Central stimulatory effect of leptin on $T_3$ production is mediated by brown adipose tissue type II deiodinase

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THYROID HORMONES have long been known to play an important role in the regulation of energy balance, in particular by stimulating thermogenesis (37). Leptin, an adipocyte-derived hormone acting on hypothalamic neurons to decrease food intake, has also been shown to stimulate energy-dissipating mechanisms (16, 42, 43). It can, therefore, be speculated that leptin may affect thermogenesis through an effect on thyroid hormones. In this respect, it is well documented that food restriction, a condition associated with low leptin levels, has profound effects on the hypothalamo-pituitary-thyroid axis in rats, including low plasma thyroxine ($T_4$) and triiodothyronine ($T_3$) levels, as well as decreased TRH and TSH synthesis in the hypothalamus and the pituitary, respectively (27). Systemic administration of leptin in rats and mice has been shown to prevent the fasting-induced reduction in proTRH mRNA levels occurring in neurons of the paraventricular nucleus, as well as to restore to normal the decreased circulating $T_4$ and $T_3$ levels due to food restriction (1, 24). Intracerebroventricular administration of leptin in rats has also been shown to reverse the marked suppression of TSH secretion measured in food-restricted animals (36). Such a stimulatory effect of leptin on spontaneous TSH secretion during food restriction was shown to depend on the thyroid status, as it was not observed in hypothyroid rats (36).

We have previously demonstrated (8) that central leptin administration in rats prevented the fall in plasma $T_3$ occurring during food restriction, whereas it was without effect on the decrease in serum TSH measured in these conditions. We also demonstrated (8) that hepatic deiodinase type I (D1) activity was decreased by food restriction, and that central leptin infusion completely prevented such a decrease. Under our experimental conditions of central leptin infusion, the peripheral plasma leptin levels were unchanged, indicating that the effects of leptin on hepatic D1 expression and activity had to be centrally elicited. Because central leptin administration has been shown to stimulate the sympathetic nervous system outflow (23), it could be hypothesized that leptin would influence hepatic D1 through an activation of this branch of the autonomic nervous system. However, D1 expression and activity are not known to be influenced by the sympathetic nervous system (or by catecholamines) (19).

In view of these considerations, we postulated that central leptin administration might stimulate deiodinase type II (D2) activity, thus resulting in an increase in plasma $T_3$ levels, which would then be sufficient to increase D1 activity (19). To test the hypothesis of a possible effect of leptin on D2, we used an in vivo approach allowing us to differentiate between the regulation of D1 and D2. This approach was based on the

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following considerations. The thyroid hormone metabolite reverse T3 (rT3) has been reported to strongly inhibit D2 activity (34). However, the rapid metabolism of rT3 by hepatic D1 in the adult rat (14, 17) hampers its in vivo use as a D2 inhibitor. By inhibiting D1 activity with propylthiouracil (PTU), we could expect to decrease rT3 degradation and to therefore obtain high plasma levels of infused rT3 that would inhibit D2 activity. The use of PTU would also reduce hepatic D1 activity to constantly low levels, excluding the possibility that leptin might affect plasma T3 levels through an activation of this enzyme. Finally, the hypothyroid state induced by PTU would be partly compensated for by moderate T4 supplementation to provide substrate for D2 activity while avoiding inhibition of D2 by T4 (25, 41).

On the basis of the aforementioned considerations and with the use of PTU-treated, T4-supplemented rats, the aims of the present study were 1) to determine whether the intracerebroventricular administration of leptin has an action on D2 in the tissues in which this enzyme is expressed, i.e., in the cerebral cortex, the hypothalamus, the pituitary gland, and brown adipose tissue (BAT); 2) to investigate what the consequences are on plasma T3 levels of such a potential effect of central leptin administration on D2; and 3) to assess whether central leptin has a concomitant stimulatory effect on the sympathetic nervous system activity, as assessed by the measurement of the mRNA expression of BAT uncoupling protein-1 (UCP1).

MATERIALS AND METHODS

Animals. Eight- to nine-week-old male Wistar rats purchased from BRL (Basel, Switzerland) were housed under conditions of controlled temperature (23°C) and illumination (7:00 AM-7:00 PM). They were allowed ad libitum access to water and standard laboratory chow (Provimi Lacta, Cossonay, Switzerland).

