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PAPER

Fat balance and serum leptin concentrations in normal, hypothyroid, and hyperthyroid rats

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OBJECTIVE: To study the influence of thyroid hormones on the relationship between serum leptin and fat mass, as well as on energy and macronutrient balance.
DESIGN: Rats with different thyroid states were obtained by 7 and 15 days of treatment with the antithyroid drug propylthiouracil or with triiodothyronine (T_3).
MEASUREMENTS: Energy balance, macronutrient balance and serum leptin concentrations.
RESULTS: In hypothyroid rats we found a decrease in metabolizable energy (ME) intake and energy expenditure together with an increase in lipid gain/lipid intake ratio and a decrease in protein gain/protein intake ratio. Consequently, body lipid percentage significantly increased compared to euthyroid rats. Hyperthyroid rats first increased energy expenditure and later ME intake, so that increased metabolism was balanced by increased intake, and energy gain was similar to that found in euthyroid rats.
CONCLUSION: These results indicate that T_3 plays a major role in the maintenance of energy and lipid balance. Our results also indicate that an inverse relationship exists between T_3 and leptin serum concentrations, and that this relationship is not only the result of changes in body fat stores induced by changed T_3 concentrations.
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Keywords: triiodothyronine; leptin; fat balance

Introduction

Thyroid hormones and leptin are both involved in the regulation of energy homeostasis. Thyroid hormones have important effects on energy balance, since they influence both energy intake and expenditure. In fact, it has been shown that different thyroid states are associated with not-able differences in energy intake,[1,2] and hypo- and hyper-thyroidism cause a decrease or an increase in energy expenditure, respectively.[2,3] The recently discovered polypeptide hormone leptin regulates body weight by signaling the size of energy stores in adipose tissue and by decreasing food intake while increasing energy expenditure.[4–7]

We have previously shown that hypothyroid rats develop obesity when fed a high-fat diet, because of their inability to attain fat balance.[8] Since eu- [9–12] and hyperthyroid[13] rats avoid excess fat deposition when fed high-fat diets, we suggested that thyroid hormones could regulate partitioning of fat fuels between oxidation and storage and could thus play a role in the achievement of fat balance and hence in body weight maintenance. Therefore, it was considered of interest to gain further insight into the effect of thyroid hormones on energy balance as well as on macronutrient balance. In fact, it is now well established that energy balance must be studied in terms of the individual energy-yielding macronutrients, since oxidative autoregulation (ie the capacity to increase substrate oxidation in response to increased substrate intake) differs between macronutrients. To this end, full energy balance measurements were carried out in rats in different thyroid states after 7 and 15 days of treatment.

In addition, since leptin increases thermogenesis,[7] and thyroid hormones increase basal metabolic rate,[2,3] it was also considered of interest to assess possible interactions between leptin and thyroid hormones. Recent reports on the relationship between leptin and thyroid hormones have given conflicting results. In fact, while some reports seem to suggest that thyroid hormones exert a negative influence on serum

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leptin concentrations,[14–17] others have shown that the influence of thyroid state on serum leptin is only indirect, through variation in fat mass.[18] To provide further insight into the relationship linking thyroid hormones and leptin, we have also measured serum leptin concentrations in rats in different thyroid states.

