Role of neonatal hyperleptinaemia on serum adiponectin and suppressor of cytokine signalling-3 expression in young rats

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(Received 9 January 2008 – Revised 14 April 2008 – Accepted 15 April 2008 – First published online 5 August 2008)

Previously we had shown that neonatal leptin treatment programmes for both hyperleptinaemia and hyperinsulinaemia, which lead to leptin resistance and low expression of the hypothalamic leptin receptor (OB-Rb) of rats aged 150 d. Here we investigated in young post-weaned rats (age 30d) if leptin treatment during lactation induces leptin and insulin resistance and if those changes are accompanied by changes in the suppressor of cytokine signalling-3 (SOCS-3) expression and serum adiponectin concentration. After delivery, the pups were divided into two groups: (1) a leptin group (Lep) that were injected with leptin daily (8 mg/100 g body weight subcutaneously) for the first 10 d of lactation; (2) a control (C) group, receiving saline. After weaning (day 21), body weight was monitored until the animals were age 30 d. They were tested for food intake in response to either leptin (0.5 mg/kg body weight intraperitoneally) (CL, LepL) or saline (CSal, LepSal) when they were aged 30 d. The CL group showed lower food intake, but no response was observed in the Lep group, suggesting leptin resistance. The Lep group had hyperleptinaemia (five-fold), hyperinsulinaemia (+42.5 %) and lower levels of serum adiponectin (−43.2 %). The hypothalamic expression of OB-Rb was lower (−22 %) and SOCS-3 was higher (+52.8 %) in the Lep group. We conclude that neonatal leptin treatment programmes for leptin resistance as soon as 30 d and suggests that SOCS-3 appears to be of particular importance in this event. In the Lep group, the lower serum adiponectin levels were accompanied by higher serum insulin, indicating a probable insulin resistance.

Hyperleptinaemia: Adiponectin: Suppressor of cytokine signalling-3: Leptin resistance

Recently, epidemiological and experimental evidence has suggested that intra-uterine undernutrition is closely associated with adulthood obesity related to detrimental metabolic sequelae(1–3), giving rise to the concept of ‘developmental origins of health and disease’(4).

The mother’s undernutrition during lactation can programme the higher body weight of their offspring in the adult life(5) and this can be mainly associated with the neonatal leptin surge. Intra-uterine undernutrition is also closely associated with obesity in adulthood. Yura et al. (6) report a mouse model in which undernutrition was applied during gestation; after birth the offspring was fed a high-fat diet and developed higher adiposity. In the neonatal period, undernourished offspring exhibited a premature onset of the neonatal leptin surge compared with offspring with normal intra-uterine nutrition. A premature leptin surge generated in control offspring by exogenous leptin administration led to accelerated weight gain with a high-fat diet. Both undernourished offspring and neonatal leptin-treated control offspring exhibited an impaired response to acute peripheral leptin administration with impaired leptin transport to the brain as well as an increased density of hypothalamic nerve terminals. The present study suggests that the premature leptin surge alters energy regulation by the hypothalamus and contributes to ‘developmental origins of health and disease’.

We have shown that leptin treatment in the first 10 d of lactation programmes for higher food intake and body weight, hyperleptinaemia(7), higher thyroid hormone serum concentration(8), hypothalamic leptin resistance, lower expression of hypothalamic leptin receptor (OB-Rb)(9) and higher medullar catecholamine content and secretion(10) in adulthood (aged 150 d).

Leptin is a cytokine-like peptide that is produced primarily by adipose tissue and inhibits food intake and increases energy expenditure in rodents(11). Leptin and insulin are key hormones involved in the regulation of energy balance and glucose homeostasis. Specific members of the suppressor of cytokine signalling (SOCS) family of proteins are now thought to have a role in the development of leptin and insulin resistance, owing to their ability to inhibit leptin- and insulin-signalling pathways(12).

Abbreviations: C group, control group; Lep group, group injected daily with leptin; NPY, neuropeptide Y; OB-Rb, leptin receptor; SOCS, suppressor of cytokine signalling.

