Cold exposure restores the decrease in leptin receptors (OB-Rb) caused by neonatal leptin treatment in 30-day-old rats

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Abstract

We had previously shown that neonatal leptin treatment programs thyroid function in adulthood. As both thyroid hormones (TH) and leptin increased thermogenesis, it was interesting to evaluate the effect of cold exposure on the thyroid function of neonate rats treated with leptin. Pups were divided into two groups: Lep, injected with leptin (8 μg/100 g/BW, s.c.) for the first 10 days of lactation and control (C), injected with saline. When they were 30 days old, the groups were subdivided into two subgroups: Lep and CC, which were exposed to 8°C for 12 h and compared with C and Lep groups, maintained at 25 ± 1°C. Serum leptin, TH, and TSH were measured by RIA. Type I liver deiodinase (D1) and mitochondrial α-glycerol-3-phosphate dehydrogenase (mGPD) activities were assayed by the release of 125I from 125I-reverse and colorimetric method respectively. Leptin receptor (OB-Rb) was evaluated by western blot. Lep group had hyperleptinemia (+22%) and lower free tri-iodothyronine (FT3: −33%). Cold exposure increased TH both in LepC and CC groups compared with respective controls free thyroxine (FT4: +63 and +39%; FT3: +75 and +40%). Liver D1 activity was lower in Lep (−22%) and increased with cold exposure (LepC +51% and CC +22%). The mGPD activity was lower in Lep (−34%) and increased (fourfold) when this group is cold exposed. Hypothalamic and thyroid OB-Rb receptors were lower in Lep group (−47 and −36% respectively) and they were restored to normal levels after cold exposure. Leptin-programmed rats had higher TH response after cold exposure. OB-Rb had a fast response to cold exposure normalizing the lower levels observed in the leptin-programmed animals and may contribute to the higher TH cold responses.


Introduction

Experimental studies have shown that environmental changes, such as nutritional, endocrine disruptors, and other stressful events, occurring in a critical period early in life, permanently program the structure and physiology of the body’s tissues and systems. Those environmental changes act to imprint hormonal, neurological, or metabolic changes that are responsible for the programming effects (Armitage et al. 2004, Moura & Passos 2005).

Malnutrition during lactation changes the pup’s leptin serum levels (Teixeira et al. 2002) and programs body weight (Passos et al. 2000) and thyroid function (Passos et al. 2002). Therefore, we hypothesized that leptin could be an imprinting factor for malnutrition programming. Further, we demonstrated in rats that leptin injection during the first half of lactation is associated with hyperleptinemia and hypothyroidism in 30-day-old animals (Toste et al. 2006a). Those rats were programed for hyperleptinemia, higher serum thyroid hormones (TH), and lower serum thyrotrophin (TSH) levels when they were 150 days old. Thus, we conclude that a 30-day period is critical for the establishment of the hormonal imprinting for this programming. Besides, we showed that these animals had central leptin resistance and lower hypothalamic OB-Rb expression in adulthood (Toste et al. 2006b).

Leptin has a well-known stimulatory role upon thyroid function, increasing thyrotrphin-releasing hormone (TRH) and TSH (Legradi et al. 1997, Nilini et al. 2000, Ortiga-Carvalho et al. 2002), or directly upon its receptors on the thyroid, increasing TH secretion (Nowak et al. 2002). Leptin receptors are present in the hypothalamus (Tartaglia 1997, Toste et al. 2006b), pituitary (Vicente et al. 2004) and thyroid glands (Nowak et al. 2002). The thyroid OB-Rb expression has been shown until now only by Nowak et al. (2002) in normally fed female rats.

thyroxine (T₄) into tri-iodothyronine (T₃; Pazos-Moura et al. 1991, Reed et al. 1994, Lisboa et al. 2003). In contrast, cold exposure reduces the circulating leptin levels (Hardie et al. 1996, Bing et al. 1998) and the expression of the ob gene in brown adipose tissue (BAT) and white adipose tissue (WAT) of rodents (Trayhurn et al. 1995, Puerta et al. 2002). These findings may be caused by the cold-induced increase in sympathetic activity in these tissues (Trayhurn et al. 1995).

