Hypothyroidism reduces ObRb–STAT3 leptin signalling in the hypothalamus and pituitary of rats associated with resistance to leptin acute anorectic action

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Abstract

Leptin has been shown to regulate the hypothalamus–pituitary–thyroid axis, acting primarily through the STAT3 pathway triggered through the binding of leptin to the long-chain isoform of the leptin receptor, ObRb. We previously demonstrated that although hyperthyroid rats presented leptin effects on TSH secretion, those effects were abolished in hypothyroid rats. We addressed the hypothesis that changes in the STAT3 pathway might explain the lack of TSH response to leptin in hypothyroidism by evaluating the protein content of components of leptin signalling via the STAT3 pathway in the hypothalamus and pituitary of hypothyroid (0.03% methimazole in the drinking water/21 days) and hyperthyroid (thyroxine 5 mg/100 g body weight /5 days) rats.

Hypothyroid rats exhibited decreased ObRb and phosphorylated STAT3 (pSTAT3) protein in the hypothalamus, and in the pituitary gland they exhibited decreased ObRb, total STAT3, pSTAT3 and SOCS3 (P<0.05). Except for a modest decrease in pituitary STAT3, no other alterations were observed in hyperthyroid rats. Moreover, unlike euthyroid rats, the hypothyroid rats did not exhibit a reduction in food ingestion after a single injection of leptin (0.5 mg/kg body weight). Therefore, hypothyroidism decreased ObRb–STAT3 signalling in the hypothalamus and pituitary gland, which likely contributes to the loss of leptin action on food intake and TSH secretion, as previously observed in hypothyroid rats.


Introduction

Leptin, a hormone produced in mature adipocytes, plays a central role in the long-term control of body weight, acting mainly at the CNS, inducing satiety and increasing energy expenditure (Zhang et al. 1994, Friedman & Halaas 1998, Ahima & Antwi 2008). In addition, leptin modulates the activity of several neuroendocrine axes, including the hypothalamus–pituitary–thyroid (HPT) axis. Similar to leptin, thyroid hormones (THs) are essential for the maintenance of basal metabolism and thermogenesis, leading to an increase in energy expenditure (Oppenheimer et al. 1987, Yen 2001, Silva 2003, Lechan & Fekete 2006, Fekete & Lechan 2007). During fasting, the reduced levels of leptin are associated with the fasting-induced suppression of the HPT axis (Ahima & Flier 2000, Seoane et al. 2000).

Several studies have demonstrated that leptin modulates thyroid function, acting at the hypothalamus, pituitary and thyroid. Leptin also modulates the activity of 5'-deiodinases (Yu et al. 1997, Ahima & Flier 2000, Seoane et al. 2000, Ortiz-Carvalho et al. 2002, Cabanelas et al. 2006, Araujo et al. 2009). Leptin has been shown to stimulate the hypothalamic production of TRH (Legradi et al. 1997, Friedman & Halaas 1998, Ahima & Flier 2000). The mechanism involves the direct action of leptin at TRH neurons in the paraventricular nucleus (PVN; Elias et al. 2000, Nilini et al. 2000, Harris et al. 2001, Guo et al. 2004) and indirect effects via the arcuate nucleus (ARC), where leptin up-regulates the activity of α-melanocyte-stimulating hormone neurons and down-regulates that of neuropeptide Y (NPY)/agouti-related peptide neurons, which have stimulatory and inhibitory projections to TRH neurons respectively (Legradi et al. 1997, Kim et al. 2000, Nilini et al. 2000, Harris et al. 2001).