Materials and methods

Animals and experimental design

Sixty-four male Wistar rats (Charles River, Calco, Como, Italy) of about 25 days of age (about 70 g) were used for the experiments. They were divided into eight groups, each composed of eight rats, with similar mean body weight and with body weight normally distributed within each group. Three groups of rats were subjected to a pretreatment, by administration of a 0.1% solution of propylthiouracil (PTU) in drinking water for 4 days. This pretreatment was done to avoid energy balance measurements being influenced by the fact that PTU-treated rats eat and drink less during the first 3–4 days of treatment, due to the bitter taste of PTU.[19] In fact, control experiments were done on rats treated with PTU plus a replacement dose of T_3 (500 ng/100 g body weight daily) or PTU plus saline for 15 days. Serum-free T_3 concentrations of PTU + T_3-treated rats were 350 ± 15 pg/100 ml. PTU had a direct action on energy intake only during the first few days of treatment, since starting from the fourth day of treatment PTU + T_3-treated rats ate and gained weight to a greater extent than PTU + saline treated rats and to a similar extent to control rats. All other groups of rats were maintained in standard conditions during this period. After the pretreatment, the experimental period started with killing one group of PTU-pretreated rats and one group of normal rats for the determination of initial body energy content and composition. Two groups of the remaining normal rats were used as controls, whereas the other two groups were made hyperthyroid by daily intraperitoneal injections of triiodothyronine (T_3) at a dose of 5 μg/100 g body weight (hyperthyroid rats). Finally, the two groups of PTU-pretreated rats were maintained on PTU treatment (hypothyroid rats). One group of control, hypothyroid and hyperthyroid rats was killed after 7 days of treatment, while the other group of control, hypothyroid and hyperthyroid rats was killed after 15 days of treatment. All rats were given free access to a standard stock diet (Mucedola 4RF21, Settimo Milanese, Milan, Italy) and water. The composition (percentage energy) of this diet was: protein 29.0, lipid 10.6, and carbohydrate 60.4; its gross energy density was 15.88 kJ/g wet weight. Rats were maintained one per cage (in metabolic cages) at 24°C under an artificial circadian 12 h light/12 h dark cycle. Animal care, housing and killing met the guidelines of the Italian Health Ministry.

At the end of the experimental period, rats were killed and used for energy balance measurements. Hyperthyroid rats were killed 16 h after the last T_3 injection.

At the time of death, the animals of each group had a mean and range of body weight equal to that of the surviving animals of the same group. Therefore, in our calculations of energy gain, we assumed that the surviving animals contained the same proportions of fat, protein and water in their carcasses as those in the killed animals.

Measurement of body composition

Rats were anesthesized with diethyl ether and blood was collected via the cava vein. After gut content removal, the carcasses were weighed, autoclaved for 90 min, chopped into small pieces, thoroughly mixed, and finally homogenized with water (volumes equal to twice the carcass weight) in a Polytron homogenizer (Kinematica AG., Lucerne, Switzerland). Aliquots of the homogenate were analyzed for lipid, protein, water and energy content. Lipid content was determined gravimetrically after extraction in chloroform–methanol and evaporation to constant weight by a rotating evaporator (Heidolh, Germany) by the method of Folch *et al*.[20] The energy as lipid was calculated from the lipid content using the coefficient of 39.2 kJ/g for the energy content of lipid. Protein content was measured using the Biuret method on samples of carcass homogenate treated with 5% SDS plus 0.5 M NaOH, as outlined by Brooks *et al*.[21] The value of 22.4 kJ/g was used for the energy content of protein. Water content was determined by the difference in weight of the homogenate before and after drying at 70°C in a vacuum oven. Then, small pellets (about 200 mg) of the dried homogenate were made and the body energy content was measured with a bomb calorimeter (Parr adiabatic calorimeter of Parr Instruments Co., Moline, Il, USA).

Energy balance measurements

Body weights and food intakes were monitored daily to allow calculations of body weight gain and gross energy intake. The feces, urine and spilled food were also collected daily, dried and ground to a powder before determining their energy content with the bomb calorimeter. The gross energy content of the stock diet was also determined by the bomb calorimeter.

Metabolizable energy (ME) intake was obtained by subtracting the energy measured in the feces, urine and spilled food from the gross energy intake as measured from daily food consumption. The gain in energy was obtained from the difference between the final body energy content at the end of each period and the energy content of the animals killed at the end of the previous period. Energy expenditure was calculated from the difference between ME intake and energy gain, and gross efficiency was calculated as the percentage of body energy retained per ME intake. Net energy expenditure was calculated from energy expenditure excluding the total cost of storage. The total cost of storage was determined, taking into account that the energy loss in storing 1 kJ of protein is 1.25 kJ,[22] while the corresponding

energy cost for fat deposition is 0.36 kJ/kJ for diets with a high percentage of carbohydrates,[22] such as the diet used in the present work.

Free triiodothyronine, leptin and free fatty acid serum concentrations

The thyroid state of the animals was monitored measuring serum free T_3 concentrations with radioimmunoassay kit (Sclavo, RIA Department, Cassina de Pecchi, Milano, Italy). Serum leptin concentrations were measured with radioimmunoassay kit (Linco Research, Inc., St Charles, MO, USA). Each hormone was measured in a single assay to remove inter-assay variations, while intra-assay coefficients of variation were 4% for T_3 and 9% for leptin. Free fatty acid (FFA) serum concentrations were measured using an enzymatic kit (Boehringer-Mannheim Biochemia, Milano, Italy).