As TH and leptin serum concentrations and hypothalamic OB-Rb expression have important changes when the animals were programmed by neonatal leptin treatment, in the present study, we evaluated the effect of cold exposure on the thyroid function as well as the expression of the OB-Rb of leptin-programmed 30-day-old animals.

Materials and Methods

Animals and experimental design

The use of the animals was in agreement with the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro, which based its analysis on the principles described in the Guide for the Care and Use of Laboratory Animals (Bayne 1996).

Wistar rats were kept in a room with controlled temperature (25 ± 1 °C) and with artificial dark–light cycles (lights on from 0700 to 1900 h). Virgin female rats aged 3 months old were caged with one male rat at a proportion of 3:1. After mating, each female was placed in an individual cage with free access to water and food until the pups’ birth. Within 24 h of birth, excess number of pups were removed, so that only six male pups were kept per dam. Such a procedure has shown maximization in the lactation performance (Fishbeck & Rasmussen 1987). The study was performed using 16 male pups, from four different dams, in each experimental group. Pups were randomly assigned to two groups: the control group (C) was given an s.c. injection of saline (NaCl 0-9%) during the first 10 days of lactation, and the leptin group (Lep) was treated with recombinant mouse leptin (provided by PeproTech Inc., London, England) in a daily dose of 8 μg/100 g body weight during the first 10 days of lactation. The recombinant leptin was dissolved in saline and all the injections were given at 1600 h.

The pups were weaned at 21 days old. After weaning, two animals of each dam were randomly chosen and placed together in the cage with free access to water and food until day 30 of life. At 30 days old, the pups treated with leptin or saline were subdivided into control cold (CC, eight animals) and leptin cold (LepC, eight animals) groups, which were kept in individual cages and exposed to 8 °C for 12 h and compared with C (eight animals) and Lep (eight animals) groups maintained at 25 ± 1 °C for the same period. Immediately after 12 h of cold exposure, the animals were killed in the cold room, and the other animals (group C and Lep) in the laboratory (25 ± 1 °C), simultaneously, with a dose of anesthetic (40 mg/kg body weight, i.p.; Thiopental, Cristália, Brazil) and blood was obtained by cardiac puncture. Blood samples were centrifuged (5600 g, 4 °C, 20 min) to obtain serum which was kept at −20 °C until the assay. Hypothalamus, thyroid gland, and liver were excised and immediately frozen in liquid nitrogen. Tissue samples were stored at −70 °C until further analysis.

Body weight, food intake, and rectal temperature

Body weight was monitored daily during lactation and every 4 days after weaning until they were 30 days old, when they were killed. The food intake was measured every 4 days after weaning until 30 days old. At 30 days old, immediately after 12 h of cold exposure, the rectal temperature, body weight, and food intake were measured. Rectal temperature was measured using a digital thermometer inserted ~1:5 cm into the anus.

Serum hormone concentrations

Leptin was measured by RIA (Linco Research Inc., St. Charles, MO, USA). This kit measures both rat and mouse leptin with an assay sensitivity of 0-5 ng/ml and a range of detection from 0-5 to 50 ng/ml. The inter- and intra-assay variations were 7-3 and 6-9% respectively.

Free serum T₃ (FT₃) and free serum T₄ (FT₄) were measured by RIA, using commercial kits (Coat-A-Coat, DPC, Los Angeles, CA, USA). The inter- and intra-assay variations were 5-4 and 3-6% for FT₃ and 6-3 and 4-4% for FT₄ respectively.

Serum TSH was determined by specific RIA, using a kit for rat TSH supplied by the NIDDKD (Bethesda, MD, USA) and data were expressed in terms of the RP provided (RP-3). The intra-assay variation was 0-6% and all measurements were performed in a unique assay.