Experimental procedure. All animals were made hypothyroid with PTU (FLUKA Chemie, Buchs, Switzerland) given in their drinking water at a dose of 0.025% during the whole experimental period. After 2 wk, the mean body weight of the animals was 165 ± 2 g, with a daily rate of weight gain of 2.3 ± 1.1 g, indicating that the PTU treatment did not result in weight loss, although the rate of body weight gain was lower than in the absence of PTU (data not shown). At that time, they were anesthetized with intramuscular ketamine (7:00 AM-7:00 PM). They were allowed ad libitum access to water and standard laboratory chow (Provimi Lacta, Cossonay, Switzerland) and with the use of PTU-treated, T4-supplemented rats. At the end of the experiments, the animals were killed by decapitation. Liver, BAT, cerebral cortex, hypothalamus, and pituitary were removed, immediately frozen, and stored at −70°C.

All procedures used were approved by the Office Vétérinaire Fédéral et Cantonal, Geneva, Switzerland. Quantitative RT-PCR procedure. Total RNA was extracted from frozen tissue samples with TRIzol reagent (Life Technologies GIBCO-BRL, Rockville, MD). RNA integrity was assessed by performing a 1% agarose gel electrophoresis in 1X Tris-borate-EDTA, and its concentration was determined by spectrophotometry. cDNA templates for RT-PCR were obtained using 2.5 μg of total RNA. Reverse transcription reaction was performed with random hexamers (Microsynth, Geneva, Switzerland), dNTPs, the RNase inhibitor RNasin (Catalys, Promega, Madison, WI), and the MMLV-RT Enzyme Kit (Life Technologies GIBCO-BRL).

The real-time PCR (Lightcycler; Roche Diagnostics, Basel, Switzerland) reaction is an automated quantitative PCR obtained by continuous monitoring of the fluorescence emitted upon binding of the SYBR Green I dye to the double-stranded DNA. Amplification of cyclophilin A, D1, D2, and UCP1 cDNAs was performed with the SYBR Green I DNA master kit (Roche Diagnostics, Mannheim, Germany) according to the light cycler standard protocol, using ~70 ng of template cDNA. Primers for cyclophilin and D1 were used at a final concentration of 0.5 μM, and primers for D2 and UCP1 were used at 0.125 μM. The annealing temperature was 57°C for the D1 and D2 and 55°C for the UCP1 primer sets. After each run, a relative quantification of amplified PCR product in the different samples was performed. This was based on the relative comparison of the PCR products during the log-linear phase of the amplification process. A standard curve was used to obtain the relative concentration of the target gene, and the results were corrected according to the concentration of cyclophilin, used as the housekeeping gene.

Primer sequences. Primers for rat cyclophilin A, D1, D2, and UCP1 were designed on-line with Primer 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by Microsynth (Balghach, Switzerland). Primers were as follows: cyclophilin A: sense primer 5’-AGCAGTTGGGGGAAAAGATT-3’ starting at 166, antisense primer
5′-CATGCCCTCCTTCACCTCC-3′ starting at 471, product size 306; D1: sense primer 5′-CTCTACACCTCTCCTC-3′ starting at 437, antisense primer 5′-TTCAGAGA-CAGCTGGAAGCT-3′ starting at 2842, antisense primer 5′-AGAGGCATTGTAAGTG-GGGTC-3′ starting at 3380, product size 539; UCP1: sense primer 5′-ACCCCGAAGACAGAAG-G-3′ starting at 369, antisense primer 5′-CAATCTGGAGGAAGCAAG-3′ starting at 459, product size 91.

The adequacy of the different PCR products was verified by nucleic acid sequencing and agarose gel electrophoresis.