Statistical analysis

Data are given as means ± s.e.m. of eight different rats. Correlation between selected parameters was performed by linear regression analysis. Significant differences between the slopes were investigated by calculating a *P*-value (two-tailed) testing the null hypothesis that the slopes are identical (the lines are parallel). The *P*-value answers this question: if the slopes really were identical, what is the chance that randomly selected data points would have slopes as different (or more different) than we observed. If the *P*-value is less than 0.05, it is concluded that the lines are significantly different. If the *P*-value for comparing slopes is greater than 0.05, the question is whether the lines are parallel or identical. A second *P*-value is calculated testing the null hypothesis that the lines are identical. If this *P*-value is low, it is concluded that the lines are not identical (they are distinct but parallel). Statistical comparison of the means was performed by two-way analysis of variance (ANOVA). *Post-hoc* comparisons between group pairs were made with the Bonferroni test. Probability values less than 0.05 were considered to indicate a significant difference. All statistical analyses were performed using GraphPad Prism 3 (GraphPad Software, San Diego, California, USA).

Materials

T_3 was purchased from Sigma Chemical Co., St Louis, MO, USA. PTU was purchased from Serva Feinbiochemica, Heidelberg, Germany. The reagents used for carcass analysis were of the highest purity commercially available.

Results

The thyroid state of the animals was monitored by measuring serum free T_3 concentrations (Table 1). Serum free T_3 concentrations in PTU-treated rats were about 10% of those found in control rats, while T_3-treated rats showed significantly increased serum free T_3 concentrations (about 50%) both after 7 and 15 days of treatment. Table 1 also shows leptin serum concentrations in rats in different thyroid states. Serum leptin concentrations significantly decreased in hyperthyroid rats after 7 and 15 days of treatment, while a significant increase was found in hypothyroid rats only after 15 days. Consequently, a highly significant inverse relationship was found between serum concentrations of T_3 and leptin (Figure 1). Finally, Table 1 reports data on FFA serum

Table 1 Serum free T_3, leptin and free fatty acids (FFA) in rats in different thyroid states

Days of treatment	*Serum free T_3 (pg/100 ml)*	*Serum leptin (ng/ml)*	*Serum FFA (μmol/l)*
7			
Euthyroid	390 ± 15	3.77 ± 0.32	124 ± 2
Hypothyroid	26 ± 1*	3.22 ± 0.42	54 ± 3*
Hyperthyroid	589 ± 28*	1.88 ± 0.29*	324 ± 7*
15			
Euthyroid	350 ± 6	4.40 ± 0.41	106 ± 6
Hypothyroid	31 ± 8*	6.51 ± 0.82*	42 ± 4*
Hyperthyroid	540 ± 28*	2.96 ± 0.14*	467 ± 1*

Values are the means ± s.e.m. of eight different rats. *$P < 0.05$ compared to euthyroid rats.

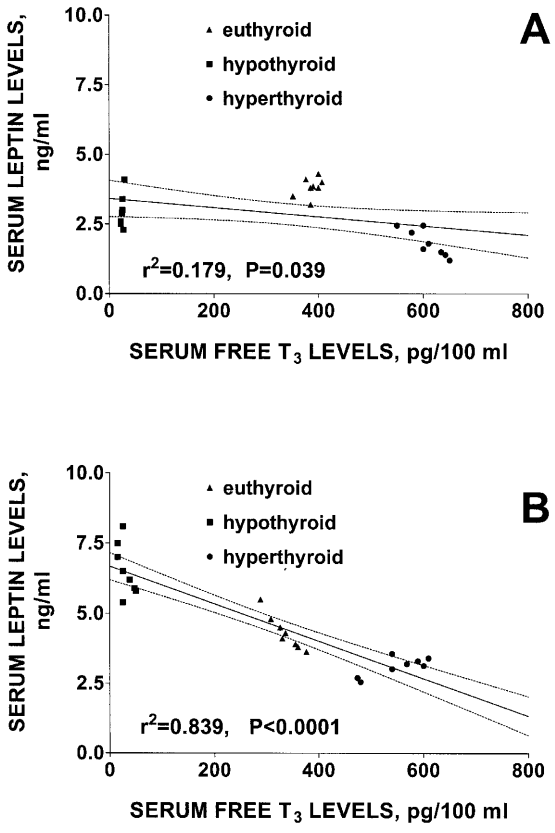

Figure 1 Correlation with 95% confidence interval between serum free T_3 and leptin concentrations in rats in different thyroid states after 7 (A) and 15 (B) days of treatment.

concentrations in rats in different thyroid states. Significantly higher FFA serum concentrations were found in hyperthyroid rats after 7 and 15 days of treatment, while hypothyroid rats exhibited significantly lower values after 7 and 15 days.