Mitochondrial α-glycerol-3-phosphate dehydrogenase (mGPD) activity determination

We followed the procedure described previously with slight modifications (Lee & Lardy 1965, Bernal et al. 1978, Recupero et al. 1983). To isolate the mitochondrial fraction, liver (250 mg) was homogenized in 5 ml sucrose magnesium solution (0-32 M sucrose, 1 mM MgCl₂) with Ultra Turrax and then centrifuged at 1000 g for 10 min at 4 °C to separate the crude nuclear pellet which was discarded. The supernatant was centrifuged at 8500 g for 10 min at 4 °C and the pellet was washed with 0-125 M potassium phosphate buffer (KBP; pH 7-5) in a vortex and followed by another centrifugation under the same conditions. The pellet containing the mitochondrial fraction was resuspended in 1 ml KBP.

In order to assay, mitochondrial suspension (100 μl) was mixed with 50 μl of 0-1 M DL-α-glycerophosphate diluted in KCN/KPB (0-32 mg/ml). Blank tubes contained only KCN.
and KPB. After 10 min at 30 °C, all tubes were transferred to a cold water bath (4 °C) and 100 µl solution of 7.9 mM INT −0.12 mM PMS was added. After 15 min at room temperature in the dark, the reaction was stopped by the addition of 50 µl TCA 10% and 1 ml absolute ethanol and tubes were centrifuged at 1000 g for 5 min. Supernatant was analyzed at 500 nm and the values were expressed as absorbance (OD)/mg of mitochondrial protein. Protein was measured using the method described by Bradford (1976).

**Hepatic deiodinase type I (D1) activity determination**

Liver tissue (250 mg) was homogenized in 50 mM Tris–HCl buffer (pH 6.8) and centrifuged at 1500 g at 4 °C for 20 min. The supernatants were stored at −70 °C until assayed. Assay for hepatic D1 activity was performed by the release of 125I from the 125I-labeled reverse T3 (rT3), with minimal modifications as previously described (Pazos-Moura et al. 1991, Dutra et al. 2003, Lisboa et al. 2003). Hepatic homogenates (35–270 µg protein) were assayed for D1 activity in phosphate buffer containing 1 mM EDTA, pH 6.9, in the presence of 1.5 mMr T3 and 10 mM dithiothreitol. Equal volumes of the 125I-rT3 (1.07 mCi/µg; Dupont-New England Nuclear, Boston, MA, USA), which was previously purified by paper electrophoresis, were added to each assay tube. They were incubated in shaking water bath at 37 °C and then stopped 30 min later by the addition of a mixture of 8% BSA and 10 mM PTU, followed by cold 20% trichloroacetic acid. The samples were then centrifuged (5600 g, 4 °C, 5 min), and 200 µl supernatants were applied to Dowex 50 W-X2 columns (100–200 mesh hydrogen from Bio-Rad). Free 125I eluted from the column with 10% acetic acid was measured in a gamma counter. The specific enzyme activity was expressed by protein nanomoles of rT3 deiodinated/h per mg. Protein was measured by the method described by Bradford (1976).

**Western blot of the hypothalamic and thyroidal leptin receptor**

Hypothalamus or thyroid was homogenized on ice-cold lysis buffer pH 6.4 (50 mM HEPES, 1 mM MgCl2, 10 mM EDTA, Triton X-100 1%, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml SBTI). The protein concentration was determined by the method of Bradford (1976).

The proteins (60 µg) were separated by SDS-PAGE (10%) and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech). The membrane was blocked for 1 h by 5% nonfat milk in TBS–T buffer (20 mM Tris, pH 7.5, 0.5 M NaCl, 0.1% Tween 20) followed by overnight incubation with primary antibody OB-Rb (m-18, goat polyclonal, Sc-1834, antihuman leptin receptor; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS-T (1:500), washed thrice with TBS–T, and incubated with secondary antibody (biotin-conjugated goat IgG, 1:10000) for 1 h. After the membrane had been washed thrice with TBS–T, it was incubated with streptavidin (avidin-conjugated peroxidase) for 1 h. Antibody binding was visualized using 3,3′-diaminobenzidine tetrahydrochloride (10 mg in 15 ml Tris buffer, 0.1 M, pH 7.4). The results were normalized with actin. Densitometry analyses of the immunoreactive bands were determined by Proplus image software (Infaimon, Barcelona, Spain).