**D1 and D2 activities.** Tissues were homogenized in 10 volumes of 0.1 M phosphate (pH 7.2), 2 mM EDTA, and 1 mM DTT (P100E2D1 buffer). D2 activity was assayed in freshly prepared homogenates. Aliquots of homogenates were snap-frozen and stored at −80°C until analysis of D1 activities. D1 and D2 activities were assayed by monitoring the release of radiolabeled diiodotyrosine from outer-ring-labeled T3 or T4, respectively. For hepatic D1 activity, liver homogenates (−50 µg protein/ml) were incubated for 30 min at 37°C with 0.1 µM T3 or T4, and 105 cpm of [3,5,125I]T3 in 0.1 ml of P100E2D10 buffer. Blank incubations were carried out in the absence of homogenate. Radiolabeled diiodotyrosine production was analyzed as previously described (32). Deiodinase activity of homogenates was corrected for nonenzymatic deiodination observed in the blanks. D2 activities in brain, pituitary, and BAT were determined by incubation of appropriately diluted homogenates for 60 min at 37°C with 1 nM (105 cpm) [3,5,125I]T4 in the presence of 0.1 µM T3 to block D3 and 0.1 mM PTU to block D1, if present. Blank incubations were carried out in the absence of homogenate. Release of 125I was determined and corrected for nonenzymatic deiodination as described. In the assay of hepatic D1 activity, the samples were diluted 4,000 times, excluding a possible in vitro effect of the PTU used in vivo to inhibit D1 activity and making the animals hypothyroid. The same holds true for any putative effect of in vivo-infused rT3 on the in vitro measurement of D2 activity. Note that D1 and D2 activities were analyzed using substrate concentrations of 0.1 µM rT3 and 1 nM T4, respectively, being approximately equal to their Km values. Very similar changes in hepatic D1 activities were observed in rats with different thyroid states by using 1 µM instead of 0.1 µM rT3 as the substrate. Similarly, relative differences in BAT D2 activities between groups of rats subjected to various treatments were identical if D2 activity was measured at 10 nM instead of 1 nM T4 as the substrate. These higher substrate concentrations provide near-maximum deiodination rates.

**Type 3 deiodinase activity.** Type 3 deiodinase (D3) activity was assayed by monitoring the production of radioactive 3,3′-diodothyronine (3,3′-T2) from outer-ring-labeled T3. Cerebral cortex homogenates (−1 mg protein/ml) were incubated for 60 min at 37°C with 1 nM (2 × 105 cpm) [3,5,125I]T3 in 0.1 ml of 100 mM phosphate (pH 7.2), 2 mM EDTA, and 50 mM DTT. Blank incubations were carried out in the absence of homogenate. The reactions were stopped by addition of 0.1 ml of ice-cold methanol on ice. The mixtures were centrifuged, and 0.1 ml of the supernatants was mixed with 0.1 ml of 0.02 M ammonium acetate (pH 4) and analyzed by HPLC. Samples of 0.1 ml were applied to a 250 × 4.6 mm Symmetry C18 column (Waters, Etten-Leur, The Netherlands) connected to an Alliance HPLC system (Waters) and eluted isocratically with a mixture of acetonitrile and 0.02 M ammonium acetate (33:67, vol/vol) at a flow of 1.2 ml/min. Radioactivity in the eluate was monitored on-line using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT). Conversion of labeled T3 to radioactive 3,3′-T2 was corrected for nonenzymatic deiodination as observed in the blanks.

**Plasma hormones and metabolites.** TSH and T4 levels were measured by RIAs (Insmulate 2000; Diagnostic Product, Los Angeles, CA; rat TSH: L2KT56; T4: L2KT4). Plasma T3 and rT3 levels were measured by in-house methods in Rotterdam (Erasmus University) with the following respective intra- and interassay coefficients of variation: T3, 2–6 and 8%; rT3, 3–4 and 9–14%. More specifically, T3 was measured by RIA using a rabbit anti-T3 antiserum (final dilution 1:250,000) and [125I]T3 (20,000 cpm) (Amersham Pharmacia Biotech, Aylesbury, UK). Sample volume was 25 µl, and incubation mixtures were prepared in 1 ml of RIA buffer [0.06 M barbitur, 0.15 M HCl, 0.1% BSA, and 0.6 g/l 8-anilino-1-naphthalenesulfonic acid (Sigma)]. Mixtures were incubated in duplicate overnight at 4°C, and antibody-bound radioactivity was precipitated using Sac-Cel cellulose-coupled second antibody (IDS, Boldon, UK). The lower limit of detection was 0.15 nmol/l. Plasma rT3 levels were determined using an in-house-produced antiserum. The limit of detection was 0.04 nmol/l rT3.