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SOCS proteins are induced by a variety of cytokines and act as a negative regulator of cytokine signalling. Among eight SOCS proteins\textsuperscript{13,14}, leptin specifically induces SOCS-3 mRNA levels at the hypothalamus\textsuperscript{15,16} and activates SOCS-3 expression in neuropeptide Y (NPY) and pro-opiomelanocortin neurons\textsuperscript{16}. Thus, an increase in SOCS-3 mRNA levels could lead to a leptin resistance, by inhibition of the Janus kinase 2–signal transducer and activator of transcription 3 (JAK2–STAT3) pathway or phosphatidylinositol 3-kinase–phosphodiesterase 3B–cyclic AMP (PI3K–PDE3B–cAMP), an alternative pathway of leptin signalling\textsuperscript{15,17}.

Although the SOCS proteins were initially thought to be primarily involved in the attenuation of cytokine signalling through classical negative feedback, there is now a growing appreciation of a role for SOCS proteins in the negative regulation of receptor tyrosine kinase signalling, such as the inhibition of insulin signalling\textsuperscript{18}.

Adiponectin acts predominantly in muscle and liver affecting fatty acids and glucose metabolism. Adiponectin has been shown to have insulin-sensitising, anti-atherogenic and anti-inflammatory properties\textsuperscript{19}. A low serum concentration of adiponectin\textsuperscript{20,21} is associated with higher insulinemia and leptinaemia. Insulin and leptin are proposed to function as adiposity signals in the feedback regulation of food intake\textsuperscript{22}. Both hormones circulate in the bloodstream in proportion to body fat mass and regulate the activity of neurons found in regions of the brain associated with body-weight regulation\textsuperscript{11,23}. Development of resistance to the action of these hormones appears to have an important role in the pathogenesis of obesity and type 2 diabetes.

Here, we demonstrate whether neonatal hyperleptinaemia changes serum adiponectin levels and expression of SOCS-3 protein in rats aged 30 d and if these factors could be associated with the development of leptin and insulin resistance.

Methods

Animals and treatment

Animal experimental protocols were approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro, which based their analysis on the principles described in the Guide for the Care and Use of Laboratory Animals\textsuperscript{24}. Wistar rats were kept in a room with controlled temperature (25 ± 1°C) and with artificial dark–light cycles (lights on from 07.00 to 19.00 hours).

Pups were divided, within 24 h of birth, into two groups: daily subcutaneously injected with 8 μg/100 g body weight\textsuperscript{7,9,25} of recombinant mouse leptin (PeproTech Inc., London, UK) (Lep group) for the first 10 d of lactation and the control group (C group) that received instead the same volume of saline (NaCl; 0·9 %). Leptin was dissolved in saline and all the injections were made at 16.00 hours.

Within 24 h of birth, excess pups were removed, so that only six male pups were kept per dam (six dams), because it has been shown that this procedure maximises lactation performance\textsuperscript{26}.

At 2 h after leptin injection, two pups of each litter (Lep and C; six rats) were randomly chosen and blood samples were obtained by decapitation and trunk blood was collected for determination of serum leptin.

The pups were weaned at age 21 d. After weaning, two animals of each litter (six litters of each experimental group) were randomly chosen and placed together in the cage with free access to water and food until they were aged 30 d, when they were killed (twelve animals per group) with a lethal dose of pentobarbital (0·15 ml/100 g bodyweight, intraperitoneally) and blood was obtained by cardiac puncture.

Body composition analysis

Body composition (fat and protein mass) was determined at 30 d by carcass analysis as reported previously\textsuperscript{19,27}. After killing, the animals were eviscerated, the carcasses were weighed, autoclaved for 1 h and homogenised in distilled water (1:1). Samples of the homogenate were stored at 4°C before analysis. Then 3 g of the homogenate were used for determining fat mass gravimetrically. The samples were hydrolysed in a shaking water-bath at 70°C for 2 h with 30% KOH and ethanol. The total fatty acids and non-esterified cholesterol were removed with three successive washings with petroleum ether. After drying overnight in a vacuum all tubes were weighed and the results were expressed as g fat/100 g carcass. Protein mass was determined in 1 g of homogenate. The tubes were centrifuged at 2000 g for 10 min. The total protein concentrations were determined by the method of Lowry et al.\textsuperscript{28}. The results are expressed as g protein/100 g carcass.

