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RESEARCH ARTICLES

Postnatal early overfeeding induces hypothalamic higher SOCS3 expression and lower STAT3 activity in adult rats

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Abstract

Postnatal early overnutrition (EO) is a risk factor for future obesity and metabolic disorders. Rats raised in small litters (SLs) develop overweight, hyperphagia, hyperleptinemia, hyperinsulinemia and hypertension when adults. As obesity is related to hyperleptinemia, leptin resistance and metabolic syndrome, we aimed to investigate body composition, plasma hormone levels, glucose tolerance and the leptin signaling pathway in hypothalamus from early overfed animals at weaning and adulthood. To induce postnatal EO, we reduced litter size to three pups/litter (SL), and the groups with normal litter size (10 pups/litter) were used as control. Rats had free access to standard diet and water postweaning. Body weight and food intake were monitored daily, and offspring were killed at 21 (weaning) and 180 days old (adulthood). Postnatal EO group had higher body weight and total and visceral fat mass at both periods. Lean mass and serum high-density lipoprotein cholesterol (HDL-C) were higher at 21 days and lower at 180 days. Small litter rats presented higher levels of globulins at both periods, while albumin levels were higher at weaning and lower at adulthood. There was higher leptin, insulin and glucose serum concentrations at 21 days old, while no glucose intolerance was observed in adulthood. Leptin signaling pathway was unaffected at weaning. However, postnatal EO induced lower JAK2 and p-STAT3, and higher SOCS3 expression in adult animals, indicating central leptin resistance in adulthood. In conclusion, postnatal EO induces obesity, higher total and visceral fat mass, lower HDL-C and central leptin resistance in adult life.

Keywords: Overnutrition; Programming; Adiposity; Leptin; Rat

1. Introduction

The prevalence of childhood obesity is increasing significantly worldwide and represents an important problem of public health [1,2], since it represents a risk factor for adult chronic diseases such as type 2 diabetes, cardiovascular diseases and metabolic syndrome [3].

Epidemiological and experimental data have shown that nutritional and hormonal status in early life permanently changes the structure and function of body tissues and systems. This association has been named programming, which is defined as basic biological phenomena that putatively underlie relationships among nutritional experiences of early life and future diseases in adulthood [3–5]. Postnatal early overnutrition (EO) can be induced by litter size reduction, which is an appropriate experimental model to study short- and long-term consequences of childhood obesity. Animals raised in small litter (SL) develop hyperphagia, obesity, hypertension and hyperinsulinemia in later life [6–11].

Adipose tissue has been shown to develop a crucial role in metabolic disorders associated with obesity [12]. Beyond secreting free fatty acids, adipose tissue produces and releases several proteins and hormones, as leptin and adiponectin, with autocrine, paracrine and endocrine functions [13–15]. Adiponectin production is inversely proportional to whole body adipose mass, and experimental studies suggest that it modulates insulin action, improving insulin sensitivity in peripheral tissues [16].

Leptin, another cytokine produced especially by adipose tissue, is known to reduce food intake and increase energetic expenditure [17,18]. Leptin acts through leptin receptors (OB-R), which belongs to the interleukin 6 (IL-6) family of class 1 cytokine receptors. Five alternatively spliced isoforms of OB-R (a, b, c, d, e) with different lengths of C-termini have been identified in mice. Long form (OB-Rb) and short form (Ob-Ra) are the most studied isoforms, and OB-Rb is fully capable of activating intracellular signaling [19]. Leptin binding to Ob-Rb initiates tyrosine phosphorylation by JAK2 (Janus tyrosine

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kinase 2). Phosphorylated JAK2 recruits STAT3 (signal transducer and activator of transcription 3) proteins, which are activated through phosphorylation by JAK2. The activated STAT3 dimerizes and translocates to the nucleus stimulating gene transcription. The JAK2/STAT3 pathway stimulates SOCS3 (suppressor of cytokine signaling 3) transcription, a leptin-inducible inhibitor of leptin signaling pathway [20].

Leptin production is directly proportional to whole body adipose mass, and higher leptin levels have been shown in obese people [21]. Leptin resistance in obesity has been associated with changes in JAK2/STAT3 pathway in hypothalamus, with a decrease in OB-R, JAK2 and STAT3 expression and/or an increase in SOCS3 content [22,23]. Although the presence of hyperleptinemia with leptin resistance and obesity has long been recognized, a causal role of elevated leptin in these biological states remains unclear.

As childhood obesity is a risk factor for metabolic disorders, in the present study, we aimed to investigate the short- and long-term effects of postnatal EO on body composition, food intake, lipid profile, total plasma proteins, insulin, leptin and adiponectin levels, as well as the glucose tolerance in adulthood. Also, we analyzed the protein expression of the leptin signaling pathway in hypothalamus from young and adult rats raised in small and normal litters (NLs).

2. Materials and methods

2.1. Experimental model

Wistar rats were housed in controlled temperature room at 23 to 25°C with 12-h light/dark cycle (7:00 am to 7:00 pm). Adult virgin female rats were caged with male rat at a proportion of 3:1. During pregnancy and lactation, mothers were housed in individual cages and had water ad libitum and standard pellet diet (commercial control diets for rodents, purine, BR). To induce postnatal EO, 3 days from birth, we adjusted litter size to three male pups per litter (SL) [10,11]. Litter containing 10 pups per mother served as control (NL). Rats had free access to standard diet and water postweaning (three animals/cage). Body weight gain was measured throughout life. Food intake was measured by determination of 24-h consumption of standard pellets from 30 to 180 days old. Normal litter and SL fasted rats were killed when they were 21 or 180 days old, and we collected blood, hypothalamus, visceral fat mass and carcass. The animals' number analyzed in our study was 12 per group per period studied from 16 different litters (SL_{21}=4, NL_{21}=4, SL_{180}=4, NL_{180}=4). At weaning, we randomly assigned three animals of each group per cage to be sacrificed at 21 and 180 days. In the cages containing SL group, all rats were killed on both periods. However, in the cages containing NL group that had a higher number of animals, we randomly assigned three rats to be killed in the two periods studied, and four rats were discarded. Our experimental design was approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEA/184/2007), which based their analysis on the principles described in the Guide for the Care and Use of Laboratory Animals [24].

