Increased Male Offspring's Risk of Metabolic-Neuroendocrine Dysfunction and Overweight after Fructose-Rich Diet Intake by the Lactating Mother

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An adverse endogenous environment during early life predisposes the organism to develop metabolic disorders. We evaluated the impact of intake of an iso-caloric fructose rich diet (FRD) by lactating mothers (LM) on several metabolic functions of their male offspring. On postnatal d 1, *ad libitum* eating, lactating Sprague-Dawley rats received either 10% F (wt/vol; FRD-LM) or tap water (controls, CTR-LM) to drink throughout lactation. Weaned male offspring were fed *ad libitum* a normal diet, and body weight (BW) and food intake were registered until experimentation (60 d of age). Basal circulating levels of metabolic markers were evaluated. Both iv glucose tolerance and hypothalamic leptin sensitivity tests were performed. The hypothalamus was dissected for isolation of total RNA and Western blot analysis. Retroperitoneal (RP) adipose tissue was dissected and either kept frozen for gene analysis or digested to isolate adipocytes or for histological studies. FRD rats showed increased BW and decreased hypothalamic sensitivity to exogenous leptin, enhanced food intake (between 49–60 d), and decreased hypothalamic expression of several anorexigenic signals. FRD rats developed increased insulin and leptin peripheral levels and decreased adiponectinemia; although FRD rats normally tolerated glucose excess, it was associated with enhanced insulin secretion. FRD RP adipocytes were enlarged and spontaneously released high leptin, although they were less sensitive to insulin-induced leptin release. Accordingly, RP fat leptin gene expression was high in FRD rats. Excessive fructose consumption by lactating mothers resulted in deep neuroendocrine-metabolic disorders of their male offspring, probably enhancing the susceptibility to develop overweight/obesity during adult life. **(*Endocrinology* 151: 4214–4223, 2010)**

Obesity is a major problem for worldwide national health systems, the epidemic level of which has been clearly identified all over the world. Obesity increases the risk of developing chronic disorders such as metabolic syndrome, type 2 diabetes mellitus and cardiovascular disease (1–3), and obesity incidence has markedly increased in childhood (4, 5). Both genes and environment play important roles for normal metabolic-endocrine functions and neuronal development of the new offspring. However, weight, like height, is a highly inheritable trait (6). Thus, we need to consider that environmentally driven changes in body weight occur, even though genetic factors could increase susceptibility to environment-facilitated early weight gain (7). Maternal nutritional disturbances during critical developmental periods such as gestation (8) and/or the early postnatal (9) are known to raise offspring's risk of developing obesity and metabolic disorders in adult life (10, 11). Diet manipulation in mothers

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Abbreviations: ADIPOQ, Adiponectin; AgRP, agouti-related protein; AUC, area under the curve; BW, body weight; CART, cocaine- and amphetamine-regulated transcript; Ct, threshold cycle; CTR, control; FRD, fructose-rich diet; iv-GTT, intravenous glucose tolerance test; LEP, leptin; LM, lactating mother; MBH, medial basal hypothalamus; NPY, neuropeptide Y; POMC, proopiomelanocortin; p-STAT-3, phosphorylated signal transducer and activator of transcription-3; RP, retroperitoneal; STAT-3, signal transducer and activator of transcription-3; T2DM, type 2 diabetes mellitus.

during these critical periods has been used to evaluate some consequences in offspring; in fact, nutrient restriction, low protein diet, and high-fat/-carbohydrate diet have been used to identify their contribution on obesity and type 2 diabetes mellitus development in offspring (10–12).

Food intake and body weight gain are processes regulated by hypothalamic neurons that are still differentiating during the rodent suckling period; thus altered nutritional status during lactation severely impacts on normal neuron development (13). Maternal diet manipulation (*e.g.* under-/overnourished mothers) induces a distorted activity of the hypothalamic circuitry controlling appetite in the offspring (14–16). This circuit involves orexigenic [*e.g.* neuroepetide Y (NPY), agouti-related protein (AgRP)] and anorexigenic (*e.g.* CRH, TRH, cocaine- and amphetamine-regulated transcript (CART), proopiomelanocortin (POMC)] pathways, contributing to maintain energy homeostasis (17). One component that has currently been modified in diets is carbohydrates, mainly sucrose and fructose. Although fructose lacks of any short-term stimulatory effect on insulin and leptin production (18), excessive fructose intake through the diet without appropriate pancreatic and adipose tissue responses could lead to long-term detrimental effects on the regulation of energy intake and body adiposity. Although nowadays the individual's total daily caloric intake rose, the per capita fructose intake (sucrose- and high fructose corn syrup-derived) increased (18) from 64 g/d (during the 1970s) to 81 g/d (in the year 1997), with an additional augment in fructose intake (2.5 g/d) resulted from increased fruit and vegetable consumption. This change in eating behavior due to fructose overload enhanced the prevalence of several metabolic disorders (19).

It was reported that offspring born to mothers consuming fructose diet during pregnancy and lactation displayed decreased body weight, hyperinsulinemia, and hypoglycemia at weaning (20). Moreover, rat pups consuming high-carbohydrate milk during lactation did develop obesity in adulthood (21). Also, rat offspring consuming fructose-rich milk during the suckling period are characterized by increased body weight, enhanced insulinemia, and augmented skeletal muscle fatty acid transport at adult life (22). Excessive insulin secretion in turn promotes key features such as enhanced lipogenesis (23) and adipogenesis (24), impaired hypothalamic leptin signaling (25), and, consequently, reduced vagus tone inhibition (26), thus resulting in additional vagus-stimulated insulin secretion (26). As a result, a vicious circle is installed assuring the persistence of several physiopathological mechanisms leading to the development of metabolic and cardiovascular disorders.

The aim of the present study was to evaluate hypothalamic signals controlling appetite and metabolic-endocrine functions in adult male rats born to primipara mothers consuming an iso-caloric fructose-rich diet (FRD) while lactating.

Materials and Methods

Animals and experimental design

Sprague Dawley rats bred in our institution were maintained under controlled conditions of temperature (21 ± 2 C) and lights (on between 0700 and 1900 h) with free access to standard commercial rat chow (Ganave Lab., Argentina) and water.

Virgin females were mated with males in 10 × 20 inches plastic cages (at a 3:1 relation) until positive detection of sperm in their vaginal smears (examined every day at 0800 h). Pregnant dams were then individually housed in plastic cages and provided with standard chow and water *ad libitum* throughout pregnancy. Immediately after delivery, litter size was adjusted to eight pups per dam (average of male pups per litter ranged between 60–65%, approximately). Lactating mothers (LM) with their offspring, fed with standard Purina chow *ad libitum*, were allocated into two groups: while one drank tap water only (control, CTR-LM; n = 7), the other drank a FRD (fructose 10% wt/vol in tap water, FRD-LM; n = 8). Fresh fructose solution was provided every 2 d. Mother body weight (BW) and food and fluid intakes by mothers were recorded every 48 h during the lactation period. Immediately after weaning, mothers were killed and plasma samples were stored (−20 C) for measurement of different metabolites. Weaned (21 d of age) male pups (raised by CTR-LM and FRD-LM: CTR and FRD, respectively) were individually housed, and fed with standard Purina chow diet and water *ad libitum* until experimentation (60 d of age). During this period, individual BW and food intake were recorded every 48 h. Animal-group constitution was consisted in the allocation of one male rat from each different litter (seven CTR and eight FRD litters), which resulted (unless indicated) in final groups of seven CTR and eight FRD male rats. Rats were killed by decapitation following protocols for animal use from the National Institutes of Health Guidelines for care and use of experimental animals. Experiments received approval from our Institutional Committee on Animal Experimentation.