**Statistical analysis.** The results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s procedure for multiple comparisons. The calculations were performed using the SigmaStat software (SPSS, Chicago, IL). A P value <0.05 was considered statistically significant.

**RESULTS**

Because the aim of the present study was to assess a potential role of leptin on the regulation of D2 with possible consequences on plasma thyroid hormone levels, the activity of hepatic D1 was inhibited by PTU, and the hypothyroid state induced by PTU was partly compensated for by moderate T4 substitution. The pathways targeted by our experimental design are schematized in Fig. 1. As shown in Table 1, hepatic D1 activity of the animals studied was consistently low and was ~90% lower than that of euthyroid rats (see legend of Table 1). This was true for the three groups of rats investigated, i.e., control animals infused intra-cerebroventricularly (icv) with vehicle and pair-fed the amount of food consumed by the leptin-infused rats (i.e., ~40% reduction of food intake compared with ad libitum-fed controls), leptin-infused rats, and leptin-infused rats receiving a 3-day subcutaneous rT3 infusion. When rats were infused with rT3, their plasma levels of this thyroid hormone metabolite reached values that were much higher than those of controls (101.8 ± 5.6 vs. 0.25 ± 0.02 pmol/ml, P < 0.001).

Figure 2 shows that, relative to pair-fed controls, the central leptin infusion resulted in a marked increase in BAT D2 activity (5-fold) and mRNA expression (2.5-fold). The activity of this BAT enzyme was decreased by rT3 administration to reach levels that were similar to those observed in the vehicle-infused, pair-fed control group. In contrast, the D2 mRNA expression remained elevated in leptin-treated animals receiving rT3. In tissues other than BAT (e.g., cortex, hypothalamus, pituitary), and as depicted in Table 2, central leptin administration had no effect on D2 activity. rT3
alongside leptin brought about a reduction in the activity of this enzyme in the pituitary only.

The effect of leptin on plasma levels of thyroid hormones is depicted in Fig. 3. Compared with pair-fed controls, central leptin administration resulted in a 50% rise in plasma T3 levels, an increase that was completely abolished when leptin was infused in animals concomitantly treated with rT3 (Fig. 3A). On the contrary, and as further illustrated by Fig. 3B, plasma T4 levels of leptin-infused rats were reduced by 30% compared with those of pair-fed control animals. Such a reduction of plasma T4 concentrations was only partly normalized by rT3 administration.

As can be noted in Table 1, the changes in plasma T3 and T4 levels were associated with a trend for plasma TSH levels to be decreased by the intracerebroventricular leptin infusion, although this change did not reach statistical significance. Plasma TSH levels of leptin-treated rats receiving rT3 were similar to those of pair-fed controls.

The expression of UCP1 mRNA in BAT was determined as a marker of sympathetic nervous system activity. As shown in Fig. 4, central leptin induced an increase in BAT UCP1 mRNA expression, an increase that remained present in the leptin-treated group that received the D2 inhibitor rT3 (Fig. 4).

D3 activity, determined in the cortex of the three groups of rats, was found to be unaltered by central leptin infusion, whether in the absence or in the presence of rT3 infusion (data not shown).

DISCUSSION

We have previously demonstrated that, when leptin was infused for 6 days into the cerebral ventricles of normal (euthyroid) rats, there was no leptin leakage in the periphery and thus no increase in plasma leptin levels, indicating that the effects produced by this hormone, among which is a sustained decrease in food intake, were centrally elicited (9). By comparing leptin-infused rats with vehicle-infused ad libitum-fed controls, and with vehicle-infused animals given the same amount of food as that consumed by the leptin-treated group (i.e., pair-fed controls to mimic leptin-induced hypophagia), we also demonstrated that central leptin administration prevented the increase in muscle UCP3 expression observed in response to pair feeding (8, 9). This central leptin effect was, at least partly, mediated by thyroid hormones, as it was not observed in hypothyroid animals (8). Additionally, in normal animals submitted to food restriction, the well-documented decreases in hepatic D1 activity and in plasma T3 levels did not occur when the animals were administered leptin intracerebroventricularly (1, 8, 24). This indicates the existence of a stimulatory influence of leptin on hepatic deiodination of T2 to T3 and on plasma T3 levels in euthyroid animals.