2.2. Body composition

The visceral fat mass (mesenteric, epididymal and retroperitoneal white adipose tissue) was excised and immediately weighed for evaluation of central adiposity [25].

Fat and protein mass were determined by carcass analysis as reported previously [26]. After sacrifice, offspring were eviscerated and the carcasses were weighed, autoclaved for 1 h and homogenized in distilled water (1:1 w/v). Samples of the homogenate were stored at 4°C for analysis.

Three grams of homogenate were used to determine fat mass gravimetrically. Samples were hydrolyzed in a shaking water bath at 70°C for 2 h with 30% KOH and ethanol. Total fatty acids and free cholesterol were removed by three successive washes with petroleum ether. After drying overnight in a vacuum, all tubes were weighed, and data were expressed as gram fat per 100-g carcass.

Protein mass was determined in 1 g of homogenate. Tubes were centrifuged at 2000×*g* for 10 min. Total protein concentrations were determined by the method of Lowry et al. [27]. Results were expressed as gram protein per 100 g carcass.

Subcutaneous fat mass was estimated from difference between total and visceral fat mass.

2.3. Plasma hormones measurement by radioimmunoassay

Blood was obtained by cardiac puncture and was centrifuged (1000×*g*, 4°C, 30 min), and plasma was stored at −20°C until assayed. All measurements were performed in a unique assay. Plasma leptin was determined by radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO, USA). The sensitivity limit and intraassay variation were 0.5 ng/ml and 6.9%, respectively. Plasma insulin was determined by RIA kit (ICN Pharmaceuticals, Orangeburg, NY, USA) with an assay sensitivity of 0.1 ng/ml and an intraassay variation of 4.1%. Total adiponectin was measured by specific RIA kit (Linco Research) with an assay sensitivity of 0.5 ng/ml and an intraassay variation of 7.1%.

2.4. Biochemical parameters

Plasma lipids [total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides] were analyzed using enzymatic colorimetric assays (Bioclin, Brazil) [28]. Low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) were calculated according to the equation of Friedwald [29]:

1) VLDL−C = tryglycerides / 5
2) LDL−C = (TC−HDL−C−tryglycerides) / 5

Castelli index I and II that correlate with atherogenicity were obtained using the following formulas [30]:

1) Castelli index I = TC / HDL−C
2) Castelli index II = LDL−c / HDL−C

Biuret reaction [31] was performed for total plasma protein measurement, and absorbance of products was determined on spectrophotometer (automated A15 BioSystems analyzer, Spain). Plasma albumin was determined by bromocresol green method [32], and globulin fraction was measured by difference between total protein and albumin. Values were expressed as milligrams per deciliter.

2.5. Intraperitoneal glucose tolerance test

Rats were fasted overnight and then injected intraperitoneally with 2 g/kg D-glucose (35% stock solution in saline). Blood samples were obtained by tail venesection before and 30, 60 and 120 min after glucose administration. Glycemia was measured by glucosimeter (ACCU-CHEK Advantage; Roche Diagnostics, Mannheim, Germany).

2.6. Western blotting analysis

To obtain the whole hypothalamus cell extracts, tissues were homogenized on ice-cold lyses buffer (50 mM HEPES, 1 mM $MgCl_2$, 10 mM EDTA, Triton X-100 1%, pH 6.4) containing the following protease inhibitors: 10 μg/μl aprotinin, 10 μg/μl leupeptin, 2 μg/μl pepstatin and 1 mM phenylmethysulfonyl fluoride (PMSF; Sigma-Aldrich, MO, USA). The Ob-R, JAK2, STAT3, p-STAT3, SOCS3, proopiomelanocortin (POMC) and neuropeptide Y (NPY) content was analyzed by Western blotting as described below, using actin as internal control.

Total protein content in the hypothalamus homogenate was determined [33], and cell lysates were denatured in sample buffer [50 mM Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue] and heated at 95°C for 5 min. Samples (50-μg total protein) were carried to 8%, 10% or 12% SDS polyacrylamide gel electrophoresis, according to the molecular weight of each protein, and transferred to polyvinylidene filters (Hybond-P; Amersham Pharmacia Biotech, NJ, USA). Rainbow markers (Amersham Biosciences, Sweden) were run in parallel to estimate molecular weights. Membranes were blocked with 5% nonfat milk in the Tween-TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20). The primary antibodies (Santa Cruz Biotechnology, CA, USA) used were anti-OB-R (1:500), anti-JAK2 (1:500), anti-STAT3 (1:500), anti-p-STAT3 (1:500), anti-SOCS3 (1:500), anti-POMC (1:500), anti-NPY (1:500) and antiactin (1:1000). Polyvinylidene filters were washed three times with Tween-TBS (0.1%), followed by 1-h incubation with appropriate secondary antibody conjugated to biotin (Santa Cruz Biotechnology). Then, filters were incubated with streptavidin-conjugated horseradish peroxidase (Caltag Laboratories, Burlingame, CA, USA). All western blots were allowed to react with horseradish peroxidase substrate (ECL-plus, Amersham Pharmacia Biotech) and then exposed to X-ray film for 10 s to 30 min. In most cases, the membranes were stripped (Restore Western Blot Stripping Buffer; Pierce, IL, USA) at 37°C for 15 min, washed with Tween-TBS (0.1%) three times and reprobed with specific primary antibody, following all the steps above. Images were scanned and the bands were quantified by densitometry, using Image J 1.34s software (Wayne Rasband National Institute of Health, MA, USA).