The action of leptin is initiated by its binding to the long form of the leptin receptor (ObRb) at the plasma membrane, triggering a specific intracellular signalling pathway mediated primarily through the activity of the protein STAT3 (Lee et al. 1996, Myers 2004). After leptin binds to ObRb, the receptor-associated Janus tyrosine kinases (JAK2) phosphorylate the leptin receptor, which results in the phosphorylation of STAT3 by JAK2 and the subsequent translocation of
phosphorylated STAT3 (pSTAT3) to the nucleus to regulate gene transcription (Ahima & Flier 2000, Bates et al. 2003, Myers 2004, Hekerman et al. 2005). pSTAT3 induces the transcription of several genes, including suppressor of cytokine signalling 3 (SOCS3), which inhibits JAK2–STAT3 signalling, leading to central leptin resistance (Bjørbaek et al. 1999, Flier 2004, Howard et al. 2004, Donato et al. 2010). In vivo and in vitro evidence indicate that STAT3 mediates the effect of leptin in the regulation of TRH (Nillni et al. 2000, Guo et al. 2004, Huo et al. 2004). After a single injection of leptin, a rapid accumulation of pSTAT3 in TRH neurons in the PVN of mice is observed (Huo et al. 2004). Moreover, it has been shown that STAT3 is recruited to the pre–TRH promoter in vivo (Huo et al. 2004) and that the murine pre–TRH promoter has regulatory sequences that are responsive to STAT3, which has the ability to regulate the transcriptional activity of the promoter (Nillni et al. 2000, Huo et al. 2004).

We have previously demonstrated that the systemic administration of leptin increased the serum TSH concentration in rats, potentially due to leptin action at the hypothalamus, as the direct pituitary effect of leptin on TSH release was inhibitory, potentially as a result of an autocrine–paracrine effect exerted by locally produced leptin (Seoane et al. 2000, Ortiga-Carvalho et al. 2002). These in vivo and in vitro pituitary effects of leptin upon TSH secretion observed in euthyroid rats were preserved in hyperthyroid rats but not in hypothyroid rats (Da Veiga et al. 2004), suggesting that hypothyroidism abolished the action of leptin at the HPT axis. Therefore, we raised the question of whether alterations in leptin signalling through the STAT3 pathway in the hypothalamus and pituitary might justify the lack of TSH response to leptin in hypothyroidism. Here, we addressed these questions by evaluating the STAT3 pathway in the hypothalamus and pituitary of animals displaying hypo- or hyperthyroidism and normal leptinaemia. Additionally, we investigated whether hypothyroidism might also affect the anorectic effect of leptin because the ObRb–STAT3 pathway is a major signalling cascade involved in the satiety effect of leptin (Friedman & Halaas 1998, Elias et al. 1999, Bates et al. 2003).

Materials and Methods

Animals

The Institutional Animal Care and Use Committee of Health Sciences Centre, Federal University of Rio de Janeiro, approved all experimental protocols. Three–month–old adult male Wistar rats (300–350 g body weight (b.w.) were maintained under a 12 h light:12 h darkness cycle (lights on at 0700 h) at 24 ± 1 °C; standard chow and water were available ad libitum, and the food intake was not monitored along the period of treatment.

Effect of hypo- and hyperthyroidism on the ObRb–STAT3 pathway in the hypothalamus and pituitary of rats

The animals were divided into three groups: euthyroid, hypothyroid and hyperthyroid (ten animals per group). Hypothyroidism was induced through treatment with methimazole (0–0.03%) in the drinking water for 21 days. Hyperthyroidism was induced using s.c. single injections of thyroxine (T4 – l-T4; Sigma) at 5 μg/100 g b.w. daily for 5 days. The animals were weighed during the treatment period once a week. At the end of the treatment, the rats were decapitated, and the serum was obtained from trunk blood for the measurements of the concentrations of hormones. Visceral (epididymal and retroperitoneal) and subcutaneous (inguinal) fat depots were dissected and weighed. The medium basal hypothalamus and the whole pituitary gland were harvested and stored at −70 °C until the protein was extracted.