D2, a selenoprotein present in the brain, pituitary, and BAT (19), is the other activating enzyme known to catalyze the 5′ deiodination of T4 to the metabolically active hormone, among which is a sustained decrease in food intake, were centrally elicited (9). By comparing leptin-infused rats with vehicle-infused ad libitum-fed controls, and with vehicle-infused animals given the same amount of food as that consumed by the leptin-treated group (i.e., pair-fed controls to mimic leptin-induced hypophagia), we also demonstrated that central leptin administration prevented the increase in muscle UCP3 expression observed in response to pair feeding (8, 9). This central leptin effect was, at least partly, mediated by thyroid hormones, as it was not observed in hypothyroid animals (8). Additionally, in normal animals submitted to food restriction, the well-documented decreases in hepatic D1 activity and in plasma T3 levels did not occur when the animals were administered leptin intracerebroventricularly (1, 8, 24). This indicates the existence of a stimulatory influence of leptin on hepatic deiodination of T2 to T3 and on plasma T3 levels in euthyroid animals.

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active product T₃. The study of a potential effect of central leptin administration on D₂ activity in euthyroid animals is difficult, because this enzyme is powerfully inhibited by T₄ (40). However, in view of the postulated key role of D₂ in the regulation of tissue-specific thyroid hormone-dependent processes, [e.g., the pituitary feedback mechanism, brain developmental processes, BAT thermogenesis, (3, 7, 22)], it was of interest to determine whether, in addition to its stimulatory effect on D₁, leptin would also affect D₂ activity. The hypothesis that leptin might stimulate D₂ activity would be in keeping with the observations that central leptin administration promotes an increased sympathetic nervous system activity (12, 23, 35) and that D₂ activity, at the level of BAT in particular, was shown to be stimulated by the sympathetic nervous system (4, 38), whereas such is not known to be the case for hepatic D₁ activity (19).

To test the hypothesis that central leptin administration regulates D₂ activity, we used an in vivo approach in which D₁ activity was strongly inhibited by the use of PTU, which also made the rats hypothyroid. However, to provide substrate for D₂ activity, hypothyroid rats were supplemented with a low T₄ substitution rate (0.6 nmol/100 g body wt/day). Vehicle-infused controls were fed the amount of food consumed by leptin-treated rats. In euthyroid rats, mean plasma levels of T₄ were 81.5 ± 8.3 pmol/ml, those of T₃ being 1.95 ± 0.15 pmol/ml. Values are means ± SE of 5–7 animals per group. *P ≤ 0.05 vs. pair fed; †P ≤ 0.05 vs. leptin.

Table 2. D₂ activity in cerebral cortex, hypothalamus, and pituitary of hypothyroid rats supplemented with 0.6 nmol/100 g body wt⁻¹·day⁻¹ of T₄ and infused with vehicle or with leptin

<table>
<thead>
<tr>
<th>D₂ Activity, fmol/min·mg⁻¹</th>
<th>Cortex</th>
<th>Hypothalamus</th>
<th>Pituitary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair fed</td>
<td>0.36 ± 0.03</td>
<td>0.031 ± 0.006</td>
<td>5.09 ± 1.08</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.55 ± 0.20</td>
<td>0.085 ± 0.035</td>
<td>7.68 ± 1.23</td>
</tr>
<tr>
<td>Leptin + rT₃</td>
<td>0.22 ± 0.04</td>
<td>0.025 ± 0.005</td>
<td>1.07 ± 0.10* †</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–7 animals per group. Leptin was infused icv at a dose of 10 μg/day. The vehicle used was isotonic saline. rT₃ was infused to inhibit the activity of D₂ (25 nmol·rat⁻¹·day⁻¹). Vehicle-infused controls were fed the amount of food consumed by leptin-treated rats (pair-fed). *P ≤ 0.05 vs. pair fed; †P ≤ 0.05 vs. leptin.