Studies performed in basal condition

After euthanization of CTR (n = 7) and FRD (n = 8) animals in basal condition (between 0800 and 0900 h), trunk blood was collected into EDTA coated tubes. Tubes were rapidly centrifuged (4 C; 3,000 rpm) and plasma samples were kept frozen (−20 C) until metabolites measurements. Immediately after euthanization, the medial basal hypothalamus (MBH) was dissected as previously reported (27) (limits: posterior border of the optic chiasm, anterior border of the mamillary bodies, and lateral hypothalamic borders, 3 mm deep, approximately); tissues were then kept frozen (−80 C) until total RNA isolation. Retroperitoneal (RP) fat pads were aseptically dissected and placed in (previously weighed) sterile Petri dishes containing 10 ml of sterile Krebs-Ringer-3[*N*-morholino]propanesulfonic acid (MOPS; Sigma Chemical Co., St. Louis, MO; 1 Krebs-Ringer:3 double distillated H_2O:1 MOPS, pH 7.4) medium. Dishes were weighed and fat mass was calculated by the difference between the recorded weights. RP adipose tissue pads were either used for cell

isolation/incubation, histological studies, or kept frozen (−80 C) until total RNA isolation.

Intravenous glucose tolerance test (iv-GTT)

Metabolic responses to high glucose load (2 g/kg BW; iv) were evaluated in 60 d-old male rats (n = 7 CTR and 8 FRD animals) bearing an indwelling iv cannulae (implanted in the right jugular vein 48 h before experimentation). On the morning of the experimental day, a small volume of blood was taken from nonfasting rats before (time 0) and 5, 15, 30, 60, and 90 min after glucose administration (28); a similar blood volume withdrawn was immediately replaced by artificial plasma. Plasma samples were kept frozen (−20 C) until determination of glucose and insulin concentrations.

Hypothalamic leptin sensitivity tests

These protocols are similar to those previously reported (29, 30), although with minor modifications. Briefly, CTR and FRD male rats were individually caged, with standard rat chow and water provided *ad libitum*. Rats were daily handled (10 min/d, between 1600 and 1700 h) for 1 wk before the study. The first test was set up on the experimental day (age 60 d). Food was withdrawn between 1600 and 1700 h. Thereafter, each group of CTR and FRD male rats received (n = 5–6 rats per group-treatment), at time 1700 h, a single ip injection of either recombinant mouse leptin solution (1 mg/kg BW; dissolved in sterile normal saline solution as vehicle) or vehicle alone (the volume of solution injected was 0.4–0.6 ml per rat). Immediately after injection, rats were back to their cages containing a known amount of rat chow. Four hours after treatment (daytime 2100 h), the remaining amount food in each cage was carefully removed and weighed, and 4-h food intake was then calculated. The second test was set up in overnight fasting rats. On the experimental day (age 60 d), each group of CTR and FRD male rats (n = 5–6 rats per group-treatment) were ip treated, at time 0800–0900 h, with a small volume of either leptin solution (1 mg/kg BW) or vehicle. Animals were then killed 45 min after treatment and the MBHs were rapidly dissected and kept frozen (−80 C) until total (STAT-3) and phosphorylated (p-STAT-3) signal transducer and activator of transcription-3 were determined by Western Blot.

Retroperitoneal adipose tissue histology

For histological studies in adipocytes, freshly dissected RP fat pads were fixed in 4% paraformaldehyde (in 0.2 M phosphate buffer), at 4 C (maximum 3 d), then washed (0.01 M PBS), and immersed in 70% ethanol (24 h) before being embedded in paraffin. Four-micrometer sections were obtained at different levels of the blocks and stained with hematoxylin-eosin then examined with a Nikon light microscope. Quantitative morphometric analysis was performed using a RGB CCD Sony camera together with the Image Pro-Plus 4.0 software (magnification, ×10). For each fat sample, seven sections and three levels were selected (n = 4 animals per group). Systematic random sampling was used to select 15 fields for each section and 2,500 cells per group were examined. Adipocyte diameter and area were measured (31); cell volume was then calculated ($4/3\pi r^3$).

Retroperitoneal adipocyte isolation and incubation

Isolated adipocytes from RP fat pads were obtained as previously and extensively described (32, 33). Isolated adipocytes were diluted to approximately 200,000 cells per 900 μl of Krebs-Ringer-MOPS medium and distributed into 15-ml plastic tubes. Substances tested (diluted in 100 μl) were as follows: medium either alone (concentration 0) or containing insulin (0.1–10 nM, Novo Nordisk Pharma AG, Switzerland) (33). Adipocytes were then incubated 45 min at 37 C, in 95% air-5% CO_2 atmosphere. At the end of incubation, media were carefully aspirated and kept frozen (−20 C) until measurement of leptin concentrations.

Peripheral metabolites measurements

Circulating glucose (Wiener Argentina Lab.), total proteins (Wiener), total cholesterol (Wiener), triglyceride (Wiener), and nonesterified fatty acid (Randox Laboratories Ltd., UK) levels were measured using commercial kits. Plasma and medium leptin (LEP) concentrations (32) and circulating levels of insulin (34) and corticosterone (19) were determined by specific RIAs developed in our laboratories. Plasma levels of other adipokines were measured (ELISA) as suggested by manufacturers (35) [Linco Research, Cat. # EZRADP-62K for adiponectin (ADIPOQ); American Diagnostica Inc., CT, IMUCLONE Cat. # 601 for plasminogen activator inhibitor factor-1; Life Diagnostics, Inc., PA, Cat. # 2210-2 for C-reactive protein; and Amersham, GE Healthcare, UK, cat. # RPN2744 for TNFα].

RNA isolation and real-time quantitative PCR

Total RNA was isolated from RP fat pads and MBH of different groups by the single-step acid guanidinium isothiocyanate-phenol-chloroform extraction method (Trizol; Invitrogen, Life Tech.; Cat. # 15596-026). One microgram of total RNA was reverse transcripted using random primers (250 ng) and Superscript III Rnase H-Reverse Transcriptase (200 U/HL Invitrogen, Life Tech; Cat. # 18989-093). Primers applied (shown, in alphabetical order, in Table 1) were β-actin, ADIPOQ, AgRP, CART, CRH, LEP, NPY, *ob*-Rb, POMC, and TRH. Two microliters of the RT mix were amplified with QuantiTect Syber Green PCR kit (Qiagen, Cat. # 204143) containing 0.5 μM of each specific primer, using LightCycler Detection System (MJ Mini Opticon, Bio-Rad). PCR efficiency was near 1. The threshold cycles (Ct) were measured in separate tubes by duplicate. The identity and purity of the amplified product were checked by electrophoresis on agarose mini-gels, and analysis of the melting curve was carried out at the end of amplification. Values of the differences between Ct were calculated in every sample for each gene of interest as followed: Ct gene of interest-Ct reporter gene. β-actin, for which mRNA levels did not differ between control and test groups, was the reporter gene. Relative changes in the expression level of one specific gene (ΔΔCt) were calculated as ΔCt of the test group minus ΔCt of the control group, and then presented as 2-ΔΔCt.