Figure 2 shows leptin/body fat correlation in rats in different thyroid states. Serum leptin concentrations were highly correlated with body fat mass in all the groups of rats. However, the regression lines obtained for rats in different thyroid states were significantly different ($P < 0.05$). A highly significant direct correlation was found between T_3 concentrations and ME intake or energy expenditure (Figures 3 and 4). On the other hand, a significant inverse correlation was found between serum leptin concentrations and ME intake or energy expenditure (Figures 3 and 4).

Figure 5 reports data on energy balance in rats in different thyroid states. In hypothyroid rats, ME intake, energy gain, lipid gain and energy expenditure significantly decreased in the period 0–7 and 7–15 days of treatment compared to euthyroid rats. T_3 administration caused a decrease in energy and lipid gain only in the period 0–7 days of treatment, while ME intake and energy expenditure significantly increased, although ME intake increased only in the period 7–15 days, compared to euthyroid rats.

Figure 6 shows changes in the percentage of body lipid and protein in rats in different thyroid states. Due to the PTU-pretreatment period, hypothyroid rats exhibited different body weight and composition compared to the other groups of rats. Therefore, percentage of body lipid and protein after 7 and 15 days of treatment are reported as the percentage of initial values of each group, to allow statistical comparison. Hypothyroid rats significantly increased their body lipid content after 7 and 15 days of treatment, while hyperthyroid rats exhibited lower lipid content only after 7 days of treatment. As for body protein content, hypothyroid rats had significantly lower values after 7 and 15 days of treatment, while no variation was found in hyperthyroid rats.

Figure 7 shows partitioning of lipid and protein intake in rats in different thyroid states. When lipid gain was expressed as a percentage of lipid intake, we found a significant increase in hypothyroid rats in all the experimental period and a significant decrease in hyperthyroid rats only in the period 0–7 days. Protein gain expressed as percentage of protein intake was significantly lower in hypothyroid rats in all the experimental periods. In addition, hyperthyroid rats exhibited lower protein gain/protein intake ratio only in the period 7–15 days.

Net energy expenditure and body energy gain, both expressed as a percentage of ME intake, in rats in different thyroid states are reported in Figure 8. Net energy expenditure expressed as a percentage of ME intake significantly increased in hypothyroid rats only in the period 7–15 days and in hyperthyroid rats in the periods 0–7 and 7–15 days. Body energy gain/ME intake ratio, called gross efficiency was significantly lower in hypothyroid rats in the period 7–15 days and in hyperthyroid rats in the periods 0–7 and 7–15 days.