Influence of hypothyroidism on the acute anorectic effect of leptin

Hypothyroid rats were obtained using the same protocol described earlier. Hypo- and euthyroid rats were housed in individual cages and divided into two groups receiving either a single i.p. injection of saline (groups: eu and hypo) or rat recombinant leptin (National Hormone and Peptide Program, Torrance, CA, USA) at a dose of 0.5 mg/kg b.w. (groups: eu lept and hypo lept) after 24 h of food deprivation. The food intake was measured at 2, 4, 6 and 24 h after saline or leptin administration. The amount of food consumed was estimated by the reduction of the mass of chow offered.

Western blot analysis

The hypothalamus and pituitary gland were homogenised in ice-cold lysis buffer (50 mM HEPES, 1 mM MgCl2, 10 mM EDTA and 1% Triton X–100, pH 6–4) containing a protease inhibitor cocktail (Complete, Roche Diagnostics) to obtain total homogenates. The total protein content in the samples was determined (Bradford 1976), and the protein content of ObRb, STAT3, pSTAT3, SOCS3 and glycer–aldehyde 3-phosphate dehydrogenase (GAPDH) was analysed using western blotting. After denaturation in sample buffer, the samples were subjected to 10 or 12% SDS–PAGE, according to the molecular weight of each protein, and subsequently transferred to polyvinylidene membranes (PVDF Hybond–P, Amersham Pharmacia Biotech). The membranes were blocked with 5% non–fat milk in Tris–buffered saline (TBS; 20 mM Tris–HCl, 500 mM NaCl, pH 7–6) and incubated with primary antibodies overnight. The primary antibodies used were obtained from Santa Cruz Biotechnology, Inc., – anti–ObRb (1:500), anti–STAT3 (1:200), anti–pSTAT3 (1:1000) and anti–SOCS3 (1:500) – and from ABR Affinity Bioreagents (Golden, CO, USA) – anti–GAPDH (1:1000). Subsequently, the membranes were washed five times with TBS–T (TBS with 0–1% Tween 20), followed by a 3–h incubation with the appropriate secondary
antibody (1:10 000) — anti-mouse Ig-G conjugated to biotin (Santa Cruz Biotechnology) or HRP anti-rabbit Ig-G (Amersham Biosciences). The membranes were incubated with streptavidin HRP (Invitrogen), when necessary, and washed five times with 0·1% TBS-T. All blots were allowed to react with HRP substrate (ECL Western Blotting System — Amersham Biosciences) and were exposed to X-ray film. The images were obtained, and the bands were quantified by densitometry using Kodak 1D v3.5.4 software. The membranes were also stained with Rouge Ponceau and further submitted to densitometry analysis for loading control. For the hypothalamus and the pituitary gland, GAPDH was used as the internal control because this protein exhibited no consistent variations with hypo- or hyperthyroidism. The densities of the specific protein bands were normalised to that of GAPDH and then normalised to the euthyroid group. The densities were also normalised to that of the Ponceau densities of the corresponding lanes, and both methods of loading correction generated similar results. The results are representatives of two or three independent experiments.

**Hormone measurements**

Serum TSH concentrations were measured using a specific RIA, employing reagents supplied by the National Hormone and Peptide Program, as described previously (Ortiga-Carvalho et al. 1996). The minimum assay detection value was 0·18 ng/ml, and the intra-assay variation coefficient was 7·5%. The total serum T4 and triiodothyronine (T3) were measured using RIA commercial kits (Mab-ICN Pharmaceuticals, Costa Mesa, CA, USA). The minimum assay detection value was 25 ng/dl for total T3 and 1 µg/dl for total T4.

Serum leptin was determined using the specific rodents RIA kit (Linco Research, St Charles, MA, USA) according to the recommendations of the manufacturer. The minimum assay detection value was 0·5 ng/ml, and the intra-assay variation coefficient was 4%. All measurements were made within the same assay.

**Statistical analysis**

The data are reported as individual values and mean±s.d. One-way ANOVA followed by a Newman–Keuls multiple comparisons post-test were employed for the assessment of all data, except for serum TSH, which was analysed employing the non-parametric Kruskal–Wallis test.