2.7. Statistical analysis

Data are reported as mean±S.E.M. GraphPad Prism 4 program was used for statistical analysis and graphics. Two-way ANOVA and Bonferroni posttest were used to analyze body weight and food intake changes. The other experimental observations were analyzed by unpaired Student's *t* test, with significance level set at $P<.05$.

3. Results

3.1. Postnatal EO effects in body weight, nasoanal length and food intake during development

During lactation, overfed offspring displayed a significant higher body mass from day 7 (+33%, $P<.05$; Fig. 1A) compared to controls, reaching the highest difference at 21 days old (+56%, $P<.05$; Fig. 1A). Small litter rats were still overweight at 180 days old (+18%, $P<.05$; Fig. 1B). Overfed animals presented higher food consumption during all development, reaching +9% at 180 days old ($P<.05$; Fig. 2B). Nasoanal length was higher in both young and adult SL animals (+27% and +4%, respectively, $P<.05$; Fig. 2A).

3.2. Body composition

Small litter offspring showed higher visceral (+176%, $P<.05$; Fig. 3A) and total body fat (+237%, $P<.05$; Fig. 3A) when they were 21 days old as well as 180 days old (visceral, +52%, and total, +38%, $P<.05$; Fig. 3B). Subcutaneous fat mass was higher only when they were 21 days old (+238%, $P<.05$; Fig. 3A). Total body protein content that represents the lean mass was higher in young SL rats, and the opposite profile was observed in adulthood (+41% and −14%, respectively, $P<.05$; Fig. 3C).

3.3. Biochemical analysis

Small litter group presented higher fasting glycemia (+25%, $P<.05$) at 21 days; however, no difference was detected at 180 days (Table 1).

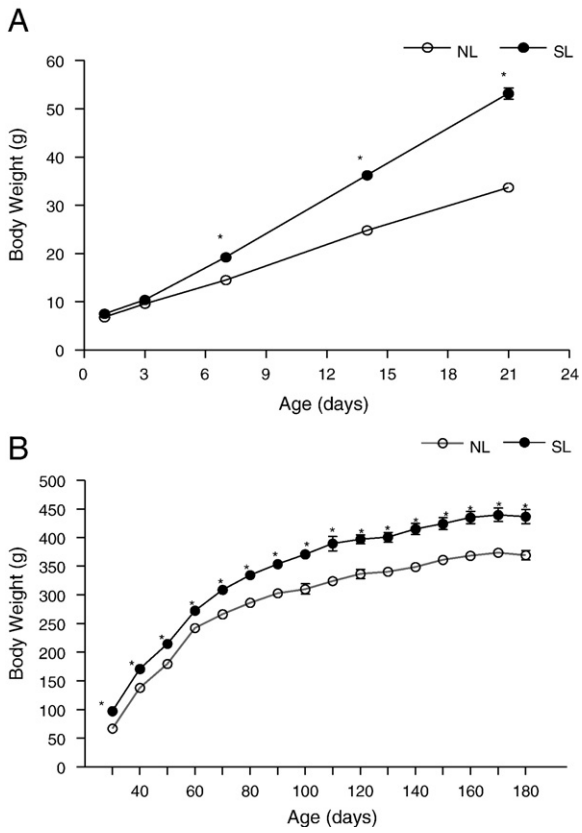

Fig. 1. Postnatal EO induces early-onset obesity and overweight in adulthood. Body weight was evaluated in NL (○; $n=12$) and SL (●; $n=12$) groups during lactation (A) and after weaning (B). Results are expressed as mean±S.E.M. *$P<.05$.

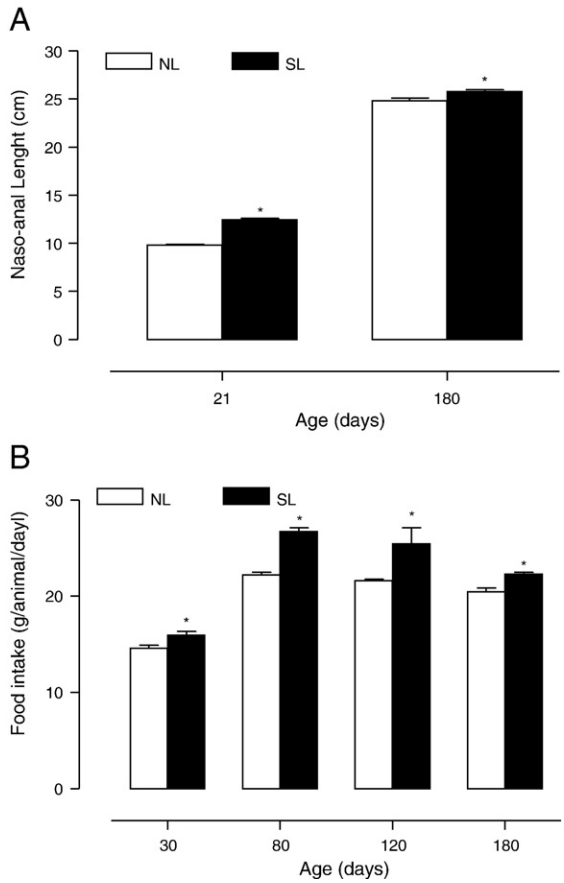

Fig. 2. Postnatal EO increases growth rate and induces persistent hyperphagia. Nasoanal length (A) and food intake (B) were evaluated in NL (white bar, $n=12$) and SL (black bar, $n=12$) groups during development. Results are expressed as mean±S.E.M. *$P<.05$.

Plasma TC and HDL-C were higher in young SL rats (+37% and +37%, respectively, $P<.05$), as depicted in Table 1. Adult SL rats showed only lower HDL-C (−12%, $P<.05$). No difference was observed in triglycerides, VLDL-C and LDL-C plasma concentrations in both periods studied. Type II Castelli index was lower at 21 days but normal at adulthood, and type I Castelli index was unaltered at both periods.