Fig. 2. D₂ activity and mRNA expression in BAT of rats infused icv for 6 days with vehicle (isotonic saline, Controls) or with leptin (10 μg/day) in the presence and absence of rT₃ infusion (25 nmol·rat⁻¹·day⁻¹) to inhibit D₂ activity. All animals were treated with PTU (0.025% in their drinking water) and supplemented with a low T₄ substitution rate (0.6 nmol·100 g body wt⁻¹·day⁻¹). Vehicle-infused controls were fed the amount of food consumed by leptin-treated rats. PF, pair feeding. Values are means ± SE of 5–7 animals per group. *P ≤ 0.05 vs. pair fed; †P ≤ 0.05 vs. leptin.

Fig. 3. Plasma T₃ (A) and T₄ levels (B) in rats infused icv for 6 days with vehicle (isotonic saline, Controls) or with leptin (10 μg/day) in the presence and absence of rT₃ infusion (25 nmol·rat⁻¹·day⁻¹) to inhibit D₂ activity. All animals were treated with PTU (0.025% in their drinking water) and supplemented with a low T₄ substitution rate (0.6 nmol·100 g body wt⁻¹·day⁻¹). Vehicle-infused controls were fed the amount of food consumed by leptin-treated rats. In euthyroid rats, mean plasma levels of T₄ were 81.5 ± 8.3 pmol/ml, those of T₃ being 1.95 ± 0.15 pmol/ml. Values are means ± SE of 5–7 animals per group. *P ≤ 0.05 vs. pair fed; †P ≤ 0.05 vs. leptin.
roid rats were supplemented with T₄, at a low substitution rate, to avoid inhibition of D2 by T₃.

We observed that, compared with control rats that were given the same amount of food as that consumed by the leptin-treated animals (pair-fed controls), central leptin administration promoted increases in D2 expression and activity in BAT but not in the brain or in the pituitary. Concomitantly, and compared with those of pair-fed controls, plasma T₃ levels of leptin-infused rats were higher, whereas their plasma T₄ concentrations were lower. Such changes in thyroid hormone levels could be the consequence of the stimulatory effect of leptin on BAT D2, in keeping with the observation made in the present study that central leptin administration could bring about an increase in T₃ production, the relative influence of these two enzymes being modulated by the nutritional state (19). Leptin action would favor the activity of hepatic D1, given the high sensitivity of this enzyme to T₃ (19). Leptin action would also favor the activity of hepatic D1, given the high sensitivity of this enzyme to T₃ (19).

Fig. 4. Expression of UCP1 mRNA in BAT of rats infused icv for 6 days with vehicle (isotonic saline, Controls) or with leptin (10 μg/day) in the presence and absence of rT3 infusion (25 nmol·rat⁻¹·day⁻¹) to inhibit D2 activity. All animals were treated with PTU (0.025% in their drinking water) and supplemented with a low T₄ substitution rate (0.6 nmol·100 g body wt⁻¹·day⁻¹). Vehicle-infused controls were fed the amount of food consumed by leptin-treated rats. Values are means ± SE of 5–7 animals per group. *P ≤ 0.05 vs. pair fed.

The present data, together with those of our previous study (8), allow us to propose that central leptin could influence the thyroid axis with the following potential series of events. It would enhance the activity of BAT D2 via a likely activation of the sympathetic nervous system, resulting in significant increases in plasma T₃ levels. Such an increase in plasma T₃ concentrations would favor the activity of hepatic D1, given the high sensitivity of this enzyme to T₃. Leptin action could therefore occur on both activity in the liver, ultimately contributing to increased plasma T₃ levels. Because in the present study PTU was used to block hepatic D1 activity, leaving D2 as the main site for peripheral T₃ production, the relative influence of these two enzymes under physiological conditions cannot be established.

In summary, our data show that central leptin administration brings about a stimulation of BAT D2 activity in T₄-substituted hypothyroid rats, thus providing yet another aspect of the well substantiated stimulatory effect of leptin on the hypothalamo-pituitary-thyroid axis (13, 15, 24, 26, 36).
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