**Statistical analysis**

The data are reported as mean ± S.E.M. Two-way ANOVA and Student’s t-test were used to analyze body weight and food intake in response to the leptin treatment. The other experimental data were analyzed by the one-way ANOVA followed by the Newman–Keuls test. The level of significance was set at P<0.05.

**Results**

**Body weight, food intake, and rectal temperature**

Body weight from birth to 30 days old is shown in Fig. 1. During that time, Lep group had a lower body weight than that of C group, from day 3 to 21 of leptin injection (approximately −5%, P<0.04). After day 21, these animals regained body weight, and reached the same weight of C group within 30 days.

Table 1 shows the effect of 12-h cold exposure (8 °C) on the body weight, food intake, and body temperature in 30-day-old rats. The body weight and food intake of the Lep
group kept at 25 ± 1 °C did not change. Cold exposure led to loss of body weight (−8.6%; \(P<0.05\)) and lower food intake (−21.8%; \(P<0.001\)) in CC group compared with the C group, whereas in the LepC group, it did not affect the body weight but led to lower food intake (−16.8%; \(P<0.01\)) compared with the Lep group. Cold exposure did not affect the body temperature in both CC and LepC groups compared with the respective controls.

**Serum hormone concentrations**

Serum leptin concentrations are shown in Fig. 2. Lep group had higher serum leptin concentrations than the C group (+22%, \(P<0.05\)). Cold exposure led to the decrease of serum leptin only in LepC group (−20%, \(P<0.05\)) compared with the Lep group, restoring those concentrations to the control values.

Figure 3 (A and B) shows the effect of cold exposure on the TH serum concentrations of rats treated with leptin on the lactation. The Lep group had a lower serum \(FT_3\) (−33%, \(P<0.05\)) and normal \(FT_4\) compared with the control. Cold exposure increases TH both at LepC (\(FT_4\): +63%, \(P<0.01\) and \(FT_3\): +75%, \(P<0.01\)) and CC (\(FT_4\): +39%, \(P<0.05\) and \(FT_3\): +40%, \(P<0.05\)) groups compared with respective controls. Serum TSH had a response compatible to a normal feedback; however, those changes were not statistically significant (Fig. 3C).

**D1 and mGPD activity**

Liver D1 activity (Fig. 4A) was lower in Lep group (−22%, \(P<0.05\)) compared with the C group and increased in both cold-exposed groups (LepC = +51%, \(P<0.001\) and CC = +22%, \(P<0.05\), compared with the Lep and C groups respectively) restoring to normal values the lower activity observed in Lep C. Liver mGPD activity was lower in the Lep group (−34%, \(P<0.05\)) compared with the C group. Cold exposure increased activity of this enzyme only in the LepC group (fourfold, \(P<0.001\)) compared with the Lep group (Fig. 4B).

**Western blot of the hypothalamic and thyroidal leptin receptor**

The levels of hypothalamic (Fig. 5) and thyroidal (Fig. 6) OB–Rb receptor expression were respectively 47% (\(P<0.01\)) and 36% (\(P<0.05\)) lower in the Lep group and restored to normal levels after cold exposure in LepC group. Quantity controls were obtained by performing parallel western blot of the housekeeping actin.

**Discussion**

We determined for the first time, the effect of the cold exposure on the body weight, thyroid function, serum leptin, and OB–Rb protein content on hypothalamus and thyroid gland of 30-day-old rats that were leptin treated during the first 10 days of life.

The data on lower body weight during lactation in the Lep group reinforces our previous finding (Oliviera Cravo et al. 2002, Toste et al. 2006b). However, after weaning, these animals recovered the body weight and had the same body weight of the control group until day 30 of life. So, the body weight during lactation was decreased by this leptin treatment.
Control cold-exposed rats had lower food intake and consequently lower body weight. In fact, cold stress may play an important role on these effects, since a more prolonged cold exposition is associated with higher food intake (Bing et al. 1998, Gasparetti et al. 2003, Torsoni et al. 2003). The LepC group when cold exposed had a lower decrease in food intake, compared with the cold-exposed controls, and did not have body weight changes. The fact that the LepC group ate relatively more than CC group could be explained by the fact that only in the LepC group leptin levels fell after cold exposure. Since leptin suppresses food intake, less leptin could counterbalance the anorectic effect of cold stress.

