. In contrast, a better blood TG profile has been related with an improvement of insulin and leptin sensitivity [5]. Hence, the dysregulation of circulating metabolic parameters in CR male rats is in agreement with the development of insulin and leptin resistance.

Unlike males, adult CR females did not display significant changes in circulating insulin and leptin levels compared to their controls. However, these animals presented other alterations related with insulin and leptin signaling at the central and peripheral level (see below), but they seem to be partially protected against the excess of fat accumulation associated with HF-diet feeding. CR female animals only showed greater size of the gonadal fat pad weight compared with their controls, but no significant changes were found in the other fat depots studied. In addition, they seem to be able to maintain normal levels of circulating parameters, such as TG levels. These results suggest that female animals are moderately protected against the detrimental effects of maternal caloric restriction during gestation. Jones and Friedman [28] also observed sex differences in the offspring of 50% caloric-restricted dams during the first 2 weeks of pregnancy, where male rats gained more weight after 5 weeks of age and became obese, but female offspring did not develop obesity. The reasons for the sex-dependent different outcomes in adult body weight and adiposity of fetal undernutrition are not clearly elucidated yet. However, the decrease in adipose tissue sympathetic innervation described in male offspring of caloric-restricted dams during gestation, but not in females, could account for the different outcomes on later adiposity [29].

To ascertain whether the manifestation of insulin and leptin resistance seen in the adult offspring of caloric-restricted animals during gestation was secondary to age and/or the obesogenic diet challenge or was the result of metabolic programmed effects, the expression of insulin and leptin receptors in selected tissues and circulating hormone levels were also studied in 25-day-old animals. At this age, these animals did not display significant changes in body weight, and no changes were found concerning insulin levels between control and CR animals either. However, circulating leptin levels were lower in CR animals compared with controls, and these changes do not seem to be attributed to lower body weight or adiposity. However, interestingly, at the age of 25 days, CR animals showed altered expression levels of insulin and leptin receptors in key target organs. Specifically, CR animals displayed lower mRNA expression levels of insulin receptor in the three tissues studied, the hypothalamus, rWAT and the liver, and lower mRNA levels of leptin receptor in the hypothalamus, with no significant changes in rWAT or liver.

The hormones insulin and leptin are able to exert their function at the central level, directly to the hypothalamus, through the regulation of the expression of different neuropeptides and key factors involved in energy homeostasis maintenance [11]. Hypothalamic alterations affecting the expression levels of insulin and leptin receptors already seen in CR animals at the age of 25 days suggest a malprogramming of the central control of appetite behavior. In agreement with these findings, we have previously described that both male and female offspring of caloric-restricted rats during gestation had, at the early age of 25 days of life, lower NPY- and αMSH-producing neurons and lower total cells in the ARC, which were accompanied by altered mRNA expression levels of these neuropeptides, as well as increased expression levels of SOCS-3, which could be indicative of lower central sensitivity to leptin and insulin [16].

We show here that adult CR animals exposed to HF diet for a 2-month period did not exhibit apparent differences in InsR mRNA levels in the hypothalamus compared with their controls. The lack of significant differences in InsR mRNA levels between control and CR animals when adult, while seen at early stages of life, could be tentatively attributed to the detrimental effects of HF feeding that may mask the effects of the early exposure to caloric restriction. In fact, HF diet has been described to contribute to central insulin resistance [30]. Concerning ObRb mRNA levels, the early differences between control and CR animals observed at the age of 25 days were maintained at the age of 6 months but only in females. Again, the lack of differences in male animals could be attributed to the overlapping effects of HF diet in these animals, whereas females could be more protected against the detrimental effects of this dietary challenge, according to the literature [20,31].

In addition, CR adult animals showed an altered response to fed/fasting conditions concerning mRNA expression levels of NPY and POMC. The NPY/POMC ratio rose in both male and female control animals under fasting conditions, but such changes were not observed in CR rats, suggesting a decreased capacity of the CNS to sense and

respond to changes in nutrient availability. This may contribute to explain the higher food intake of CR rats. Previous studies have also shown that obesity is characterized by an impaired response to feeding conditions, which has been observed both when studying the mRNA expression and the protein levels of a number of genes involved in energy metabolism [32,33] as well as for hundreds of genes measured by transcriptomics in peripheral tissues [34,35]. Moreover, it is interesting to highlight that CR females, which also exhibited lower ObRb mRNA expression, also showed lower POMC mRNA levels than their controls, which would be closely involved in their dysregulated food intake. A decrease in the expression of ObRb has been described to be involved in the development of leptin resistance [36]. Therefore, central insulin and leptin resistance appears to be an early malprogramming effect of moderate caloric restriction during gestation, which may be responsible for impaired food intake control and hence hyperphagia in both male and female CR animals.