Adiponectin, insulin and leptin serum quantification

All assays were performed using RIA kits (Linco Research, Inc., St Charles, MO, USA). The adiponectin kit had a lower detection limit of 0·78 ng/ml and the inter-assay and intra-assay CV were 7·31 and 4·09 %, respectively. The insulin kit had an assay sensitivity of 0·1 ng/ml and a range of detection from 0·1 to 10 ng/ml. The inter-assay and intra-assay CV were 4·2 and 8·47 %, respectively. The leptin kit had an assay sensitivity of 0·5 ng/ml and a range of detection from 0·5 to 50 ng/ml. The inter-assay and intra-assay CV were 6·9 and 7·3 %, respectively.

Glucose measurement

Glycaemia was determined in blood sample from the tail vein of fasting rats using a glucometer (ACCU-CHEK® Advantage; Roche Diagnostics, Mannheim, Germany).

Insulin resistance evaluation

We evaluated insulin resistance by the product of insulin and glucose:

\[
\text{Insulin resistance index} = \frac{\text{fasting insulin (} \mu \text{IU/ml)} \times \text{fasting glucose (mmol/l)}}{}
\]

Leptin resistance test

Peptide. Recombinant mouse leptin (PeproTech, Rocky Hill, NJ, USA) was dissolved in saline vehicle (0·9 %, w/v) and was given as a bolus injection at a dose of 0·5 mg/kg body weight intraperitoneally.
Feeding study. At 30 d, the rats of each experimental group (Lep and C) were randomly assigned to one of the following groups: leptin (CL, LepL) or saline (CSal, LepSal) group. The animals were food-deprived for 24 h while maintaining free access to water before the test. After an intraperitoneal injection of leptin or saline, they were returned to their home cages and provided with a supplementary amount of a standard diet. They were housed singly and food intake was measured by weighing the food cups 2, 4, 6 and 24 h after leptin or saline injections\(^{(5,9,29)}\).

Western blotting analysis
The hypothalamus, collected under basal conditions, was homogenised in ice-cold lysis buffer (50 mM-HEPES (pH 6.4), 1 mM-MgCl\(_2\), 10 mM-EDTA, Triton X-100 (1 %), plus aprotinin, leupeptin and soyabean trypsin inhibitor, each one at 1 mg/ml). The homogenates were stored at \(-20^\circ\text{C}\). The protein concentration was determined by the method of Bradford\(^{(30)}\).

The proteins (60 μg) were separated by SDS-PAGE (12 %) and transferred to a nitrocellulose membrane (Hybond™ ECL\(^{\text{TM}}\); Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). The membrane was blocked for 30 min with 2 % bovine serum albumin in Tris-buffered saline Tween 20 (TBS-T) buffer (20 mM-2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) (pH 7.5), 0.5 M-NaCl with 0.1 % Tween 20), incubated with OB-R-goat polyclonal IgG primary antibody (1:1000)/SOCS-3-goat polyclonal IgG primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS-T overnight, washed three times with TBS-T, and incubated with secondary antibody (peroxidase-conjugated donkey IgG, 1:1000), for 1 h. After the membrane was washed three times with TBS-T, the antibody binding was visualised using diaminobenzidine (5 mg in 10 ml 0.1M-Tris buffer, pH 7–8). Actin antibody (I-19, SC-1616; Santa Cruz Biotechnology) was used as an internal control. Densitometry analyses of the immunoreactive bands were determined by Image-Pro\(^{\text{®}}\) Plus software (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical analysis
The data are reported as mean values with their standard errors. Two-way ANOVA followed by the Newman–Keuls multiple comparisons test were used to analyse body-weight evolution and food intake in response to the acute leptin treatment. Other experimental observations were determined by Student’s \(t\) test with the significance level set at \(P<0.05\).