In respect to the programming effects of leptin on body weight regulation, our data disagree with some reports (Stocker et al. 2004, Picó et al. 2007). It is possible that these differences occur mainly because of the amount, period, and route of leptin administered. In the study by Stocker et al. (2004), the administration of leptin from day 14 of pregnancy and throughout lactation to rats fed on a low-protein diet reduces the susceptibility of a high-fat diet to induce higher weight gain. Contrary to our study, Stocker et al. (2004) report the effect leptin given to the mother from the third part of gestation did not study the effect of leptin treatment in mothers fed a standard chow diet. The model of Stocker et al. (2004) is a more complex one, which involves several variables such as: two different periods of development (pregnancy and lactation), undernutrition during the treatment, and a higher fat diet after weaning. Our model is a more physiological one, since the animals received a normal diet during the study period. In other

Figure 3  Serum concentrations of (A) free T4, (B) free T3, and (C) TSH of animals treated with saline and kept at 25 °C when 30 days old (C group, black bar), treated with saline and exposed to 8 °C for 12 h (CC group, white bar), treated with leptin on the first 10 days of lactation and kept at 25 °C when 30 days old (Lep group, gray bar), and treated with leptin on the first 10 days of lactation and exposed to 8 °C for 12 h when 30 days old (LepC group, hatched bars). Values are given as the mean±S.E.M. of eight animals per group. (*) Significant differences between Lep and CC groups and C, (#) between LepC and Lep. The level of significance was set at P<0.05.

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Acute cold regulates leptin receptor

Figure 5 (A) Western blot analysis and (B) densitometric analysis of the immunoreactive bands of the hypothalamic OB-Rb of animals treated with saline and kept at 25 °C (C group, black bar) when 30 days old, treated with saline and exposed to 8 °C for 12 h (CC group, white bar), treated with leptin on the first 10 days of lactation and kept at 25 °C when 30 days old (Lep group, gray bar), and treated with leptin on the first 10 days of lactation and exposed to 8 °C for 12 h when 30 days old (LepC group, hatched bars). Also shown is the western blot analysis of β-actin. Values are given as the mean ± S.E.M. of five animals per group. (*) Significant differences between Lep and CC groups and C, (#) between LepC and Lep. The level of significance was set at P<0.05.

Figure 6 (A) Western blot analysis and (B) densitometric analysis of the immunoreactive bands of the thyroidal OB-Rb of animals treated with saline and kept at 25 °C when 30 days old (C group, black bar), treated with saline and exposed to 8 °C for 12 h (CC group, white bar), treated with leptin on the first 10 days of lactation and kept at 25 °C when 30 days old (Lep group, gray bar), and treated with leptin on the first 10 days of lactation and exposed to 8 °C for 12 h when 30 days old (LepC group, hatched bars). Also shown is the western blot analysis of β-actin. Values are given as the mean ± S.E.M. of five animals per group. (*) Significant differences between Lep and CC groups and C, (#) between LepC and Lep. The level of significance was set at P<0.05.

Previous reports of our group (Lins et al. 2005, Passos et al. 2007), we injected leptin into the mother for a shorter period (3 days at the end of lactation) and observed that the offspring were programmed for higher body weight and higher food intake, when they were 180 days old. In the study of Picó et al. (2007), the daily oral dose of leptin (fivefold the leptin milk concentration) was given to suckling male rats during lactation. After weaning, the pups were fed with a normal fat or a high-fat diet. Leptin-treated animals had, in adulthood, lower body weight, fat content, food intake, and hypoleptinemia. However, with this amount of leptin, they did not observe body weight changes during lactation. We showed previously that leptin injection reduces pups body weight, even when leptin was injected in the mother (Oliviera Cravo et al. 2002, Lins et al. 2005, Toste et al. 2006b).