Small litter rats presented higher levels of globulins at both ages (weaning, +22%, and adult, +8%, $P<.05$; Table 1), while albumin levels were higher at weaning and lower at adulthood (+47% and −12%, respectively, $P<.05$; Table 1). Consequently, plasma total proteins were higher just at weaning (+27%, $P<.05$; Table 1).

3.4. Plasma insulin, leptin and adiponectin concentrations

Animals raised in SL presented higher plasma insulin and leptin concentrations when they were 21 days old (+88% and +56%, respectively, $P<.05$; Fig. 4A and B); however, no difference was observed in adult rats (Fig. 4A and B). Despite increased visceral fat mass at both periods, no change was observed in plasma adiponectin levels in SL groups compared to NL group (Fig. 4C).

3.5. Glucose tolerance test

Glucose tolerance test was evaluated when the animals were 180 days old, after intraperitoneal glucose load in overfed and control groups during different periods. After 30 min from glucose injection, both groups exhibited the same increase in glucose levels. However,

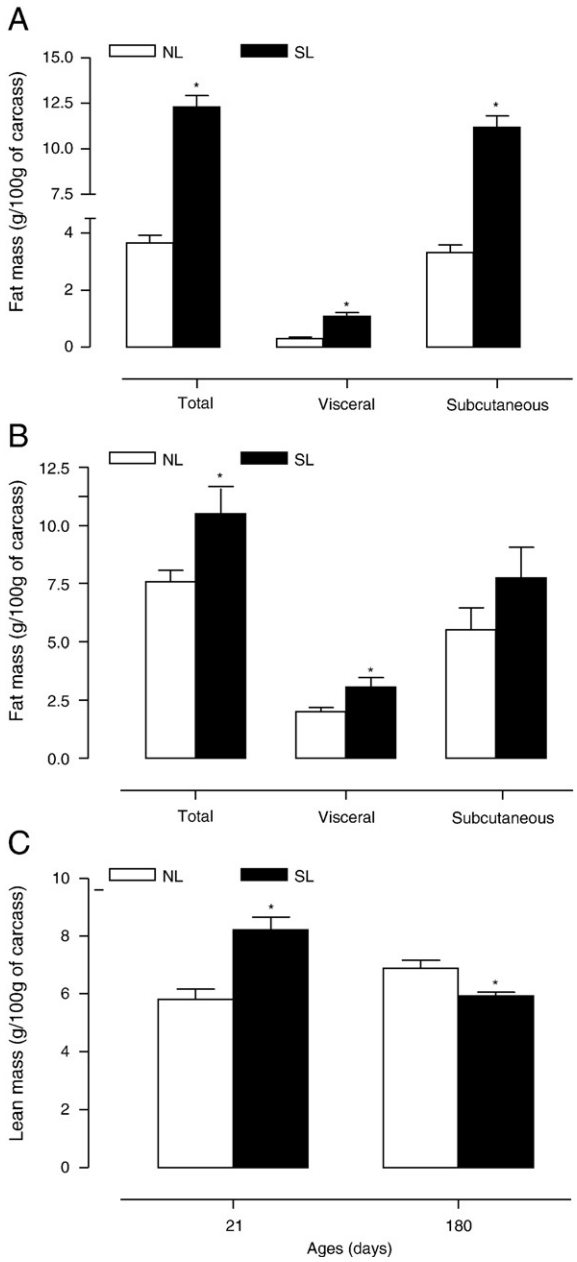

Fig. 3. Effect of postnatal EO in body composition of young and adult NL and SL rats. Body composition was determined by direct carcass analysis. Total, visceral and subcutaneous fat mass in young (A) and adult (B) NL and SL groups. (C) Lean mass from NL and SL rats at 21 and 180 days old. White bars represent control group (NL, $n=7$) and black bars represent overfed group (SL, $n=7$). Results are expressed as mean± S.E.M. *$P<.05$.

60 and 120 min after, SL rats presented a slightly higher glucose levels compared to NL rats (+10% and +7%, respectively, $P<.05$; Fig. 5), which could suggest lower insulin sensitivity. However, there was no significant difference of the area under the curve (AUC), which weakens this hypothesis.

3.6. Postnatal EO effects in hypothalamic leptin signaling pathway

At weaning, no change was detected in the expression and activity of the leptin signaling proteins in hypothalamus from animals raised in SL compared to controls (Figs. 6 and 8A). Despite no change in Ob-R and STAT3 content (Fig. 7A and C), SL group showed lower JAK2 (−59%, $P<.05$; Fig. 7B) and higher SOCS3 (+32%, $P<.05$; Fig. 7D), which resulted in lower STAT3 phosphorylation at 180 days old (−53%, $P<.05$; Fig. 8B), indicating leptin resistance at this age.

3.7. Long-term effect of postnatal EO in hypothalamic neuropeptides related to orexigenic/anorexigenic pathways

At adulthood, no change was detected in the POMC content in hypothalamus from SL group (Fig. 9A). However, these animals presented lower NPY expression (−50%, $P<.05$; Fig. 9B).

4. Discussion

In the present study, we observed that postnatal EO induced by litter size reduction causes a dramatic increase in body mass gain during lactation and programs for hyperphagia and overweight in later life. These findings are in accordance with earlier studies [8,10,11,34–36].

We also showed that early overfeeding induces an increase in rat growth (higher body length) that could be related to higher total body protein mass in young animals. However, the higher body weight in SL rats was due to higher fat mass, since protein mass was lower in adult SL animals. Adipose tissue distribution in obese subjects is very important, once regional fat deposition confers different cardiovascular risk [35,37,38] and SL animals presented higher central and total adiposity on both ages. Interestingly, the estimated subcutaneous fat mass was higher just in young SL rats, indicating that in adulthood, higher body weight and fat mass are mainly due to increased visceral fat mass. Recently, we reported higher epididymal fat mass in rats raised in SL when they were 90 days old [10], and previous studies also showed higher epididymal and visceral fat mass as well as an increase in adipocyte numbers in the same experimental model [39,40].