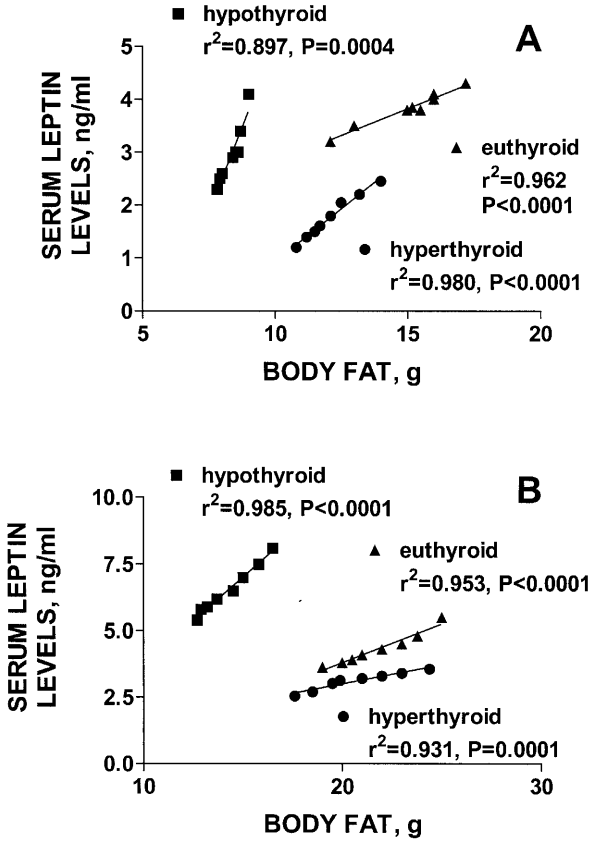

Figure 2 Correlation with 95% confidence interval between body fat mass and serum leptin concentrations in rats in different thyroid states after 7 (A) or 15 (B) days of treatment.

Discussion

In the present work we have studied energy and macronutrient balance in rats in different thyroid states. The above parameters were measured at two time points, ie after 7 and 15 days from the beginning of the treatment. This experimental approach allowed us to gain further insight into the time course of the variations elicited by altered thyroid state. In fact, the period 0–7 days of treatment allows us to determine some changes peculiar to a transition period from the euthyroid to the hypo- or hyperthyroid state, which are partly reversed after 15 days of treatment.

In hypothyroid rats a decrease in ME intake and energy expenditure together with a shift in lipid and protein partitioning was found after 7 and 15 days of treatment. In agreement with this shift, hypothyroid rats showed an increase in lipid gain/lipid intake ratio and a decrease in

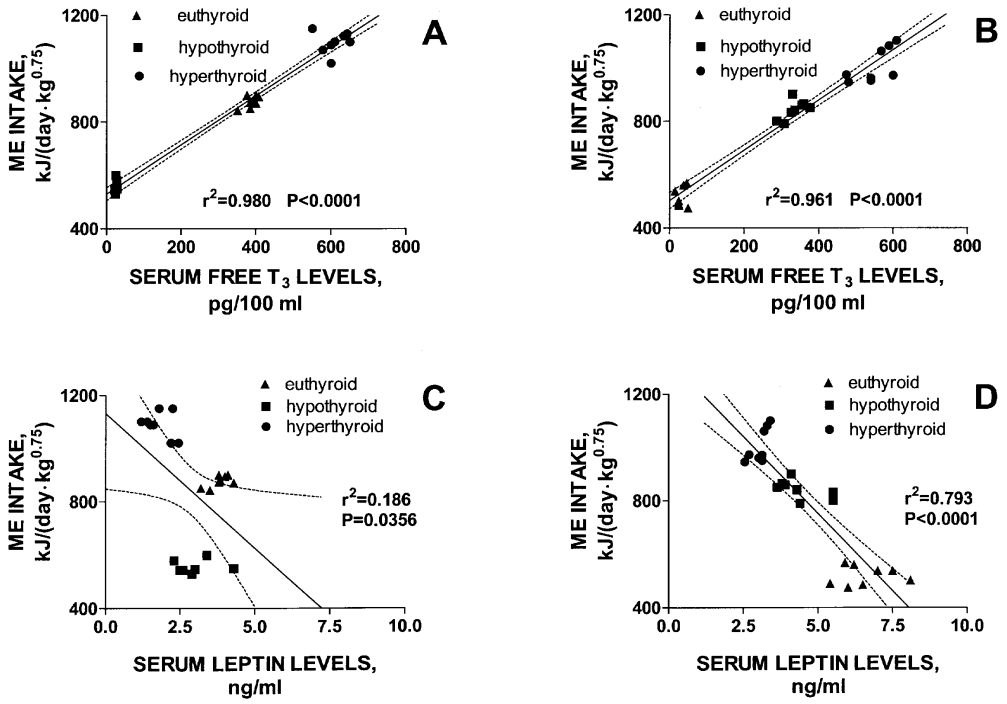

Figure 3 Correlation with 95% confidence interval between metabolizable energy (ME) intake and serum-free T_3 or leptin concentrations in rats in different thyroid states after 7 (A–C) and 15 (B–D) days of treatment.