At 30 days of age, leptin-programmed rats had lower serum T3, hepatic mGPD, and D1 activities. Nevertheless, those animals showed a higher response of TH when cold exposed compared with the controls, with a consequent higher increase in mGPD and hepatic D1 activities.

One week after weaning, neonatal leptin-treated rats presented a persistent hyperleptinemia, resistance to the anorectic effect of this hormone (Toste et al. 2006b), and a lower expression of hypothalamic and thyroidal OB-Rb receptors. Some nutritional or hormonal mechanism might link the anorectic effect of leptin on the first 10 days of lactation with an increment of leptin after weaning. Maybe those animals, which had a decrease in food intake in those early days of life, when they were given chow ad libitum, after weaning, increases their intake and they quickly gain more adipose tissue, producing more leptin. Those with hyperleptinemia, according to the known phenomena of down-regulation, can be responsible for the lower expression of OB-Rb receptors both at the thyroid and hypothalamus. The stimulatory effect of leptin on the thyroid function is well known, through its effects at the hypothalamic level, increasing TRH, or its direct effects on the thyroid gland (Legradi et al. 1997, Nowak et al. 2002, Ortiga-Carvalho et al. 2002). In the present situation, it is possible that down-regulated receptors cause a resistance to the leptin effect both at hypothalamic and thyroidal levels, leading to a thyroid hypofunction. Consequent to the decrease on the thyroid function, those animals had decreased activity of liver D1 and mGPD enzymes that are thyroid hormone dependent.

Cold exposure is associated with rapid activation of the hypothalamus–pituitary–thyroid axis (Hefti et al. 1975, Joseph-Bravo et al. 1998), peripheral conversion of T4 into T3 (Pazos-Moura et al. 1991, Reed et al. 1994, Lisboa et al. 2003), and low levels of circulating leptin (Hardie et al. 1996, Bing et al. 1998). Increase in catecholamine secretion can play
a role on these changes. Catecholamine has a stimulatory effect on thyroidal $T_3$ production (Shimura et al. 1990) as well as D1 (Aceves & Rojas-Huidobro 2001) and D2 activities (Silva & Larsen 1983). Also, it mediates the cold-induced suppression of leptin expression (Trayhurn et al. 1995). Norepinephrine deficiency is also associated with a resistance in leptin action on brown and adipose tissues (Commins et al. 1999), suggesting that norepinephrine stimulates OB-Rb expression on these tissues. In fact, we showed recently that those programmed animals presented higher catecholamine adrenal content and higher basal and caffeine-stimulated in vitro secretion (Trevenzoli et al. 2007). Thus, in this present study, catecholamine may have a higher increase when those leptin–programmed animals are cold exposed, increasing the OB-Rb protein content at the hypothalamus and thyroid gland, and this factor could contribute to the higher TH cold response.

Higher TH response in leptin–programmed cold-exposed animals is associated with a higher liver mGPD and D1 activities. This effect is more marked over mGPD. The animals may have a better adaptation to cold environment than controls when mGPD have a thermogenic effect (Dos Santos et al. 2003).

Leptin increases D1 activity in liver (Lisboa et al. 2003) and D2 activity in BAT (Cettour-Rose et al. 2002), stimulating $T_3$ production. Thus, in leptin-programmed animals exposed to cold, the higher serum $T_3$ seems to be, at least in part, due to the peripheral conversion of $T_4$ into $T_3$. In this study, leptin–programmed cold-exposed animals showed lower serum leptin concentration, suggesting that the main source of $T_3$ could be the thyroidal supply. We cannot discard that the same phenomena observed for thyroidal OB-Rb receptors, with a rapid recovery of its lower levels observed at room temperature, could be happening with the hepatic leptin receptors.

In conclusion, the main finding of this paper is to show that the physiological changes observed in leptin-treated neonatal animals, 1 week after weaning, can be corrected by cold exposure, both at the leptin and thyroid functional level, with probable consequences for cold adaptation and body weight regulation of those programmed animals.

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