Some authors suggest that the higher fat mass in animals raised in SL can be due to higher lipogenic enzymatic activity in adipocytes [41]. Velkoska et al. [35] showed that rats submitted to postnatal EO present higher 11-β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) mRNA in adipose cells at 120 days old. This enzyme converts corticosterone in cortisol, increasing lipogenesis in abdominal fat [42]. Interestingly, transgenic mice overexpressing 11β-HSD1 in adipose tissue present higher fat mass associated with hypertension and leptin and insulin resistance [43]. In addition, it has been shown that postnatal EO results in hypertension in rats at 60 and 240 days [11,44]. Considering this finding and the higher central adiposity and lower plasma HDL-C levels observed in adult SL group, we suggest that neonatal obesity induced by postnatal EO could be a risk factor for cardiovascular disease and metabolic syndrome [45].

Table 1
Plasma biochemical parameters in young (21 days) and adult (180 days) NL and SL rats

	21 days		180 days	
	NL	SL	NL	SL
Glycemia (mg/dl)	72.17±3.11	90.08±2.08*	82.88±2.38	82.44±3.12
TC (mmol/l)	81.09±2.70	93.60±1.70*	58.65±1.00	57.50±1.70
LDL-C (mmol/l)	48.00±1.40	49.00±1.40	32.78±1.30	31.94±1.30
HDL-C (mmol/l)	23.17±1.10	31.80±1.60*	16.52±0.60	14.53±0.70*
VLDL-C (mmol/l)	11.77±0.90	13.00±1.10	11.19±0.70	10.60±0.70
Triacylglycerols (mmol/l)	59.18±4.30	70.90±7.20	55.48±3.60	52.50±3.20
Castelli index I	3.45±0.18	3.11±0.12	3.75±0.12	3.99±0.18
Castelli index II	1.92±0.10	1.52±0.08*	2.06±0.11	2.27±0.16
Total protein (g/dl)	4.01±0.18	5.08±0.16*	7.74±0.14	7.50±0.17
Albumin (g/dl)	2.73±0.14	3.34±0.14*	3.88±0.11	3.40±0.14*
Globulin (g/dl)	1.19±0.09	1.71±0.14*	3.86±0.07	4.16±0.08*

Results expressed as mean±SD of 12 rats per group.
* $P<.05$.

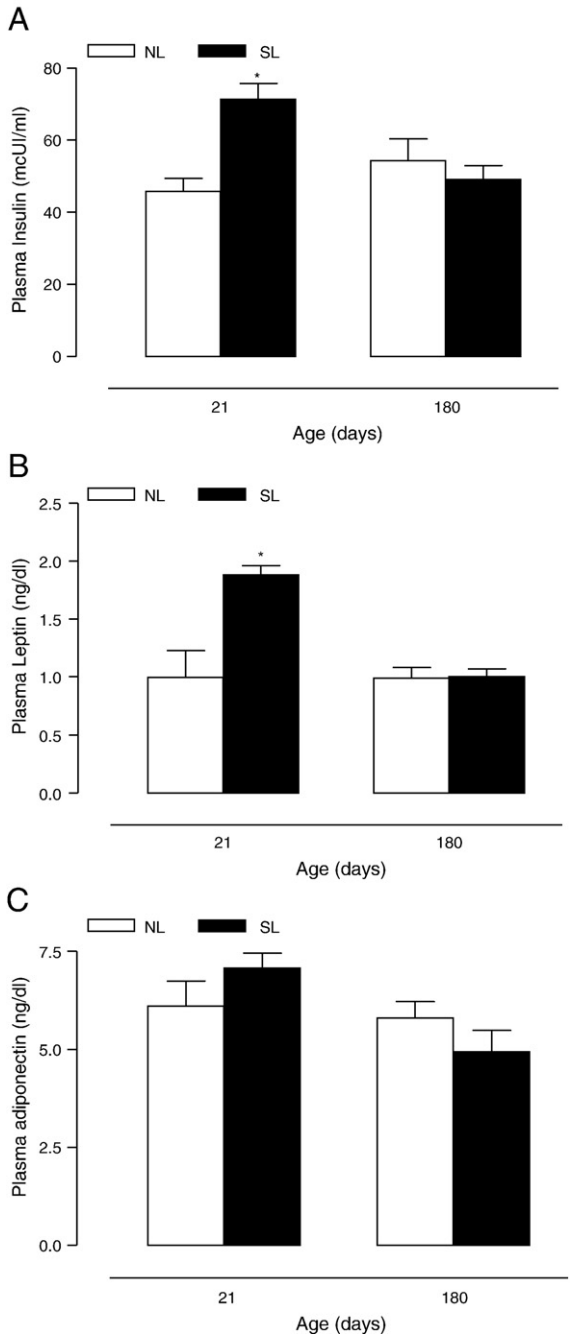

Fig. 4. Effect of postnatal EO on hormonal profile of young and adult NL and SL rats. Plasma hormones were measured from young and adult NL (white bar, $n=12$) and SL (black bar, $n=12$). Animals were fasted 12 h before the blood sampling. Insulin (A), leptin (B) and adiponectin levels (C) were analyzed by RIA. Results are expressed as mean±S.E.M. *$P<.05$.

Corroborating previous studies, animals raised in SL presented hyperinsulinemia and hyperleptinemia at weaning [34,46]. This hormonal profile can be responsible for the hypothalamic changes in neural circuitry present in young early overfed animals [47,48], as both hormones are important to brain development during critical periods [49–51]. Also, Plagemann et al. [52] showed that weaned SL animals present higher intrahypothalamic insulin levels. Possibly, this occurs because neonatal blood–brain barrier is not yet mature, allowing increase insulin leakage from circulation into hypothalamus [53].