**Results**

As depicted in Table 1, hypothyroid rats exhibited decreased serum total T4 (P<0·0001), serum total T3 below detection limits and increased serum TSH (P<0·0001) compared with euthyroid rats. Conversely, hyperthyroid rats exhibited a 3– and 2·7-fold increase in serum T4 and T3 (P<0·0001), respectively, and the TSH levels were reduced compared with the euthyroid rats (P<0·0001). The body weight of the hypothyroid rats was significantly lower compared with the euthyroid rats (~15%; P<0·001), while the hyperthyroid rats showed body weights similar to the euthyroid rats at the end of the treatment (Table 1). The white adipose tissue mass, visceral (epididymal and retroperitoneal pads) and subcutaneous (inguinal) depots normalised to the body weight were not changed by the hypo- or hyperthyroidism (Table 1). The serum leptin levels were similar among all groups (Table 1).

As depicted in Fig. 1, the ObRb expression in the basomedial hypothalamus of hypothyroid rats was significantly reduced compared with the euthyroid and hyperthyroid rats (P<0·01 and P<0·001 respectively; Fig. 1B). Despite the unchanged expression of STAT3 (Fig. 1C), the content of pSTAT3 in the hypothalamus of hypothyroid rats was reduced by ~20% compared with euthyroid rats (P<0·05; Fig. 1D). No changes were observed in the SOCS3 content in the hypothalamus of hypothyroid rats. The hypothalamic content of the proteins involved in the leptin signalling cascade was not affected by the hyperthyroidism (Fig. 1).

The hyperthyroid animals showed a reduced content of ObRb in the pituitary gland, compared with the euthyroid and hyperthyroid rats (P<0·05 and P<0·01 respectively — Fig. 2B), and a reduced STAT3 (P<0·001 vs the euthyroid group, Fig. 2C), and pSTAT3 content compared with euthyroid and hyperthyroid rats (P<0·01; Fig. 1D). These changes were associated with a significantly reduced content of SOCS3 (P<0·05 vs euthyroid group; Fig. 2E). Compared with euthyroid rats, hyperthyroid rats exhibited a lower STAT3 content (P<0·05), with no alterations in the other proteins of the leptin signalling pathway in the pituitary gland (Fig. 1B, C, D and E).

Because the hypothalamic STAT3 pathway is also importantly involved in the anorexigenic effect of leptin, we investigated the food ingestion of hypothyroid rats in response to a single injection of leptin. The hypothyroid rats, similar to those in the other experiments, exhibited lower serum THs.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Body weight, adiposity and serum hormone concentrations of euthyroid, hypo- and hyper-thyroid rats. Data are reported as the mean±s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Euthyroid</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>327·6±30·78</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>382·2±32·93</td>
</tr>
<tr>
<td>Viseral fat mass (g)</td>
<td>2·29±0·11</td>
</tr>
<tr>
<td>Subcutaneous fat mass (g)</td>
<td>1·63±0·58</td>
</tr>
<tr>
<td>T4 (ng/dl)</td>
<td>64·70±8·24</td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>4·30±0·53</td>
</tr>
<tr>
<td>TSH (ng/ml)</td>
<td>3·27±1·89</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>4·80±1·14</td>
</tr>
</tbody>
</table>

(i), first day of methimazole or T4; (f), killing day; *P<0·001 and †P<0·0001 vs euthyroid; n=10 per group.

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and a lower body weight compared with the euthyroid animals (Table 2). The serum leptin, measured at 24 h after leptin administration, was similar among all groups. As depicted in Fig. 3, in euthyroid rats, leptin injection reduced the food intake from 2 to 6 h after its injection \((P<0.01)\), and the effect disappeared by 24 h. However, leptin had no effect on food ingestion in the hypothyroid rats. Thus, hypothyroid rats, regardless of being injected with saline or leptin, exhibited lower food ingestion than euthyroid rats \((P<0.001)\).