Although the hypothalamus is the central controller of appetite behavior and energy balance, the peripheral response to both circulating insulin and leptin hormones may also determine the susceptibility to develop obesity and other related metabolic alterations. In this sense, WAT is a key tissue involved in both fat accumulation and mobilization, and these processes are regulated, among other hormones, by insulin and leptin; alterations in the action of these hormones due to malprogramming effects on early life [18,37] or in their adaptability under obesogenic environments such as HF diet feeding may affect the propensity to develop overweight [20]. At the age of 25 days of life, CR rats already presented lower WAT InsR mRNA levels, with no changes in ObRb expression levels, but a tendency to lower levels. When adult and under HF diet, CR males, but not females, also displayed lower InsR mRNA levels, and both male and female CR animals presented lower ObRb mRNA levels than their controls.

Levels of proteins involved in insulin and leptin signaling in adult animals have been measured as they may provide evidence of the presumed altered sensitivity to these hormones. Results obtained for PKC are supportive as CR female animals showed lower levels of PKCζ compared with their controls. This decrease was more marked under fasting conditions. PKCζ is a downstream effector in the insulin signaling pathway and plays an important role in activating the glucose transport response; in fact, overexpression of PKCζ or constitutively active PKCζ has an insulin-like effect on glucose transport during *in vitro* incubation of different kinds of cell lines (rev. [38]). Thus, the decrease in PKCζ protein levels in rWAT of CR females suggests a decrease in insulin signaling and glucose uptake by the adipose tissue. Decreased PKCζ levels in insulin target organs, such as muscle, have also been found in different experimental models of undernutrition during gestation and related with major propensity to insulin resistance development in adulthood [39].

However, despite changes in PKCζ in female CR animals, the protein levels of total IRS1 or pIRS1 were not significantly different between control and CR animals. In the same way, STAT3 and pSTAT3 levels did not decrease in CR animals either; conversely, CR male animals showed higher protein concentration of total STAT3 in this tissue, but no differences in the pSTAT3/STAT3 ratio. The higher protein levels of STAT3 in CR males could be the result of their hyperleptinemia, which allows certain leptin signaling in these animals. IRS1 plays a critical role in insulin signaling, being the initial step, while STAT3 mediates leptin signaling [40]. Leptin is also able to induce an insulin-like signaling pathway involving IRS/PI3K, making this a relevant point of cross talk between the insulin and leptin signaling pathways (rev. [41]). Protein levels of pIRS1 have been found to be altered in other models of maternal malnutrition during gestation. In particular, the offspring of 60% protein-restricted mice during gestation showed, at the age of 21 days, lower levels of pIRS1 and of other insulin signaling proteins in muscle, suggesting a predisposition of these animals to insulin resistance [39]. Here, although no effect of maternal caloric restriction during gestation was observed in IRS1 protein levels in adult animals, we cannot rule out that other key molecules of the insulin signaling cascade could be affected. In addition, as previously discussed, we cannot rule out either that the negative effects of HF diet on insulin resistance could be masking the effect of these maternal conditions during gestation.

Even though changes in proteins involved in insulin and leptin signaling in adult CR animals under HF diet did not prove a decreased adipose tissue sensitivity to these hormones compared with controls, it is interesting to highlight that both male and female CR rats showed higher expression levels of the proinflammatory cytokine TNFα. TNFα is overexpressed in adipose tissue in many rodent models of obesity and affects insulin sensitivity [42]. In fact, a link between obesity, insulin resistance and inflammation has been proposed [43]. TNFα has been involved in the JNK-mediated serine phosphorylation of IRS1, which inhibits the normal tyrosine phosphorylation of IRS1 in response to insulin [43]. In adult CR males, obesity was clearly manifested, but CR females did not present overweight at that moment of life, but these results, in accordance with other alterations in the expression of other key genes in WAT, such as the ObRb gene, suggest a dysregulation of energy homeostasis in these animals. Since females have been described to be more protected against the detrimental effects associated to obesity compared to males [20] and CR females presented severe alterations in other factors involved in the control of energy balance and appetite behavior at the central and peripheral level, it could be thought that CR females might be in a previous stage of the disease and could develop excessive fat accumulation, together with other endocrine alterations, later on in life. In favor of the detrimental programming effects of maternal caloric restriction during gestation in the female offspring is the lack of increase in ATGL mRNA expression levels in rWAT under fasting conditions, which occurs in control animals, in agreement with the literature [19]; this could be indicative of an impaired capacity to mobilize TG from this tissue under a negative energy balance situation. On the other hand, and in accordance with their higher fat accumulation in the adipose tissue, CR male animals did not exhibit an increase in CPT1 mRNA levels under fasting conditions, while this increase was found in control animals.