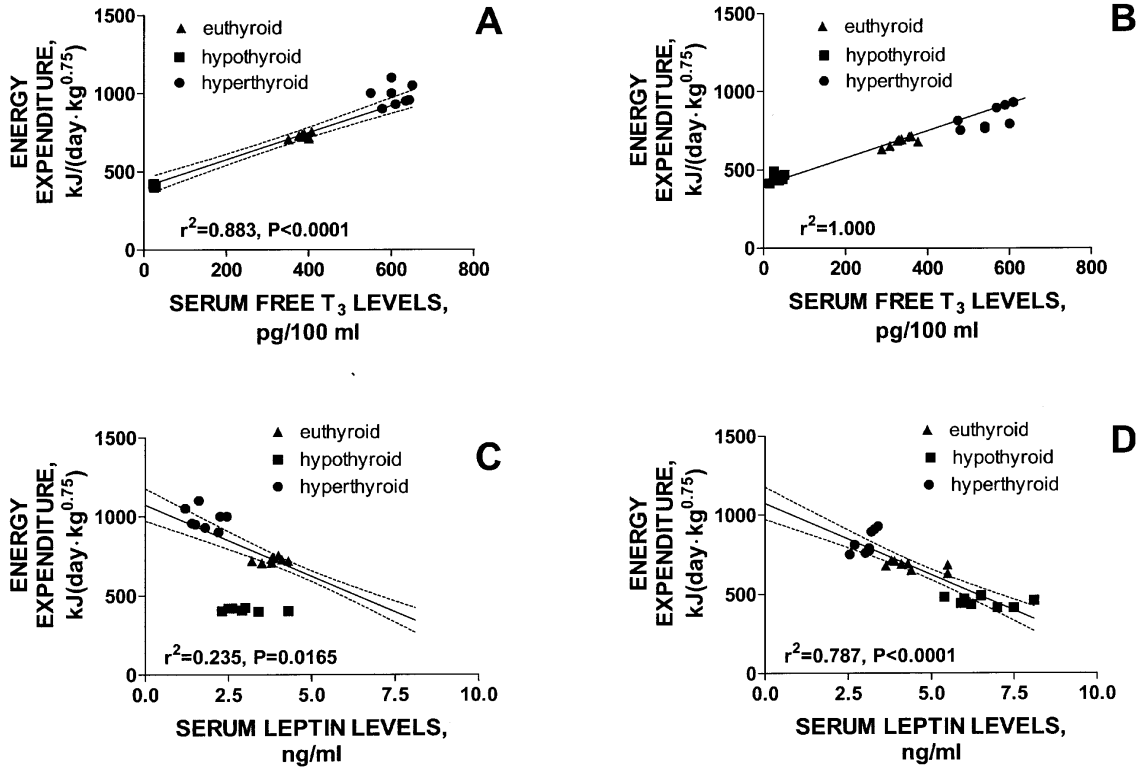

Figure 4 Correlation with 95% confidence interval between energy expenditure and serum-free T_3 or leptin concentrations in rats in different thyroid states after 7 (A–C) and 15 (B–D) days of treatment.