Results

Body composition
Body weight from birth until age 30 d is shown in Fig. 1. During that time, the Lep group had a lower body weight than the C group, from the third day of leptin injection to the 21st day (approximately \(-5\ %; \ P<0.04\)). After day 21, these animals regained body weight, and reached the same weight of the C group at the end of the experimental period. Despite their normal body-weight gain, their fat mass was lower (\(-22.2\ %; \ P<0.004\)) and protein mass was higher (+100 %; \(P<0.0002\)) compared with controls (Table 1).

Serum leptin, insulin, adiponectin, blood glucose and insulin resistance index
The Lep group had a higher serum leptin (+136 %; \(P<0.05\)) concentration 2 h after the last leptin injection, which confirmed the hyperleptinaemia. Table 1 shows serum hormone concentrations for offspring aged 30 d. The Lep group had higher serum leptin (5-fold; \(P<0.0001\)), higher glycaemia (+8 %; \(P<0.05\)) and hyperinsulinaemia (+43 %; \(P<0.05\)), but lower serum adiponectin (–43.2 %; \(P<0.05\)) compared with the control group. The Lep group showed a significantly higher (+65 %; \(P<0.05\)) insulin resistance index, which may indicate insulin resistance (Table 1).

Leptin resistance test
Fig. 2 shows the leptin resistance test for animals aged 30 d. The effectiveness of the test is demonstrated by the result of the control group, where leptin treatment significantly suppressed food intake at 2 h (\(-26.3\ %; \ P<0.05\)), 4 h (\(-21\ %; \ P<0.02\)) and 6 h (\(-17.6\ %; \ P<0.05\)) after leptin injection compared with the group that received saline (Fig. 2 (a)). However, the LepL group did not show a similar decrease, in all periods studied, suggesting a resistance to the anorexigenic effect of leptin (Fig. 2 (b)).
Western blotting of the hypothalamic leptin receptor and suppressor of cytokine signalling-3

Western blotting of total protein lysates from the hypothalamus of rats aged 30 d is shown in Fig. 3. The level of OB-Rb was lower (−21.6%; \( P < 0.05 \)), while SOCS-3 expression was higher (þ52.8%; \( P < 0.002 \)) when compared with the control group.

Discussion

The findings of the present study confirm in young (age 30 d) rats similar changes observed in adult ones (age 150 d) caused by neonatal hyperleptinaemia and leptin resistance probably due to lower hypothalamic OB-Rb expression (9). Here we evaluated other parameters, showing lower adiponectinaemia and higher SOCS-3 expression. The major finding of the present study is that this programming occurs as soon as 1 week after weaning.

In agreement with other studies (31–33) and our previous findings (7–9,34), leptin injected peripherally during the first 10 d of lactation did increase serum leptin in the neonatal period at age 10 d and it is still higher at age 30 d.

Table 1. Body composition and serum hormone concentrations of animals at age 30 d that were given daily injections of leptin for the first 10 d of lactation and control animals