Western blot analysis

Briefly, frozen hypothalami were homogenized in lysis buffer: RIPA (Santa Cruz Biotechnology), protease inhibitor cocktail (0.35 mg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin), and phosphatase inhibitor cocktail (10 mM sodium fluoride, 20 mM sodium β-glycerophosphate, and 10 mM benzamidine). After lysis (90 min in ice), samples were centrifuged (10,000 × g at 4 C for 10 min), and soluble protein concentrations of the resulting lysates were determined by Lowry. Proteins (50 μg per lane) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred onto polyvinylidene fluoride membranes and incubated overnight at 4 C with

TABLE 1. Primers used (designed for a high homology region of different genes) for real-time RT-PCR

	Primers (5′–3′)	GBAN	bp
ACTB	se, AGCCATGTACGTAGCCATCC	NM_031144	115
	as, ACCCTCATAGATGGGCACAG		
ADIPOQ	se, AATCCTGCCCAGTCATGAAG	NM_144744	159
	as, TCTCCAGGAGTGCCATCTCT		
AgRP	se, GGGCGTGGCACCACTGAAGG	NM_033650	183
	as, ACACAGCGACGCGGAGAACG		
CART	se, AAGGCGGCAACTTCGGGCTC	NM_017110	167
	as, CGATCCTGGCCCCTTTCCGC		
CRH	se, GCTAACTTTTTCCGCGTGTT	NM_031019	175
	as, GGTGGAAGGTGAGATCCAGA		
LEP	se, GAGACCTCCTCCATCTGCTG	NM_013076	192
	as, CTCAGCATTCAGGGCTAAGG		
NPY	se, TACTCCGCTCTGCGACACTA	NM_012614	115
	as, GGGCATTTTCTGTGCTTTCT		
ob-Rb	se, TGTGGAATCTGGAGTGGTCA	AF287268	115
	as, TCTGGAGCCTGAACCAGTTT		
POMC	se, CCTATCGGGTGGAGCACTT	NM_139326	123
	as, TCTTGATGATGGCGTTCTTG		
TRH	se, GGGACCTCCTCAGAAAGGAAGGGT	NM_013046	165
	as, CCCCCACCCCAAGGACATATCTAA		

se, Sense; as, antisense; GBAN, GenBank Accession Number; amplicon length, in bp.

specific primary antibodies directed toward the signal transducer and activator of transcription-3 (anti-p-STAT-3 or antitotal STAT-3, from Santa Cruz Biotechnology, Inc.) followed by a 1-h incubation at room temperature with secondary antibody (goat antirabbit IgG horseradish peroxidase conjugates; Upstate, Millipore). Following membrane washing, immune complexes were revealed using enhanced chemiluminescence reagents (Amersham Life Science). The intensity of bands was quantified (Section Image Software), and the p-STAT-3/total-STAT-3 ratios were calculated.

Statistical analysis

Data (expressed as mean ± SEM) were analyzed by ANOVA, followed by post hoc comparisons with Fisher's test. The nonparametric Mann-Whitney test was used to analyze data from mRNA expression and Western blot analysis (36). *P* values <0.05 were considered statistically significant.

Results

Body weight, energy intake, and peripheral metabolites in lactating mothers

The mothers' BW was similar in both groups at the beginning and at the end of the lactation period (Table 2). Moreover, no differences among groups were found in the average of 48-h accumulated calorie intake throughout (expressed as average) the lactation period (Table 2). CTR-LM and FRD-LM, when evaluated at the end of the lactation period, showed no significant difference in circulating levels of several metabolites (Table 2).

Effects of FRD consumption by the lactating mother on male rat body weight and food intake

When weaned, male FRD rats were heavier between ages 21 and 60 d (Fig. 1, upper panel). No differences in 48-h accumulated food intake were observed in individual male pups from both groups between d 21 and 48 of age. Conversely, between d 49 and 60 of age, FRD male pups ate a significantly ($P < 0.05$) higher amount of food than age-matched CTR male rats (Fig. 1, lower panel; 27.45 ± 1.11 and 31.49 ± 1.33 g/rat in the last 24 h in CTR and FRD groups, respectively; $P < 0.05$).

Impact of FRD intake by lactating mothers on peripheral levels of several metabolites in male offspring

Sixty-day-old male offspring of the two groups of mothers displayed similar circulating levels of glucose, triglycerides, total cholesterol, nonesterified fatty acids, total proteins, and corticosterone (Table 3). Conversely, FRD male rats had significantly ($P < 0.05$) higher plasma

TABLE 2. Anthropometric characteristics and circulating levels of several metabolites in mothers from both groups (CTR-LM and FRD-LM) evaluated on the weaning day

	CTR-LM	FRD-LM
Initial body weight (g)	275.11 ± 6.54	283.19 ± 6.18
Final body weight (g)	313.58 ± 13.21	317.72 ± 9.29
Energy intake (Kj/d)	619.40 ± 41.76	687.22 ± 32.17
Glucose (mM)	5.69 ± 0.26	5.47 ± 0.35
Triglyceride (mM)	0.44 ± 0.03	0.61 ± 0.06
Total cholesterol (mM)	1.84 ± 0.10	1.98 ± 0.18
Total proteins (g/liter)	10.83 ± 1.32	10.36 ± 0.63
Leptin (ng/ml)	0.82 ± 0.19	0.92 ± 0.25
Corticosterone (nM)	205.79 ± 29.44	170.58 ± 32.89
Insulin (nM)	0.26 ± 0.06	0.31 ± 0.07

Values are means ± SEM (n = 7 and 8 rats, respectively).

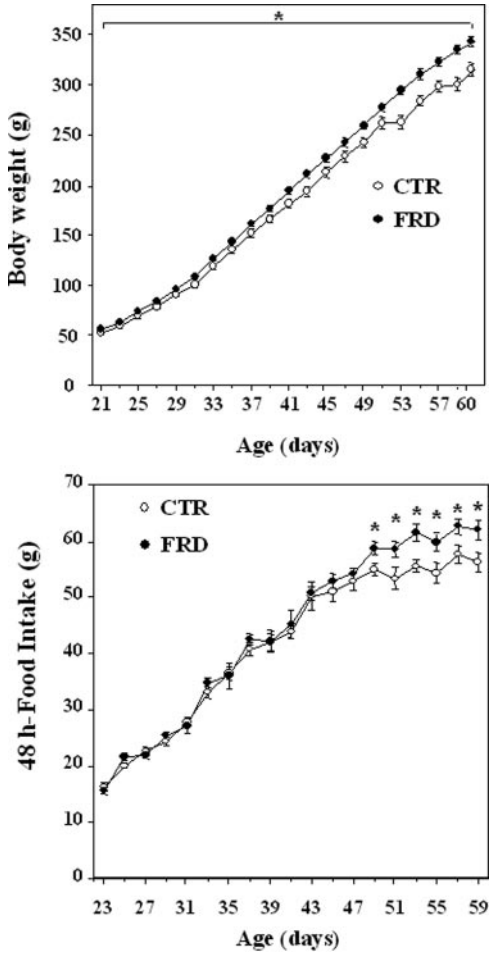

FIG. 1. Body weight (*upper*) and 48-h accumulated food intake (*lower*) in CTR and FRD male rats after weaning. These parameters were recorded on alternate days of age (between d 21–60 of age for BW and between d 23–59 of age for food intake). Values are means ± SEM (n = 25–30 rats per group, randomly selected from different litter-groups, respectively). *, $P < 0.05$ *vs.* CTR values on the same day.

concentrations of insulin than age-matched CTR rats (Table 3). It is important to remark that in separate experiments these high peripheral insulin levels were already observed at age 30 d and that they were accompanied by a significant decrease in glycemia and an increase in triglyceridemia, although these alterations no longer persisted at age 60 d (data not shown).