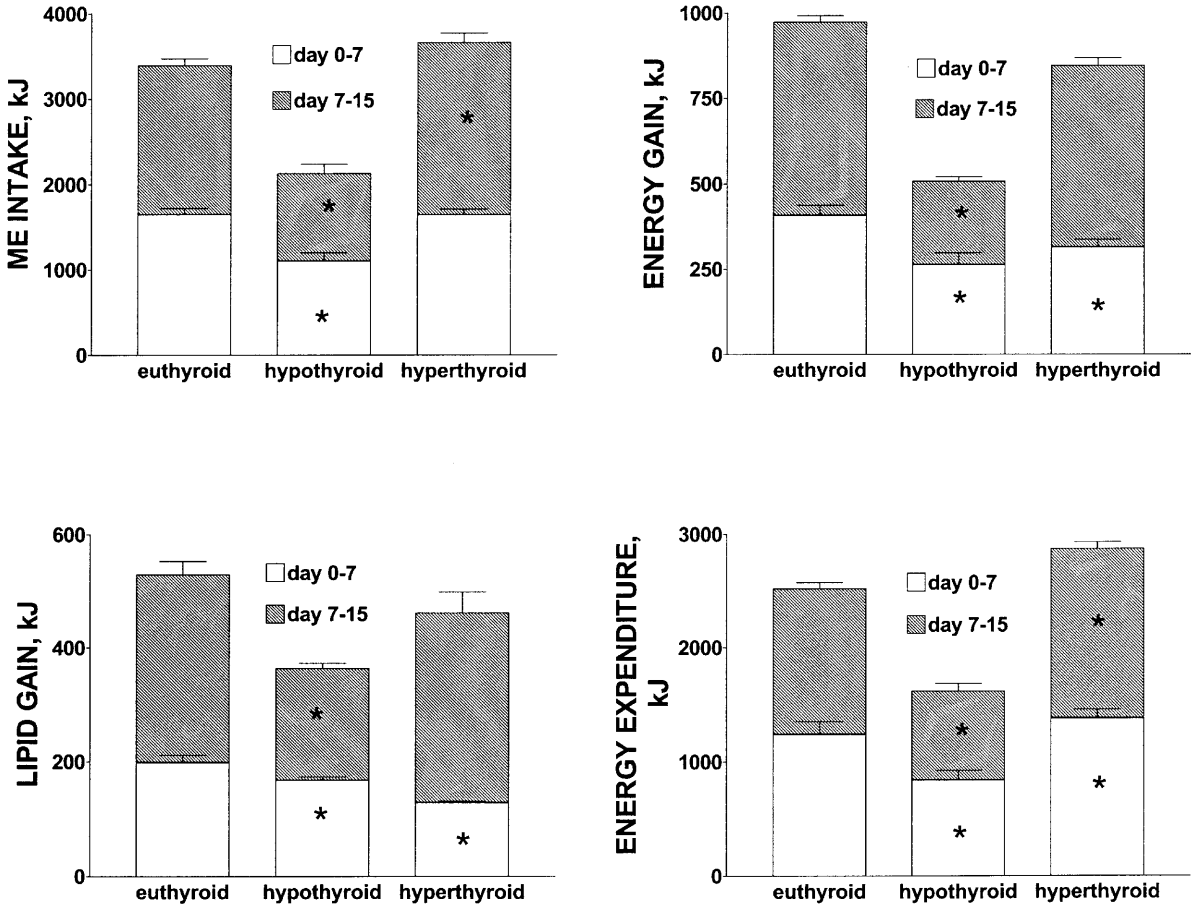

Figure 5 Metabolizable energy (ME) intake, energy expenditure, and energy and lipid gain in rats in different thyroid states. Values are the means ± s.e.m. of eight different rats. $*P < 0.05$ compared to euthyroid rats.

protein gain/protein intake ratio. Consequently, body lipid percentage significantly increased compared to euthyroid rats. The increased lipid gain found in hypothyroid rats is in agreement with the decrease in fatty acid oxidation activity found in these rats[23] and indicates a decrease in the utilization of fat as metabolic fuels, as also suggested by lower serum FFA concentrations found in hypothyroid rats. On the other hand, the lower protein gain can be explained by the reduced protein synthesis characteristic of the hypothyroid state,[23] as well as by the fact that reduced fat utilization for metabolic needs implies an increase in the utilization of amino acids as fuels. In addition, the present results on energy balance measurements are similar to our previous ones, obtained on hypothyroid rats fed a high-fat diet.[8] Taking together previous and the present results shows that, in the absence of T_3, energy and lipid balance is not maintained despite the increased serum leptin concentrations found in hypothyroid rats. It appears therefore that T_3 plays a major role in the maintenance of energy and lipid balance.

Concerning hyperthyroid rats, after 7 days of treatment there was an increase in energy expenditure while body energy gain and gross efficiency significantly decreased. However, unlike hypothyroid rats, hyperthyroid ones mainly utilize dietary lipids for metabolic needs, so that they are able to maintain a normal body protein content. In fact, we found a decrease in lipid gain/lipid intake ratio in hyperthyroid rats, compared to euthyroid ones, with no variation in protein gain/protein intake ratio. The increased lipid oxidation of hyperthyroid rats is in agreement with results showing that T_3 stimulates fatty acid oxidation[2] and enhances lipolysis by increasing the sensitivity of the process to catecholamines,[2] as well as with the increased serum FFA concentrations found here. In the period 7–15 days, hyperthyroid rats increased ME intake, so that increased metabolism is balanced by increased intake, and energy gain is similar to that found in euthyroid rats. These results are similar to those found previously by us in euthyroid rats fed a high-fat diet,[9–12] which exhibited higher serum T_3 concentrations.[9] In fact, these rats exhibited increased ME