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<thead>
<tr>
<th></th>
<th>Control</th>
<th>Leptin-injected</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Body fat (g/100 g carcass)</td>
<td>5.61 ± 0.34</td>
<td>4.33* ± 0.21</td>
</tr>
<tr>
<td>Body protein (g/100 g carcass)</td>
<td>5.59 ± 0.35</td>
<td>11.23* ± 0.98</td>
</tr>
<tr>
<td>Serum leptin (ng/ml)</td>
<td>0.79 ± 0.12</td>
<td>4.79* ± 0.67</td>
</tr>
<tr>
<td>Serum insulin (mIU/ml)</td>
<td>27.72 ± 1.80</td>
<td>39.51* ± 4.80</td>
</tr>
<tr>
<td>Serum glucose (mg/l)</td>
<td>97.40 ± 1.79</td>
<td>105.90* ± 3.05</td>
</tr>
<tr>
<td>Serum adiponectin (µg/ml)</td>
<td>4.31 ± 0.20</td>
<td>3.47* ± 0.05</td>
</tr>
<tr>
<td>Insulin resistance index (µIU insulin/ml × mmol glucose/l)</td>
<td>147.10 ± 7.13</td>
<td>232.30* ± 27.84</td>
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*Mean value was significantly different from that of the control group (\( P < 0.05 \)).
Ahima et al. \(^{(31)}\) demonstrated in normal lean mice that during the neonatal period leptin rises transiently, which was termed as the ‘neonatal leptin surge’. In their experiment, leptin increased 5- to 10-fold to a peak level of 50 (SD 10) ng/ml in mice aged 10 d.

In the neonatal period, leptin alters hypothalamic neuropeptide expression and the metabolic rate before exerting its anorectic effect\(^{(32,35)}\). Bouret et al. \(^{(36)}\) demonstrated that leptin treatment in ob/ob mice in the neonatal period (4–12 d of life) reverts the reduction in nervous fibres that express pro-opiomelanocortin; the contrary occurred with NPY neurons. However, the treatment during adulthood did not result in the formation of anorexigenic and orexigenic neurons, confirming the existence of a critical period, where the neurons are leptin-dependent for their development in mice.

Several groups reported that exogenous leptin does not significantly inhibit growth, food intake, or energy expenditure during the first 2–3 postnatal weeks\(^{(32,35)}\). More specifically, Mistry et al. \(^{(32)}\) have shown that leptin does not alter significantly O\(_2\) consumption (a marker of energy expenditure) or food intake in normal lean mice until postnatal day 17. So, this suggests that the neonatal brain is relatively insensitive to leptin and may present leptin resistance\(^{(32)}\). However, leptin receptors are expressed in the arcuate nucleus of the hypothalamus (ARH) of rats\(^{(37)}\) before the development of ARH pathways.

Leptin also acts as a trophic signal that directs key developmental events in the early hypothalamic pathways that convey leptin signals to brain regions regulating body weight. Events imposed by prenatal nutrition that alter leptin levels may have consequences for the formation and function of circuits that regulate food intake and body weight throughout the life of an animal\(^{(36)}\).

Plagemann et al. \(^{(38)}\) showed in the small-litter model that the offspring developed overweight, hyperleptinaemia and hyperinsulinaemia, and did not find the expected decrease in NPY content in the arcuate nucleus and paraventricular nucleus when the rats were age 21 d. Since the NPY system is functionally mature already at this age, these findings might indicate an acquired resistance of the hypothalamic NPY system to increased levels of leptin and insulin in early postnatal overfed rats. In another model studied by our group, the prolactin blockage caused a higher transfer of leptin through the milk\(^{(39)}\) and the offspring developed overweight, hyperleptinaemia and hyperinsulinaemia. These data are similar to our previous study where the lactating rats were submitted to a protein-restricted diet\(^{(40)}\). Offspring with a protein-restricted diet had, in adulthood, lower body weight and this can be mainly associated with a lower milk concentration of protein. It is possible that in the study of Picó et al. \(^{(41)}\) that the animals present malnutrition, since oral doses of leptin could lead to a accentuated higher satiety and lower milk intake with consequent protein restriction and lower body weight in adulthood. However, it is also possible, that oral leptin could induce some gastrointestinal mediators that were bypassed by subcutaneous injection.

Vickers et al. \(^{(42)}\) showed that leptin injection on the first day of lactation in female rats whose mothers were submitted to energy restriction during pregnancy normalises the body-weight gain, the food intake and body fat of the offspring in adulthood. However, in this study the neonatal leptin treatment did not cause changes in the female adult offspring from mothers with a normal diet during pregnancy. Moreover, our present findings confirm the recent data of Vickers et al. \(^{(45)}\), which showed that the administration of leptin on the first days of lactation in male rats, whose mothers received a normal diet during pregnancy, programmed for higher body weight, hyperinsulinaemia and higher total adiposity. Our findings also corroborate the study of Yura et al. \(^{(6)}\) where neonatal leptin-treated rats developed pronounced weight gain, adiposity and leptin resistance later in life.