Peripheral levels of several adipokines in 60-d-old male CTR and FRD rats

Maternal FRD intake throughout lactation significantly ($P < 0.05$) enhanced and reduced peripheral concentrations of leptin and adiponectin, respectively (Table 3). This difference held when circulating leptin and adiponectin concentrations were expressed in relation to individual BW (0.79 ± 0.13 and 1.82 ± 0.37 ng LEP/ml · 100 g BW in CTR and FRD rats, respectively, $P < 0.05$; and 1.61 ± 0.16 and 1.09 ± 0.07 μg ADIPOQ/ml · 100 g BW in CTR and FRD rats, respectively, $P <$ 0.05). Conversely, the circulating levels of other adipokines such as plasminogen activator inhibitor factor-1, C-reactive protein, and TNFα were unmodified in FRD rats (Table 3) regardless of their BWs.

TABLE 3. Basal circulating levels of several metabolites in adult CTR ($n = 7$) and FRD ($n = 8$) male rats

	CTR	FRD
Glucose (mM)	6.60 ± 0.16	6.55 ± 0.22
Triglyceride (mM)	1.44 ± 0.15	1.51 ± 0.16
Total cholesterol (mM)	1.58 ± 0.08	1.42 ± 0.10
NEFA (mM)	1.05 ± 0.13	1.03 ± 0.12
Total proteins (g/liter)	7.58 ± 0.53	7.29 ± 0.59
Corticosterone (nM)	134.79 ± 31.17	240.09 ± 48.49
Insulin (nM)	0.31 ± 0.02	0.45 ± 0.03*
LEP (ng/ml)	2.41 ± 0.35	6.26 ± 1.56*
ADIPOQ (μg/ml)	5.87 ± 0.66	3.59 ± 0.27*
PAI-1 (ng/ml)	1.44 ± 0.06	1.45 ± 0.18
CRP (mg/ml)	0.36 ± 0.07	0.26 ± 0.22
TNFα (pg/ml)	84.11 ± 0.73	88.93 ± 1.99

Each rat assigned to a group derived from a different litter. Values are means ± SEM.

*, $P < 0.05$ *vs.* CTR values.

iv-GTT in adult CTR and FRD male rats

The pattern of circulating glucose levels (Fig. 2A) and the area under the curve (AUC) of peripheral glucose levels (Fig. 2B) throughout the iv-GTT were similar in the two groups of male rats. Basal (time zero) values had already been recovered 60 min after glucose administration in all groups examined.

Conversely, significantly ($P < 0.05$ *vs.* CTR values) higher circulating insulin levels were found in FRD male rats on several times throughout the iv-GTT (Fig. 2C). Moreover, whereas CTR male rats restored basal (time zero) circulating levels of insulin 30 min after high glucose load, the recovery of basal insulinemia by FRD male rats was delayed up to 60 min after glucose load (Fig. 2C). Accordingly, the AUC of insulin values was significantly ($P < 0.05$) higher in FRD than in CTR male rats (Fig. 2D).

RP adipose tissue characteristics and functionality

We found that RP fat mass was similar in both groups of male rats (Table 4). However, RP adipocyte diameter, area, and volume were significantly ($P < 0.05$) higher in FRD than in CTR male rats (Table 4).

Enhanced peripheral levels of leptin found in FRD male rats correlated with the expression of LEP mRNA in RP fat pads: this parameter was approximately 3.7 times higher ($P < 0.05$) in RP fat pads from FRD than from CTR male rats (Table 4). Conversely, no group differences were found in RP fat ADIPOQ mRNA expression (Table 4).

Figure 3 shows the results of *in vitro* leptin release by isolated RP fat adipocytes incubated in absence (spontaneous: insulin concentration zero) or presence of insulin

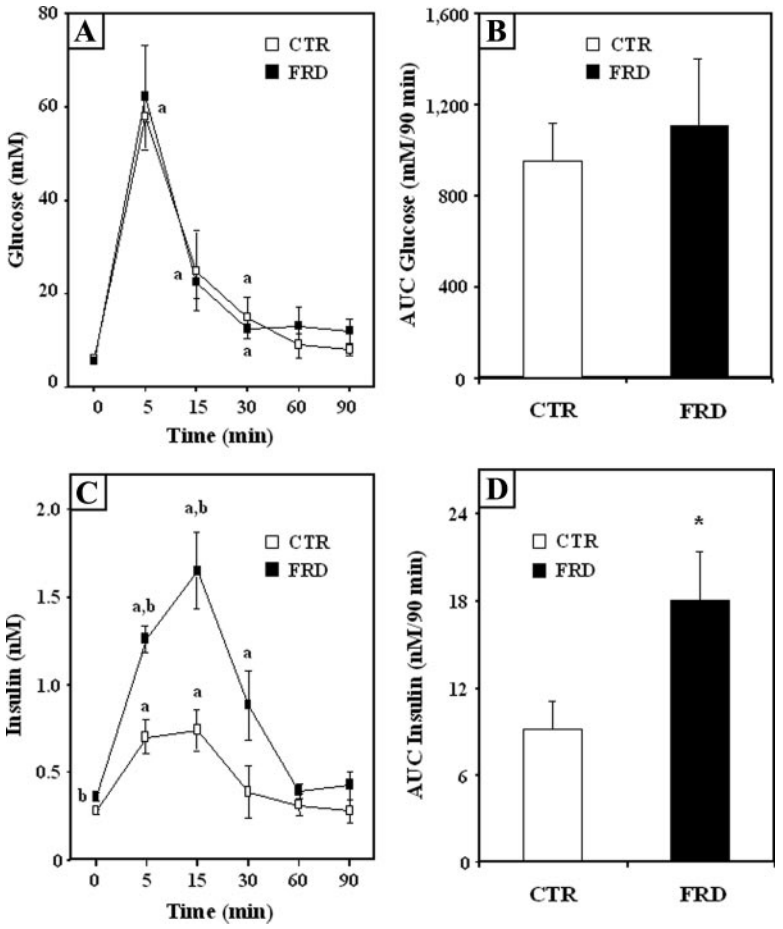

FIG. 2. Plasma glucose (A) and insulin (C) concentrations before (time zero) and several times after high glucose load in 60-d-old CTR (n = 7) and FRD (n = 8) male rats. Each rat assigned to a group derived from a different litter. The area under the curves (AUC) of glucose and insulin values throughout the iv-GTT in both groups is shown (B and D, respectively). Data are means ± SEM. [a], $P < 0.05$ *vs.* time 0 values in the same group; [b], $P < 0.05$ *vs.* CTR values at same time; *, $P < 0.05$ *vs.* CTR values.