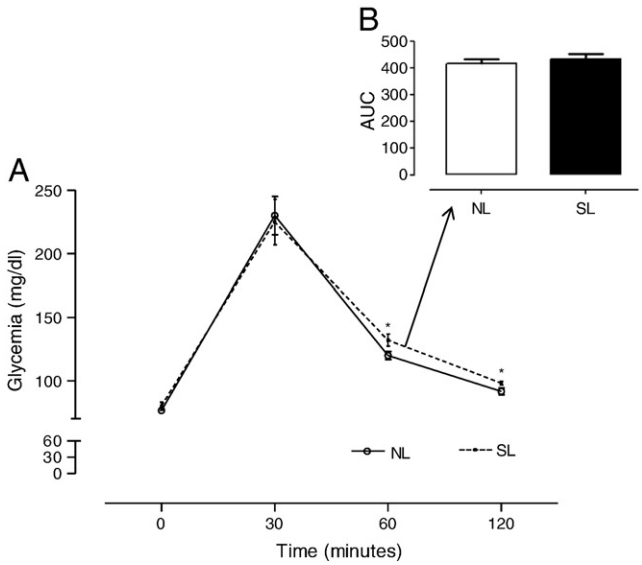

Fig. 5. Intraperitoneal glucose tolerance test of NL and SL groups in adulthood (180 days). Blood glucose levels were evaluated at fasting (12 h) period and 30, 60 and 120 min post intraperitoneal glucose loading of NL (○, $n=12$) and SL (●, $n=12$) groups (A). The insert represents the AUC (B). Results are expressed as mean±S.E.M. *$P<.05$.

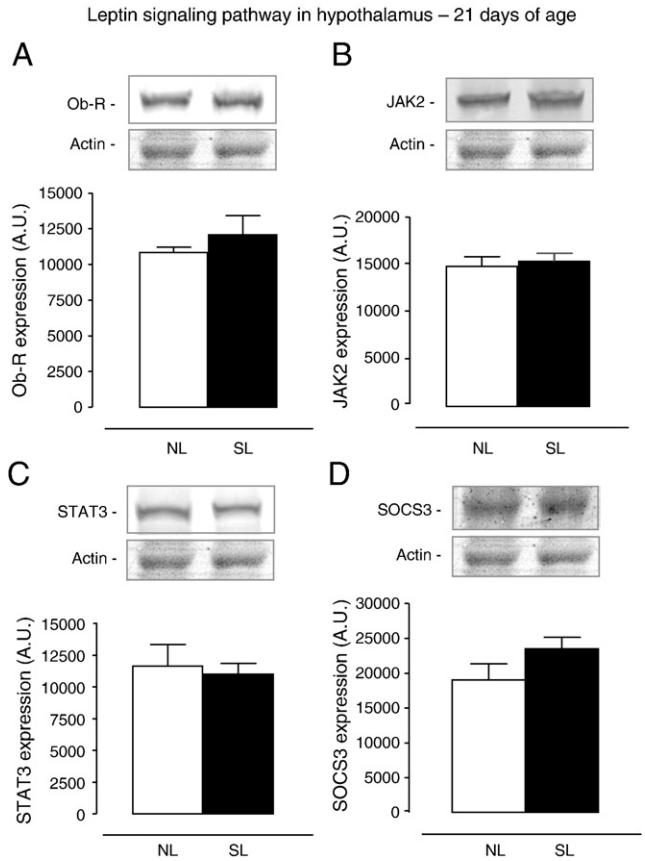

Fig. 6. Effect of postnatal EO on protein expression of leptin signaling pathway in hypothalamus of young rats (21 days). Homogenates of hypothalamus from weaned NL (white bar, $n=7$) and SL (black bar, $n=7$) rats were obtained, and ObR (A), JAK2 (B), STAT3 (C) and SOCS3 (D) contents were performed by Western blotting. Protein contents were quantified by scanning densitometry of the bands [arbitrary units (AU)]. Actin content was used as control loading, and results are expressed as mean±S.E.M. A representative experiment is shown from three independent experiments.

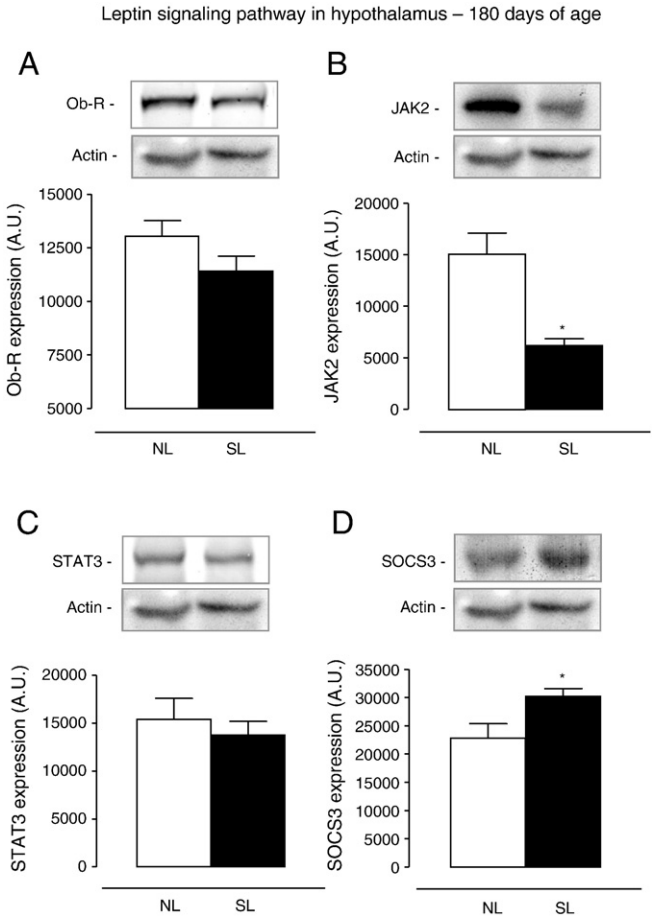

Fig. 7. Effect of postnatal EO on protein expression of leptin signaling pathway in hypothalamus of adult rats (180 days). Homogenates of hypothalamus from adult NL (white bar, $n=7$) and SL (black bar, $n=7$) rats were obtained, and ObR (A), JAK2 (B), STAT3 (C) and SOCS3 (D) contents were performed by Western blotting. Protein contents were quantified by scanning densitometry of the bands [arbitrary units (AU)]. Actin content was used as control loading, and results are expressed as mean±S.E.M. A representative experiment is shown from three independent experiments.