Regarding the liver, at the age of 25 days, CR rats presented lower InsR mRNA levels and no significant changes in ObRb mRNA levels compared to controls. Interestingly, when adult and under HF diet, CR males showed lower ObRb mRNA levels than controls, but only under fed conditions, with no changes in InsR mRNA levels. Nevertheless, CR males also showed lower PKCζ protein levels than their controls. In addition, although hepatic levels of IRS1 and STAT3 did not change between control and CR animals, it must be noted that their phosphorylated levels (Tyr632 and Tyr705, respectively) showed a diminished response to fasting conditions compared with their controls. All in all, these results agree with impaired leptin and insulin signaling in liver, which could be associated with the impaired response to changes in fed/fasting conditions concerning lipogenesis. In fact, CR males displayed a lower or even a lack of response to fasting conditions concerning the expression levels of SREBP1c and GPAT, respectively. Unlike males, no changes were found in females between control and CR animals concerning the expression of insulin and leptin receptors, or of other genes related with lipid metabolism. CR females even showed higher ObRb mRNA levels than their controls under fasting conditions. This does not seem to be indicative of higher leptin signaling in these animals since they displayed lower protein levels of total STAT3 (only under fed conditions) and of their phosphorylated (Tyr705) form, which suggest lower leptin signaling. The function of leptin in liver is not clearly established, but it has been shown to have antisteatotic

effects by lowering the expression of SREBP1. In fact, mice with ablated hepatic leptin signaling have increased lipid accumulation in the liver [44]. Thus, leptin appears to act as a negative regulator of insulin action in liver [45], and therefore, impaired leptin action in liver may affect whole energy homeostasis.

Thus, these results concerning the expression levels of insulin and leptin receptors in peripheral tissues such as WAT and liver at early stages of life, together with the apparent impaired action of these hormones found in adult life, suggest that early programming of peripheral insulin resistance may be a direct consequence of fetal caloric restriction, while peripheral leptin resistance, which appears in adulthood and under HF diet, might be secondary to insulin resistance or to central leptin resistance. In fact, it is recognized that elevated insulin levels promote both insulin resistance and increased leptin biosynthesis and secretion from adipose tissue, which may further desensitize leptin signaling and increase leptin resistance [46]. However, although the leptin–obesity–insulin resistance link is established, which alterations are causes or consequences in this particular situation and why the phenotypic outcomes are different between males and females need further clarification.

All in all, we show here that maternal caloric restriction during gestation results in early effects on the expression of leptin and insulin receptors in key tissues involved in energy homeostasis, particularly hypothalamus, which may compromise the proper functioning of the leptin and insulin systems. Moderate caloric restriction has also been associated with malprogramming of central hypothalamic structures involved in energy balance [16] and with a reduction in adipose tissue sympathetic innervation [29]. However, the mechanisms or factors responsible for the detrimental effects of these perinatal conditions are not clearly established. Leptin, which is naturally present in maternal milk, is known to play a key role during the suckling period [12]. Leptin supplementation (physiological doses) in rats during the suckling period has later effects in the offspring, preventing overweight in adulthood [13] as well as other alterations related with the metabolic syndrome [47], and also improves later insulin and leptin sensitivity [14]. These beneficial effects of leptin during lactation seem to be related, at least in part, with a better control of food intake, associated with epigenetic changes in the promoter methylation of POMC [48]. Moreover, in rodents, it is known that plasma leptin levels rise transiently during the neonatal period, peaking at around day 10 of lactation, a process that has been termed as “neonatal leptin surge” [49–51]. This surge in leptin levels seems to be important for programming the structural and functional development of hypothalamic orexigenic and anorexigenic centers [52], and its potential alteration by maternal caloric restriction has been checked here. In control animals, we observed the expected peak in circulating leptin levels at this period (9 days of life), but it is suggestive that this was absent in CR animals. A premature or delay in leptin peak, as occurring in mice with severe fetal undernutrition [53] or in protein-restricted rats during gestation and during both gestation and lactation [54], has also been associated with obesity in adulthood. Nevertheless, whether the malprogramming effects observed by caloric restriction could be simply attributed to the lack of the leptin surge needs to be specifically addressed. In any case, these results underscore the importance of leptin during lactation and the critical consequences that leptin deficiency may have during a critical period in postnatal life, being responsible for the common, detrimental outcomes of different adverse perinatal conditions.

In conclusion, results show that 20% maternal caloric restriction during the first 12 days of gestation programs the offspring for a lower capacity to respond to insulin and to central leptin action, which is already present at early ages, and this leads to hyperphagia in both genders and higher body weight in males but not in females. Males show higher and earlier harmful effects by caloric restriction during fetal life than females, while females appear more resistant to the detrimental effects of gestational caloric restriction, in terms of maintenance of body weight, in spite of the altered profile of gene expression in key tissues involved in energy homeostasis. The lack of leptin surge during a critical window of developmental plasticity, such as the suckling period, appears to be closely associated with the adverse health effects observed in the offspring of caloric-restricted dams.

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