(0.1–10 nM). Spontaneous leptin output by isolated RP adipocytes was significantly ($P < 0.05$) higher in the FRD than in the CTR group. While 0.1 nM insulin did not enhance leptin secretion in either group, 1 and 10 nM insulin significantly ($P < 0.05$) enhanced leptin release over the baseline only in cells from CTR male rats. Conversely, when testing adipocytes from FRD male rats, only the highest insulin concentration (10 nM) was able to significantly ($P < 0.05$) increase leptin secretion over the respective baseline.

Hypothalamic expression of genes modulating food intake in adult male rats

In view of the increase in both BW (21–60 d of age) and daily food intake (48–60 d of age) characterizing FRD male rats, we decided to examine the hypothalamic profile of different appetite-controlling signals. We found that the hypothalamic expression of several genes was distorted in the adult FRD male rats. Specifically, CRH, TRH, and *ob*-Rb (Table 5) mRNA expression was significantly ($P <$ 0.05) lower in FRD than in CTR male rats. Conversely, the hypothalamic mRNA expression of other appetite controlling factors, such as AgRP, CART, NPY, and POMC remained at the same level in both experimental groups (Table 5).

Effect of peripheral leptin treatment on food intake and hypothalamic p-STAT-3 in CTR and FRD rats

Finally, and because we observed enhanced leptinemia and decreased hypothalamic expression of *ob*-Rb mRNA in adult FRD male rats, two different peripheral leptin test-studies in rats from both groups were performed.

Our data indicate that ip injection of leptin (1 mg/kg BW) in experimental rats induced a differential effect on food intake: while leptin treatment significantly ($P < 0.05$ *vs.* vehicle-injected CTR rats) reduced 4-h food intake in CTR male rats, it failed to induce any significant hypophagia in FRD rats (Fig. 4, upper panel).

Data from leptin (1 mg/kg BW, ip)-dependent STAT-3 phosphorylation in the hypothalamus of fasted rats, from different experimental groups, are depicted in Fig. 4 (lower panel). In each group, STAT-3 phosphorylation levels were normalized to total-STAT-3, and value 1 was attributed to the p-STAT-3/total-STAT-3 ratio measured in vehicle-injected animals of the same experimental group (CTR and FRD). A significant increase of this ratio in leptin-treated rats was taken as an index of the hypothalamic responsiveness toward leptin. As depicted, while leptin treatment significantly ($P < 0.05$) increased STAT-3 phosphorylation in CTR rats, this effect was absent in FRD rats (Fig. 4, lower panel).

Discussion

Our study shows for the first time deleterious effects of the intake of an iso-caloric FRD by the lactating mother rat on several functions in males from the first progeny when they reached adulthood. Specifically, the adult male offspring suckled by mothers consuming FRD dur-

TABLE 4. Retroperitoneal fat pad mass, adipocyte characteristics, and leptin (LEP) and adiponectin (ADIPOQ) mRNA expression (in arbitrary units, AU) of adult CTR ($n = 4$) and FRD ($n = 5$) male rats

	CTR	FRD
Pad mass (g/rat)	2.09 ± 0.18	2.05 ± 0.24
Adipocyte diameter (μm)	37.21 ± 0.09	45.45 ± 0.25*
Adipocyte area (μm^2)	1,182.34 ± 10.64	1,807.18 ± 20.29*
Adipocyte volume ($\mu m^3 \times 10^3$)	26.97 ± 1.84	49.18 ± 3.39*
LEP mRNA (AU)	1.09 ± 0.19	3.69 ± 0.85*
ADIPOQ mRNA (AU)	1.27 ± 0.41	2.36 ± 0.62

Each rat assigned to a group derived from a different litter. Values are means ± SEM.

*, $P < 0.05$ *vs.* CTR values.

TABLE 5. Hypothalamic mRNA expression (in arbitrary units) of several genes involved in the local circuitry controlling food intake in adult CTR ($n = 4$) and FRD ($n = 6$) male rats

	CTR	FRD
CRH	1.02 ± 0.12	0.41 ± 0.16*
TRH	1.01 ± 0.11	0.44 ± 0.09*
ob-Rb	1.03 ± 0.29	0.27 ± 0.06*
AgRP	0.99 ± 0.12	1.19 ± 0.11
CART	1.00 ± 0.21	0.62 ± 0.28
NPY	1.01 ± 0.51	0.87 ± 0.23
POMC	1.02 ± 0.33	1.35 ± 0.41

Each rat assigned to a group derived from a different litter. Values are means ± SEM.

*, $P < 0.05$ *vs.* CTR values.

ing lactation displayed the following: 1) increased body weight, food intake, and leptinemia, facts partially related to disrupted hypothalamic activity; 2) impaired insulin sensitivity, and 3) distorted retroperitoneal adipose tissue function.

Several studies focused on effects of excessive carbohydrate consumption throughout the lactation period on the offspring's metabolism, but changes in diet were applied directly to pups (22, 37). Other researchers used diet manipulation on lactating mothers and, as in our design, avoided changes in the diet provided to offspring. In those studies 1) lighter male offspring was found when raised by mothers fed a protein restricted diet (38), 2) accelerated increase in offspring's BW was induced by reducing litter size (a pup-overfed model), although BW values normalized after weaning (39), and 3) offspring's BW was found normal when suckled by mothers fed with a high-fat diet (40). Now, we show that FRD-fed lactating mothers modified the offspring's phenotype by significantly enhancing male BW between ages 21 and 60 d. The early increase in BW later correlated with enhanced food intake (49–60 d of age). These data strongly support that FRD intake by lactating dams induced overweight in their male offspring.

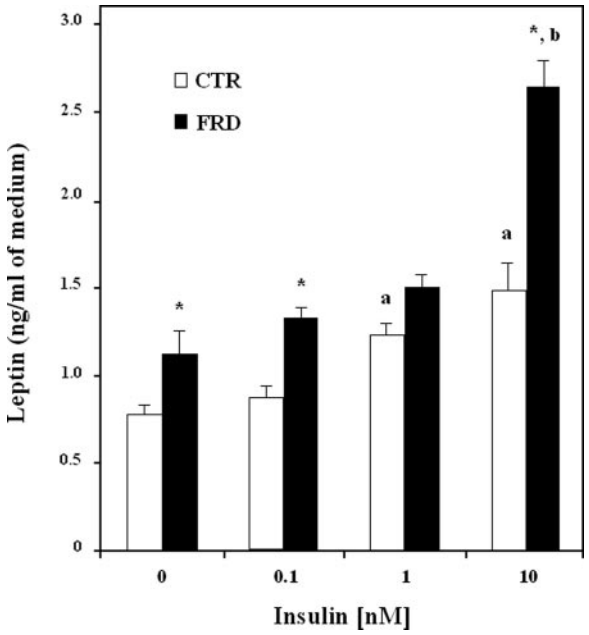

FIG. 3. Spontaneous (insulin 0 nM) and insulin (0.1–10 nM)-induced leptin release by isolated RP fat adipocytes obtained from 60-d-old CTR and FRD male rats. Data are means ± SEM (n = 3 different experiments using rats from different litters; 5 replicates per condition were run in each experiment). [a], $P < 0.05$ *vs.* insulin 0 nM values in the CTR group; [b], $P < 0.05$ *vs.* insulin 0 nM values in the in the FRD group; *, $P < 0.05$ *vs.* CTR values in similar condition.