On the other hand, no change in plasma insulin and leptin was detected between the groups at 180 days old. Two previous studies showed that postnatal EO causes hyperleptinemia when the animals were 60 days old [9,35], and one of them reported that this change was not observed in SL animals when they were 120 days old [35]. To the best of our knowledge, there are no data reporting leptin and insulin levels in animals raised in SL at 180 days old. This result is interesting once overfed offspring that became an adult exhibit higher adiposity, but leptin, which is mainly produced by adipocytes, was unchanged. Regional differences in leptin secretion and gene expression in adipocytes from subcutaneous compared with omental adipose tissue are well established. It has been shown that leptin is produced mainly by subcutaneous adipose tissue [54,55], and human omental adipocytes has less leptin mRNA than subcutaneous adipocytes [54,56]. Small litter group showed hyperleptinemia only at weaning, when we observed higher subcutaneous adipose tissue, reinforcing the concept that serum leptin is more dependent of subcutaneous tissue. Despite the higher total and visceral fat in SL rats at 180 days, subcutaneous fat mass was normal, which could explain the unchanged serum leptin levels.

Recently, Lopez et al. [9] had shown that leptin levels in cerebrospinal fluid did not differ between SL and NL groups, despite that SL animals presented hyperleptinemia at 60 days old, suggesting that the rate of leptin influx into central nervous system (CNS) is not proportional to plasma leptin levels, indicating leptin resistance. This situation is similar to observations in obese humans that also exhibit hyperleptinemia with no change in cerebrospinal fluid leptin levels compared to nonobese subjects [57]. In this case, the signal from adipose tissue cannot indicate the real body fat mass to the brain, possibly due to impaired leptin transport across the blood–brain barrier [58,59], maybe by Ob-Ra deficiency.

Since postnatal EO induces an increase in visceral fat mass and considering the correlation between adipose tissue and insulin resistance, we analyzed the plasma adiponectin levels. Curiously, despite the higher body fat mass, adiponectin levels did not differ between the groups in both periods studied. Ours results are in accordance with other study that showed unchanged adiponectin levels in SL animals at 120 days old [35]. However, recently, Boullu-Ciocca et al. [60] reported hypoadiponectinemia in rats submitted to postnatal EO at 150 days old. At the moment, no study has shown adiponectin level in rats raised in SL at 180 days old, and adiponectin level found in younger SL animals is still controversial [35,60].

In our study, we also showed that SL group presented an increase in fasting glucose levels and hyperinsulinemia at weaning and normoglycemia and normal insulin levels when adults. Thus, SL animals are insulin resistant at weaning. Moreover, earlier studies corroborates our finding of elevated glucose and higher insulin levels at weaning [46,52]. Other groups showed that older animals other

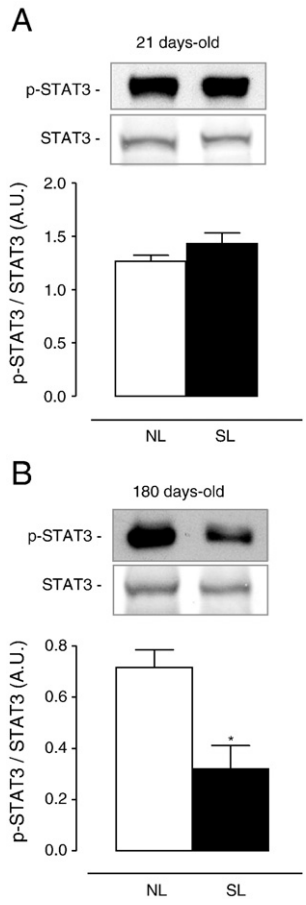

Fig. 8. Effect of postnatal EO on STAT3 activity in hypothalamus of young and adult rats. Homogenates of hypothalamus from young (A) and adult (B) NL and SL rats were obtained, and the STAT3 activity was performed by Western blotting. White bars represent control group (NL, $n=7$) and black bars represent overfed group (SL, $n=7$). Protein contents were quantified by scanning densitometry of the bands [arbitrary units (AU)], and results are expressed as mean±S.E.M. from the ratio between p-STAT3 and total STAT3 content. *$P<.05$. A representative experiment is shown from three independent experiments.

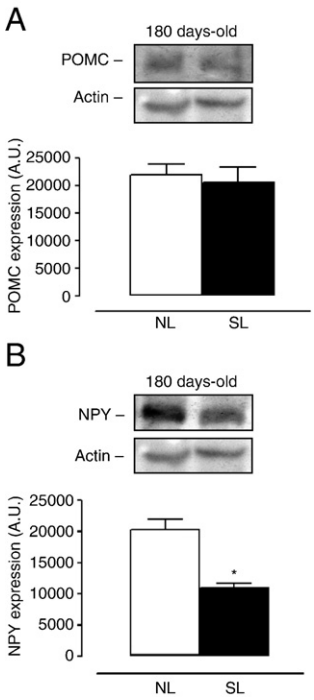

Fig. 9. Effect of postnatal EO on protein expression of hypothalamic neuropeptides related to orexigenic/anorexigenic pathways of adult rats (180 days). Homogenates of hypothalamus from adult NL (white bar; $n=7$) and SL (black bar; $n=7$) rats were obtained, and POMC (A) and NPY (B) content were performed by Western blotting. Protein contents were quantified by scanning densitometry of the bands [arbitrary units (AU)]. Actin content was used as control loading, and results are expressed as mean±S.E.M. A representative experiment is shown from three independent experiments.

than those used in our study (8 months old) still continued to be insulin resistant [6,11]. However, in our 6-month-old animals, we did not find any remarkable signal of insulin resistance.