**Discussion**

The main findings of the present paper are that hypothyroidism reduces the expression of the ObRb–STAT3 signalling pathway in the basomedial hypothalamus and pituitary of rats, and in addition, hypothyroid rats are resistant to the acute anorectic action of leptin. To the best of our knowledge, there are no previous reports on the influence of hypo- and hyperthyroidism on the expression of this signalling pathway.

Our finding that hypothyroidism was associated with the down-regulation of proteins of the leptin signalling pathway in the hypothalamus, namely reducing the content of ObRb and pSTAT3, suggests that the action of leptin might be reduced at the hypothalamic level in hypothyroidism. This result is consistent with our previous study (Da Veiga et al. 2004), showing that in hypothyroid rats, leptin lost its ability to increase serum TSH, because, as demonstrated by others, the STAT3 pathway is the primary intracellular mediator of the direct and indirect effects of leptin to stimulate TRH expression in PVN neurons (Legradi et al. 1997, Friedman & Halaas 1998, Ahima & Flier 2000, Guo et al. 2004, Huo et al. 2004). However, it remains to be demonstrated whether
Table 2 Body weight, adiposity and serum hormone concentrations of euthyroid saline-injected (eu), euthyroid leptin-injected (eu lep), hypothyroid saline-injected (hypo) and hypothyroid leptin-injected (hypo lep) rats. The data are reported as the mean ± s.d.

<table>
<thead>
<tr>
<th></th>
<th>Eu</th>
<th>Eu lep</th>
<th>Hypo</th>
<th>Hypo lep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>307.5±35.26</td>
<td>260±9</td>
<td>293.4±23.35</td>
<td>292.9±44.92</td>
</tr>
<tr>
<td>Body weight (%)</td>
<td>336.3±34.52</td>
<td>308.3±31.39</td>
<td>273.6±23.26†</td>
<td>285±34.08*</td>
</tr>
<tr>
<td>Visceral fat mass (% of b.w.)</td>
<td>1.54±0.42</td>
<td>1.30±0.19</td>
<td>1.30±0.36</td>
<td>1.75±0.27</td>
</tr>
<tr>
<td>Subcutaneous fat mass (% of b.w.)</td>
<td>1.27±0.15</td>
<td>1.15±0.63</td>
<td>1.20±0.16</td>
<td>1.42±0.14</td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>78±2.94</td>
<td>78.62±14.19</td>
<td>21.66±12.02*</td>
<td>18.85±5.38²</td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>5.41±0.58</td>
<td>5.58±1.13</td>
<td>2.73±0.46²</td>
<td>2.74±0.50²</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>3.58±0.3</td>
<td>3.12±0.54</td>
<td>3.26±1.32</td>
<td>3.40±1.34</td>
</tr>
</tbody>
</table>

(i), first day of methimazole; (f), killing day; *P<0.05, †P<0.01 and ‡P<0.001 vs euthyroid; n=8–12 per group. The rats were killed at 24 h after a single injection of leptin (0.5 mg/kg b.w.).

modifications of these signalling proteins occur in the TRH neurons of the PVN or in other neurons of the PVN or ARC that are involved in the regulation of TRH neurons by leptin (Legradi et al. 1997, Nilini et al. 2000, Guo et al. 2004, Huo et al. 2004).

Moreover, we demonstrated that the acute appetite-suppressing action of leptin was lost in hypothyroid rats. This fact might also be justified, at least in part, by our findings in the ObRb–STAT3 pathway in the hypothalamus of hypothyroid rats, as this signalling cascade is crucial to leptin action (Legradi et al. 1997, Nilini et al. 2000, Guo et al. 2004, Huo et al. 2004).

Our model of hypothyroid rats did not exhibit alterations in serum leptin, which excludes the possibility that hyperleptinaemia, known to induce central leptin resistance (Howard et al. 2004, Rodrigues et al. 2009), might have influenced our findings.