Moreover, we found that the adult male offspring suckled by FRD lactating dams displayed enhanced insulinemia, although without changes in peripheral glucose metabolism or peripheral lipid profile. Mothers consuming excess carbohydrates during gestation and lactation displayed no modifications in plasma levels of triglycerides, free fatty acids, and cholesterol (20, 37). However, sucrose consumption during gestation and lactation, although it did not modify the lipid profile, did increase liver triglyceride content (37). Conversely, offspring nursed with carbohydrate-rich artificial milk displayed no changes in circulating levels of triglycerides (22, 41) or free fatty acids (41), despite increased lipogenesis (42). Therefore, although they were not examined in the present study, changes in the lipogenic process [*e.g.* due to enhanced FRD-induced oxidative stress (43)] cannot be ruled out in our adult FRD male rats. While other studies also revealed hyperinsulinemic offspring, this dysfunction was found after direct nutritional intervention in the offspring by either: nursing them with a high-carbohydrate milk formula (44); pup overfeeding, due to drastic litter-size re-

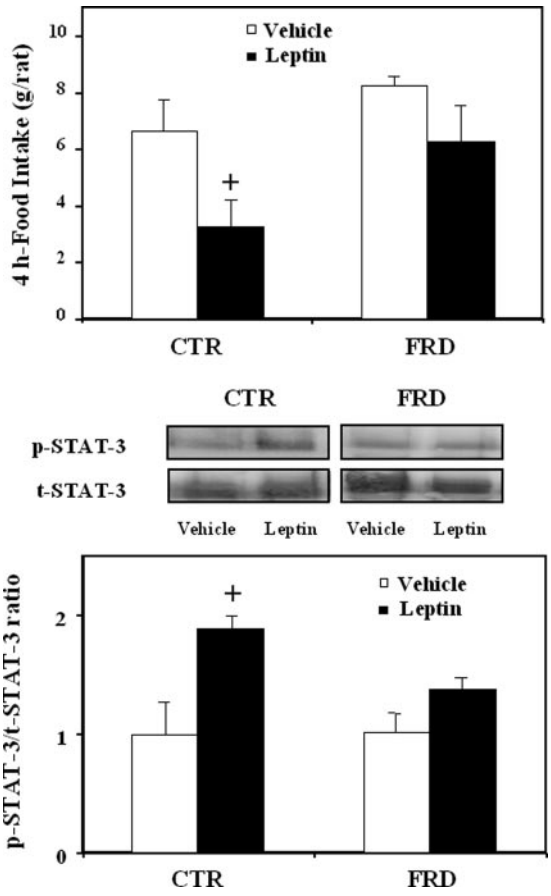

FIG. 4. Four-hour food intake by adult CTR and FRD male rats after either leptin (ip 1 mg/kg BW injection; n = 5 CTR and 6 FRD animals) or vehicle (n = 5 CTR and 6 FRD animals) treatment (*upper*). In addition, Western blot analysis of phosphorylated and total STAT-3 (p- and t-STAT-3, respectively) in hypothalamic protein extracts from fasted 60-d-old CTR and FRD male rats, 45 min after ip treatment with either vehicle (n = 5 CTR and 6 FRD animals) alone or containing leptin (1 mg/kg BW; n = 5 CTR and 6 FRD animals) is shown (*lower*). Each rat assigned to a group treatment derived from a different litter. Values are means ± SEM. $^{+}$, $P < 0.05$ *vs.* vehicle values.

duction (45); providing pups with a diet containing 62% fructose for 2 wk (46); or moderate FRD (15% fructose) intake by pups for several months (47). Another study (21) did report a clear increment in the offspring's peripheral insulin levels when mothers were fed a carbohydrate rich diet, although diet manipulation was applied during gestation and lactation. The origin of the increment in peripheral insulin levels could be due to changes in either β cell function or peripheral insulin sensitivity, or in both. In this sense, some authors found a profound impact on the offspring's pancreatic function when pups consumed a carbohydrate rich diet during the preweaning period (48, 49). Our data clearly indicate that the enhanced insulinemia developed by male offspring could serve as an adaptive role for normal management of peripheral carbohydrate metabolism, such as after the high glucose load test. We also observed that decreased adiponectin and increased leptin peripheral levels characterized our adult FRD male rats, whereas other adipokines remained the same. Although obesity has been considered a state of chronic inflammation in which several peripheral adipocytokines contribute to impair insulin sensitivity (50), it could be speculated that in our model TNFα appears to not be involved in the changes in peripheral insulin sensitivity. It is accepted that adiponectin is a well-known endogenous signal enhancing insulin sensitivity (51) and leptin is able to affect the insulin signaling mechanism (52, 53). Thus the unbalanced adipokine status we found (enhanced leptin and decreased adiponectin plasma concentrations) could contribute to the impaired insulin sensitivity characterizing our FRD male rats. As presently demonstrated, retroperitoneal adipose tissue dysfunction has occurred in animals bearing enlarged adipocytes with enhanced LEP gene expression, both facts being clear indicators of enhanced adipose tissue LEP production (54). Moreover, these characteristics concord with our functional *in vitro* observations. In fact, retroperitoneal adipocytes from FRD rats spontaneously released more leptin than CTR rat-derived adipocytes and also displayed impaired response to insulin stimulation. However, these changes occurred without any modification in retroperitoneal fat mass, thus suggesting that a modified adipogenic process could take place in adult male offspring nursed by FRD lactating dams, a point deserving further research. It should be mentioned that FRD intake by lactating mothers could possibly have an impact on the male offspring by modifying the activity of other adipokines able to modify adipocyte function (55). Recently, it was found (56) that circulating levels of zinc-α2-glycoprotein, derived from human adipocytes, directly correlate with those of insulin, suggesting that it may be an important marker of insulin resistance and obesity.

Maternal consumption of a FRD while lactating resulted in increased food intake by male offspring, observed at age 49 d and older. Recently, consumption of a carbohydrate-rich diet by pups between postnatal d 4 and 24 was reported to increase orexigenic and decrease anorexigenic signals at the hypothalamic level, resulting in distorted insulin sensitivity (44). In our experimental design, we also found changes in hypothalamic gene expression of several factors involved in control of food intake. Although the male offspring nursed by FRD lactating mothers developed a modest hyperphagia in adulthood, this seems to occur depending on a weak satiety signaling. Decreased hypothalamic *ob*-Rb gene expression and STAT-3 phosphorylation in response to peripheral leptin treatment are characteristics of our FRD hyperleptinemic male rats. This impairment in the leptin signaling system is a clear indicator of the development of hypothalamic leptin-resistance (57) in these rats. Interestingly, we found

that down-regulated hypothalamic *ob*-Rb gene expression coexists with impaired CRH- and TRH-ergic (mainly paraventricular nucleus-derived anorexigenic signals) (45) functions in our FRD male rats; however, disruption of other signals of the same origin and with similar activity in FRD male rats should not be discarded (58). Conversely, other hypothalamic factors controlling food intake (mainly from arcuate nucleus origin) such as AgRP, CART, NPY, and POMC remained unchanged in our FRD male rats. Only a few experimental models have been developed to study the consequences of nutritional manipulation in mothers during lactation only. Earlier reports indicate that as a result of drastic litter size reduction, overfed pups developed hyperinsulinemia, hyperleptinemia, and obesity at adult age (54) without changes in hypothalamic NPY (59). Others have shown, however, that isolated hypothalamic neurons from overfed pups displayed abnormal responses to several stimuli, underlining the relevance of the impact of overfeeding (60, 61) during lactation and the quality of milk (62) on pups' neuron development.