We analyzed glucose tolerance in adult SL rats through intraperitoneal glucose tolerance test (IGTT). Our data showed a slight impairment in glucose tolerance 60 and 120 min after glucose injection, suggesting insulin resistance. Recently, we demonstrated unchanged IGTT in 90-day-old overfed animals [10], despite changes in insulin signaling pathway in adipocytes. At 90 days old, SL rats presented lower insulin receptor substrate 1, phosphatidylinositol 3-kinase and glucose transporter 4 (GLUT-4) content, followed by a decrease in Akt (serine kinase Akt) activity and GLUT-4 translocation in adipocytes. Boullu-Ciocca et al. [7] showed, in the same experimental model, higher glucose and insulin levels and glucose intolerance at 120 days old. Therefore, our and others' studies indicate that in later life, the programmed changes in adipose tissue induced by postnatal EO, and possible in other tissues, may cause a glucose homeostasis imbalance. Thus, changes in insulin sensitivity in this experimental model seem to be age dependent.

We also evaluated the total plasma proteins as well as plasma albumin and globulin concentrations. The evaluation of these proteins is important to help the nutritional assessment in the malnourished animal. Weaned SL group presented higher albumin and globulin and, consequently, higher total plasma proteins. It is possible that an increase in liver production of these proteins was consequence of higher food intake during lactation. In adulthood, despite unchanged total plasma proteins levels, SL rats presented lower albumin and higher globulin levels. Interesting, the same profile of plasma proteins has been reported in humans with chronic inflammation, which is reported to be associated with obesity [61]. In addition, Boubred et al. [44] showed that postnatal EO rats presented increase in proteinuria at 1 year old and glomeruloesclerosis at 2 years old, which is a chronic inflammatory state and can be consequence of the hypertension observed since the rats were 2 months old.

Beside possible changes in leptin transport across blood–brain barrier, leptin resistance in obesity has also been related with alterations in the leptin signaling pathway in hypothalamus [23]. Then, we analyzed the leptin signaling in hypothalamus from young and adult rats submitted to postnatal EO. Young SL rats had no alteration in hypothalamic leptin signaling proteins. Lopez et al. [62] showed lower hypothalamic Ob-Rb mRNA expression evaluated by in situ hybridization in early overfed animals when they were 24 days old. It is possible that those animals synthetize a functionally abnormal protein that could have less bioactivity and turnover but still maintain their immunological property, presenting normal protein content by Western blotting. In adulthood, we observed lower JAK2 and higher SOCS3 content, which induced a decrease in basal STAT3 phosphorylation, indicating hypothalamic leptin resistance. This result could explain the hyperphagia observed in adult overfed animals. In other models of programming during lactation, our group showed that leptin injections on the first 10 days of life program for hyperleptinemia when they were 30 days old, lower Ob-Rb and higher SOCS3 content in hypothalamus [63], similar to our present results for adult SL rats. As stated before, SOCS3 does an intracellular feedback regulation on leptin signaling pathway; blocking JAK2 phosphorylation and increase in hypothalamic SOCS3 expression have been clearly related with leptin resistance in obesity [64–67].

In adult animals, we also analyzed the protein expression of the central neuropeptides related to orexigenic/anorexigenic pathways. Interestingly, SL rats showed lower NPY expression and no change of POMC content. In fact, recently, it was observed that reduction in NPY expression in 18-week animals raised in SLs [68]. These authors suggested that the excessive nutrition during a critical window of postnatal development could change the neural density or activity, affecting the appetite regulation. In the present study, we measured the protein expression by Western blotting in total hypothalamus; however, we cannot discard that mRNA expressions of both appetite regulators are altered. In addition, perhaps agouti-related peptide (AgRP) and α-melanocyte-stimulating hormone (MSH) or cocaine- and amphetamine-regulated transcript (CART) expressions are also changed in SL rats. Thus, the persistent hyperphagia in adult SL rats may have the involvement of other hypothalamic mechanisms in obesity development, highlighting the complexity of the feeding system.

Adipose tissue and macrophages infiltrate in adipose tissue in obesity have been known to produce several cytokines [69]. Acute effects of some proinflammatory cytokines in obesity are produced at CNS level, particularly in the hypothalamus [64]. Interestingly, animals raised in SL showed high content of tumor necrosis factor (TNF)-α and IL-6 in mesenteric adipose tissue at 150 days old [60]. TNF-α has been shown to impair the anorexigenic effects of insulin and leptin in obesity [70,71]. Fat-rich diets increases insulin resistance through the activation of proinflammatory response [72]. Also TNF-α has previously been reported to induce reactive oxygen species formation in a variety of tissues [73,74]. Reactive oxygen species is known to activate nuclear factor κ-B (NFκ-B), which has been involved in diet-induced insulin resistance in hypothalamus [72]. It is possible that NFκ-B as well as TNF-α and IL-6 could also regulate the leptin signaling protein expression in hypothalamus, inducing leptin resistance observed in adult SL rats.

In summary, postnatal EO induces obesity, higher total and visceral fat mass as well as central leptin resistance and lower HDL-C in adulthood. Therefore, our results show that endocrine and metabolic dysfunctions in adult life, as metabolic syndrome and diabetes, can have the origins in the nutritional environment in early life.

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