It is possible that the differences in these studies occur mainly because of the dose, different periods of development (pregnancy and lactation), sex, undernutrition during the treatment and a higher-fat diet after weaning.

In the present study we showed the consequences of neonatal leptin treatment at an early age. The Lep animals presented higher serum leptin and insulin concentrations and, different from observed before in adult animals, the young Lep group had hyperglycaemia and a higher insulin resistance index, suggesting insulin resistance. The development of resistance to the action of these hormones appears to have an important role in the pathogenesis of obesity and type 2 diabetes. The serum adiponectin concentration has been considered an appropriate parameter for evaluation of insulin resistance\(^{(47)}\). The Lep group presented, at age 30 and 150 d, lower serum adiponectin; this could be related to their insulin resistance at those ages.

Leptin resistance may occur for one of several reasons: leptin may fail to cross the blood–brain barrier, the hypothalamic receptors may be down-regulated or there may be abnormalities in the leptin receptor signalling pathway, such as SOCS-3 activation\(^{(48–50)}\).

We demonstrated that leptin treatment during lactation increases serum leptin concentration in adulthood, which leads to leptin resistance by reducing the expression of the hypothalamic OB-Rb\(^{(9)}\), since hyperleptinaemia can down-regulate its receptors. But here we add another level of complexity to this programming effect, showing the increase in SOCS-3 protein, that can be caused by both hyperleptinaemia
or hyperinsulinaemia and may be one of the causes of both leptin and insulin resistance.

It is interesting that in this model the first thing to occur is leptin resistance, even with a lower adiposity. Thus, the primary defect seems to be hypothalamic and we suggest that higher SOCS-3 could have an important role blocking the leptin’s signalling transduction.

Those animals had hyperleptinaemia even with lower adiposity. Leptin can be produced by other tissues, such as skeletal muscle\(^2\), that is probably higher in those animals that presented higher protein mass. In fact, the hyperleptinaemia in this model seems to be not related to the mass of adipose tissue. Even though in most of the models leptin dose correlates with adipose tissue mass, this relationship seems to be not so simple\(^2\); in some cases the relationship is curvilinear, reflecting more the process of fat accumulation than the total fat mass. Since leptin is produced by other tissues, such as muscle\(^2\), the increase of leptin production in those tissues could be the main explanation for these findings. Another explanation could be differences in the leptin clearance of these two groups.

In conclusion, the present results showed that neonatal hyperleptinaemia has a role as a neurotrophic factor that alters energy regulation by the hypothalamus and contributes to developmental programming of leptin and insulin resistance in rats as soon as 1 week after weaning. These effects may be explained by a higher expression of SOCS-3 protein and lower serum adiponectin. Our findings also suggested that the resistance to the anorexigenic effect of leptin at 30 d can be one of the ‘imprinting’ factors that associate the hyperleptinaemia during the first 10 d of lactation with the programming of higher risk to develop diabetes mellitus with ageing.

Acknowledgements

Research was supported by grants from the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico; CNPq), Coordination for the Enhancement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; CAPES) and the State of Rio de Janeiro Carlos Chagas Filho Research Foundation (Fundação de Amparo à Pesquisa do Rio de Janeiro; FAPERJ). Fernanda P. T. and P. A. T. were recipients of a CNPq fellowship and Fabiane P. T. was a recipient of a CAPES fellowship. All authors are grateful to Ms Monica Gaspar de Moura, Tiago Gomes and Mr Carlos Roberto for their technical assistance. All authors are grateful to Ms Monica Gaspar de Moura, Tiago Gomes and Mr Carlos Roberto for their technical assistance.

The authors declare no conflict of interest.

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