Our study leads to alert the population on the deleterious effect of high-fructose diet intake by lactating mothers on the offspring's health. Adequately nourished mothers during lactation could reduce the incidence of overweight/obesity in their adult male progeny.

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References

1. **Bray GA, Bellanger T** 2006 Epidemiology, trends, and morbidities of obesity and the metabolic syndrome. Endocrine 29:109–117
2. **Reaven G, Abbasi F, McLaughlin T** 2004 Obesity, insulin resistance, and cardiovascular disease. Recent Prog Horm Res 59:207–223
3. **Gluckman PD, Hanson MA, Cooper C, Thornburg KL** 2008 Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 359:61–73
4. **Rocchini AP** 2002 Childhood obesity and a diabetes epidemic. N Engl J Med 346:854–855
5. **Breier BH, Vickers MH, Ikenasio BA, Chan KY, Wong WP** 2001 Fetal programming of appetite and obesity. Mol Cell Endocrinol 185:73–79
6. **Barsh GS, Farooqi IS, O'Rahilly S** 2000 Genetics of body-weight regulation. Nature 404:644–651
7. **Farooqi S, O'Rahilly S** 2006 Genetics of obesity in humans. Endocr Rev 27:710–718
8. **Hamilton JK, Odrobina E, Yin J, Hanley AJ, Zinman B, Retnakaran R** 2010 Maternal insulin sensitivity during pregnancy predicts infant weight gain and adiposity at 1 year of age. Obesity (Silver Spring) 18:340–346
9. **Patel MS, Srinivasan M** 2010 Metabolic programming due to alterations in nutrition in the immediate postnatal period. J Nutr 140: 658–661
10. **Taylor PD, Poston L** 2007 Developmental programming of obesity in mammals. Exp Physiol 92:287–298
11. **McMillen IC, Robinson JS** 2005 Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev 85:571–633
12. **Plagemann A** 2006 Perinatal nutrition and hormone-dependent programming of food intake. Horm Res 65(Suppl 3):83–89
13. **Grove KL, Grayson BE, Glavas MM, Xiao XQ, Smith MS** 2005 Development of metabolic systems. Physiol Behav 86:646–660
14. **Plagemann A, Harder T, Melchior K, Rake A, Rohde W, Dörner G** 1999 Elevation of hypothalamic neuropeptide Y-neurons in adult offspring of diabetic mother rats. Neuroreport 10:3211–3216
15. **Plagemann A, Harder T, Rake A, Melchior K, Rohde W, Dörner G** 2000 Hypothalamic nuclei are malformed in weanling offspring of low protein malnourished rat dams. J Nutr 130:2582–2589
16. **Davidowa H, Plagemann A** 2000 Decreased inhibition by leptin of hypothalamic arcuate neurons in neonatally overfed young rats. Neuroreport 11:2795–2798
17. **Schwartz MW, Baskin DG, Kaiyala KJ, Woods SC** 1999 Model for the regulation of energy balance and adiposity by the central nervous system. Am J Clin Nutr 69:584–596
18. **Elliott SS, Keim NL, Stern JS, Teff K, Havel PJ** 2002 Fructose, weight gain, and the insulin resistance syndrome. Am J Clin Nutr 76:911–922
19. **Bray GA, Nielsen SJ, Popkin BM** 2004 Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. Am J Clin Nutr 79:537–543
20. **Rawana S, Clark K, Zhong S, Buison A, Chackunkal S, Jen KL** 1993 Low dose fructose ingestion during gestation and lactation affects carbohydrate metabolism in rat dams and their offspring. J Nutr 123:2158–2165
21. **Srinivasan M, Dodds C, Ghanim H, Gao T, Ross PJ, Browne RW, Dandona P, Patel MS** 2008 Maternal obesity and fetal programming: effects of a high-carbohydrate nutritional modification in the immediate postnatal life of female rats. Am J Physiol Endocrinol Metab 295:E895–E903
22. **Huynh M, Luiken JJ, Coumans W, Bell RC** 2008 Dietary fructose during the suckling period increases body weight and fatty acid uptake into skeletal muscle in adult rats. Obesity 16:1755–1762
23. **Kreier F, Fliers E, Voshol PJ, Van Eden CG, Havekes LM, Kalsbeek A, Van Heijningen CL, Sluiter AA, Mettenleiter TC, Romijn JA, Sauerwein HP, Buijs RM** 2002 Selective parasympathetic innervation of subcutaneous and intra-abdominal fat–functional implications. J Clin Invest 110:1243–1250
24. **Gregoire FM, Smas CM, Sul HS** 1998 Understanding adipocyte differentiation. Physiol Rev 78:783–809
25. **Münzberg H, Myers Jr MG** 2005 Molecular and anatomical determinants of central leptin resistance. Nat Neurosci 8:566–570
26. **Lustig RH** 2003 Autonomic dysfunction of the β-cell and the pathogenesis of obesity. Rev Endocr Metab Disord 4:23–32
27. **Spinedi E, Giacomini M, Jacquier MC, Gaillard RC** 1991 Changes in the hypothalamo-corticotrope axis after bilateral adrenalectomy:

evidence for a median eminence site of glucocorticoid action. Neuroendocrinology 53:160–170
28. **Perelló M, Castrogiovanni D, Moreno G, Gaillard RC, Spinedi E** 2003 Neonatal hypothalamic androgenization in the female rat induces changes in peripheral insulin sensitivity and adiposity function at adulthood. Neuro Endocrinol Lett 24:241–248
29. **Niimi M, Sato M, Yokote R, Tada S, Takahara J** 1999 Effects of central and peripheral injection of leptin on food intake and on brain Fos expression in the Otsuka Long-Evans Tokushima Fatty rat with hyperleptinaemia. J Neuroendocrinol 11:605–611
30. **Férézou-Viala J, Roy AF, Sérougne C, Gripois D, Parquet M, Bailleux V, Gertler A, Delplanque B, Djiane J, Riottot M, Taouis M** 2007 Long-term consequences of maternal high-fat feeding on hypothalamic leptin sensitivity and diet-induced obesity in the offspring. Am J Physiol Regul Integr Comp Physiol 293:R1056–R1062
31. **Moreno G, Perelló M, Camihort G, Luna G, Console G, Gaillard RC, Spinedi E** 2006 Impact of transient correction of increased adrenocortical activity in hypothalamo-damaged, hyperadipose female rats. Int J Obes (Lond) 30:73–82
32. **Giovambattista A, Piermaria J, Suescun MO, Calandra RS, Gaillard RC, Spinedi E** 2006 Direct effect of ghrelin on leptin production by cultured rat white adipocytes. Obesity (Silver Spring) 14:19–27
33. **Giovambattista A, Gaillard RC, Spinedi E** 2008 Ghrelin gene-related peptides modulate rat white adiposity. Vitam Horm 77:171–205
34. **Perello M, Castrogiovanni D, Giovambattista A, Gaillard RC, Spinedi E** 2007 Impairment in insulin sensitivity after early androgenization in the post-pubertal female rat. Life Sci 80:1792–1798
35. **Alzamendi A, Castrogiovanni D, Ortega HH, Gaillard RC, Giovambattista A, Spinedi E** 2010 Parametrial adipose tissue and metabolic dysfunctions induced by fructose-rich diet in normal and neonatal-androgenized adult female rats. Obesity (Silver Spring) 18:441–448
36. **Zar JH** 1974 Biostatistical Analysis. Englewood Cliffs, NJ: Prentice-Hall
37. **Sedová L, Seda O, Kazdová L, Chylíková B, Hamet P, Tremblay J, Kren V, Krenová D** 2007 Sucrose feeding during pregnancy and lactation elicits distinct metabolic response in offspring of an inbred genetic model of metabolic syndrome. Am J Physiol Endocrinol Metab 292:E1318–E1324
38. **Zambrano E, Bautista CJ, Deás M, Martínez-Samayoa PM, González-Zamorano M, Ledesma H, Morales J, Larrea F, Nathanielsz PW** 2006 A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. J Physiol 571:221–230
39. **Chen H, Simar D, Morris MJ** 2009 Hypothalamic neuroendocrine circuitry is programmed by maternal obesity: interaction with postnatal nutritional environment. PLoS One 4:e6259
40. **Férézou-Viala J, Roy AF, Sérougne C, Gripois D, Parquet M, Bailleux V, Gertler A, Delplanque B, Djiane J, Riottot M, Taouis M** 2007 Long-term consequences of maternal high-fat feeding on hypothalamic leptin sensitivity and diet-induced obesity in the offspring. Am J Physiol Regul Integr Comp Physiol 293:R1056–R1062
41. **Aalinkeel R, Srinivasan M, Song F, Patel MS** 2001 Programming into adulthood of islet adaptations induced by early nutritional intervention in the rat. Am J Physiol Endocrinol Metab 281:E640–E648
42. **Hiremagalur BK, Vadlamudi S, Johanning GL, Patel MS** 1993 Long-term effects of feeding high carbohydrate diet in pre-weaning period by gastrostomy: a new rat model for obesity. Int J Obes Relat Metab Disord 17:495–502
43. **Alzamendi A, Giovambattista A, Raschia A, Madrid V, Gaillard RC, Rebolledo O, Gagliardino JJ, Spinedi E** 2009 Fructose-rich diet-induced abdominal adipose tissue endocrine dysfunction in normal male rats. Endocrine 35:227–232
44. **Srinivasan M, Mitrani P, Sadhanandan G, Dodds C, Shbeir-ElDika S, Thamotharan S, Ghanim H, Dandona P, Devaskar SU, Patel MS** 2008 A high-carbohydrate diet in the immediate postnatal life of rats induces adaptations predisposing to adult-onset obesity. J Endocrinol 197:565–574
45. **Boullu-Ciocca S, Dutour A, Guillaume V, Achard V, Oliver C, Grino M** 2005 Postnatal diet-induced obesity in rats upregulates systemic and adipose tissue glucocorticoid metabolism during development and in adulthood: its relationship with the metabolic syndrome. Diabetes 54:197–203
46. **Fields M, Lewis CG, Lure MD** 1996 Responses of insulin to oral glucose and fructose loads in marginally copper-deficient rats fed starch or fructose. Nutrition 12:524–528
47. **Blakely SR, Hallfrisch J, Reiser S, Prather ES** 1981 Long-term effects of moderate fructose feeding on glucose tolerance parameters in rats. J Nutr 111:307–314
48. **Vadlamudi S, Hiremagalur BK, Tao L, Kalhan SC, Kalaria RN, Kaung HL, Patel MS** 1993 Long-term effects on pancreatic function of feeding a HC formula to rats during the preweaning period. Am J Physiol 265:E565–E571
49. **Aalinkeel R, Srinivasan M, Kalhan SC, Laychock SG, Patel MS** 1999 A dietary intervention (high carbohydrate) during the neonatal period causes islet dysfunction in rats. Am J Physiol 277:E1061–E1069
50. **Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J, Feve B** 2006 Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw 17:4–12
51. **Berg AH, Combs TP, Du X, Brownlee M, Scherer PE** 2001 The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nat Med 7:947–953
52. **Walder K, Filippis A, Clark S, Zimmet P, Collier GR** 1997 Leptin inhibits insulin binding in isolated rat adipocytes. J Endocrinol 155:R5–R7
53. **Krempler F, Hell E, Winkler C, Breban D, Patsch W** 1998 Plasma leptin levels: interaction of obesity with a common variant of insulin receptor substrate-1. Arterioscler Thromb Vasc Biol 18:1686–1690
54. **Couillard C, Mauriège P, Imbeault P, Prud'homme D, Nadeau A, Tremblay A, Bouchard C, Després JP** 2000 Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia. Int J Obes Relat Metab Disord 24:782–788
55. **Hirai K, Hussey HJ, Barber MD, Price SA, Tisdale MJ** 1998 Biological evaluation of a lipid-mobilizing factor isolated from the urine of cancer patients. Cancer Res 58:2359–2365
56. **Mracek T, Ding Q, Tzanavari T, Kos K, Pinkney J, Wilding J, Trayhurn P, Bing C** 2010 The adipokine zinc-α2-glycoprotein is downregulated with fat mass expansion in obesity. Clin Endocrinol (Oxf) 72:334–341
57. **Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS** 1999 Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. Endocr Rev 20:68–100
58. **Oh-I S, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, Eguchi H, Yamamoto M, Imaki T, Hashimoto K, Tsuchiya T, Monden T, Horiguchi K, Yamada M, Mori M** 2006 Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 443:709–712
59. **Plagemann A, Harder T, Rake A, Waas T, Melchior K, Ziska T, Rohde W, Dörner G** 1999 Observations on the orexigenic hypothalamic neuropeptide Y-system in neonatally overfed weanling rats. J Neuroendocrinol 11:541–546
60. **Davidowa H, Li Y, Plagemann A** 2003 Altered responses to orexigenic (AGRP, MCH) and anorexigenic (α-MSH, CART) neuropeptides of paraventricular hypothalamic neurons in early postnatally overfed rats. Eur J Neurosci 18:613–621
61. **Davidowa H, Plagemann A** 2004 Hypothalamic neurons of postnatally overfed, overweight rats respond differentially to corticotropin-releasing hormones. Neurosci Lett 371:64–68
62. **Bautista CJ, Boeck L, Larrea F, Nathanielsz PW, Zambrano E** 2008 Effects of a maternal low protein isocaloric diet on milk leptin and progeny serum leptin concentration and appetitive behavior in the first 21 d of neonatal life in the rat. Pediatr Res 63:358–363