It is well known that THs have orexigenic effects, although the involved mechanisms are not completely elucidated. Evidence suggests the direct action of THs in NPY neurons of the ARC (Coppola et al. 2005); in addition, THs might act by inhibiting the activity of the ventromedial nucleus neurons, which play a suppressive role in appetite (Kong et al. 2004). Therefore, the reduced food ingestion of hypothyroid rats in our study is most likely associated with the lack of the orexigenic effect of TH. However, the resistance to the acute anorectic effect of leptin and the lower activity of the ObRb–STAT3 pathway in the hypothalamus of hypothyroid rats cannot justify, per se, the lower food intake of these animals. Recently, it has been proposed that the hyperphagia of hyperthyroid animals is associated with the TH-induced up-regulation of hypothalamic mTOR signalling (Varela et al. 2012). The mechanisms underlying appetite suppression in hypothyroidism remain unknown.

The decrease in the leptin signalling pathway induced through hypothyroidism was even more apparent in the pituitary gland. The reduced ObRb, STAT3, pSTAT3 and SOCS3 contents were detected in the pituitary of hypothyroid rats (Fig. 2). Previous studies have indicated that STAT3 activation through tyrosine phosphorylation plays an important role in leptin signal transduction at the pituitary gland (Tsumanuma et al. 2000, Lloyd et al. 2001). Although pituitary cells other than thyrotropes also express leptin receptors (Sone et al. 2001), our findings suggest that reduced leptin signalling in the whole pituitary gland might contribute to the abolishment of the direct effect of leptin on TSH secretion from the isolated pituitaries of the hypothyroid rats that were previously studied (Da Veiga et al. 2004). The mechanisms of leptin resistance are not completely understood, but it has been shown that high

Figure 3 Effect of acute leptin administration on food intake of euthyroid and hypothyroid rats. Euthyroid (eu) and hypothyroids (hypo) rats received a single i.p. injection of leptin at a dose of 0.5 mg/kg b.w. (eu lep and hypo lep) or saline and had their food intake monitored at 2 (A), 4 (B), 6 (C) and 24 h (D) after leptin administration. The data are reported as the mean±s.d., with significance levels **P<0.01 and ***P<0.001. n=8–12 rats per group. The data represents two independent experiments.
SOCS3 in the hypothalamus of hyperleptinaemic rats might exert a central role in the leptin resistance of obesity models (Bjorbaek et al. 1999, Mori et al. 2004). In our study, hypothyroid animals displayed normal serum leptin and reduced SOCS3; therefore, the aforementioned mechanism can be discarded as a cause of resistance to leptin action on TSH secretion in the pituitaries of hypothyroid rats.

To the best of our knowledge, there are no other reports showing that the thyroid status can affect the ObRb–STAT3 signalling pathway. It has been well demonstrated that the STAT3 pathway is necessary for leptin action in the regulation of food ingestion and in the thyroid axis (Bates & Myers 2003, Bates et al. 2003); therefore, we propose that the reduced STAT3 pathway in hypothyroid animals is involved in the lack of response to leptin, both at the thyroid axis and on appetite regulation. The physiological meaning of these findings is unclear, and because leptin levels were normal, one possibility is that the reduction in leptin signalling might represent an early adaptation to the decrease in energy intake exhibited by hypothyroid animals.

Hyperthyroidism had no major effect on the STAT3 pathway in hypothalamic or pituitary tissues. However, there was a small decrease in the STAT3 content in hypothalamic pituitaries, and pSTAT3 and the ObRb receptors were unaltered; therefore, they may be able to respond normally to leptin. These results are consistent with our previous study in which, employing the same protocol of short-term hyperthyroidism in rats, we observed that hyperthyroid rats or the isolated pituitary glands from hyperthyroid rats exhibited the same response as those in the euthyroid condition (Da Veiga et al. 2004).

In conclusion, this study suggests that hypothyroidism in rats results in impairment of the leptin signalling pathway via ObRb–STAT3 in the hypothalamus and pituitary gland, which is likely to be involved in the resistance to the effects of leptin on food ingestion and TSH secretion that we observed in hypothyroid condition.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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