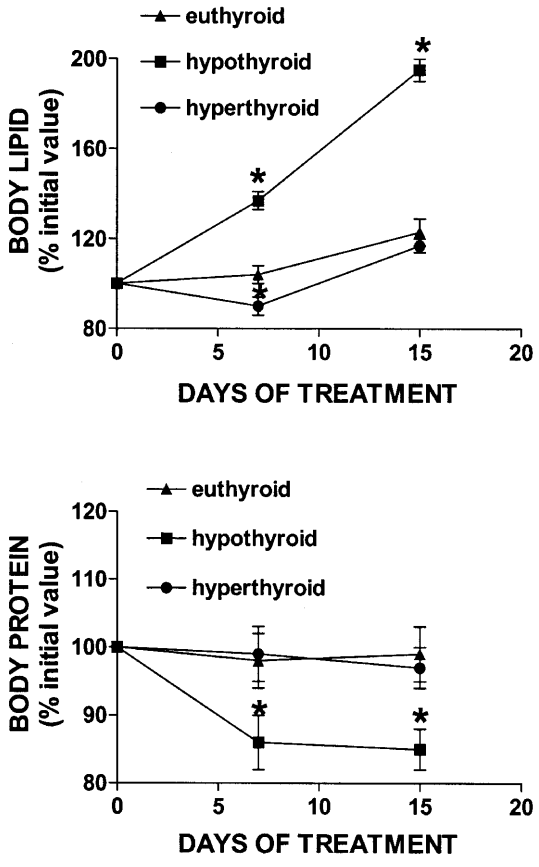

Figure 6 Lipid and protein body mass in rats in different thyroid states after 7 and 15 days of treatment. Values are the means ± s.e.m. of eight different rats and are reported as a percentage of initial values of each group. *$P < 0.05$ compared to euthyroid rats. Baseline values were: body weight (g): 103 ± 4 for eu- and hyperthyroid rats, and 67 ± 3 for hypothyroid rats; body lipid (g/100 g body weight): 8.2 ± 0.4 for eu- and hyperthyroid rats and 5.4 ± 0.2 for hypothyroid rats; body protein, (g/100 g body weight): 17.5 ± 0.8 for eu- and hyperthyroid rats and 19.9 ± 0.9 for hypothyroid rats.

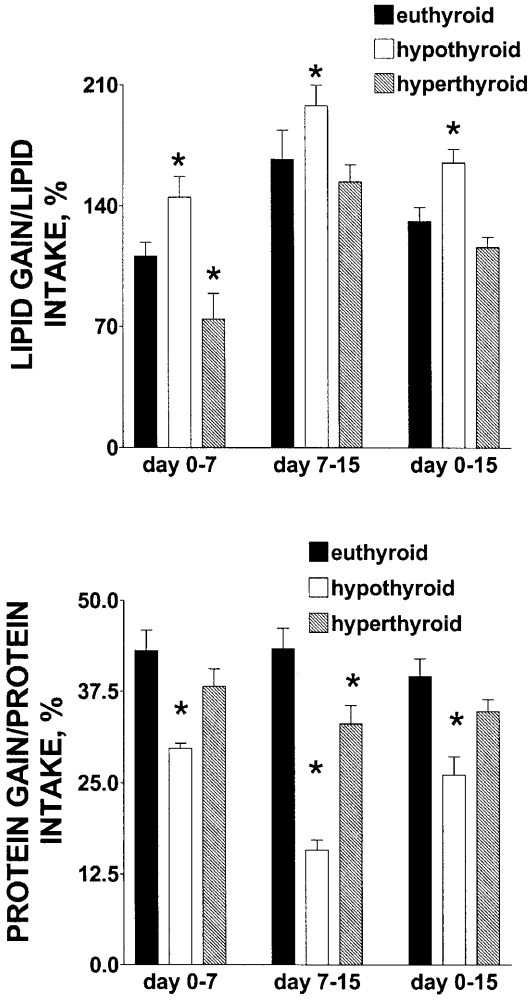

Figure 7 Lipid and protein gain, expressed as a percentage of lipid and protein intake, in rats in different thyroid states. Values are the means ± s.e.m. of eight different rats. *$P < 0.05$ compared to euthyroid rats.

intake and energy expenditure, with no variation in energy gain.[9–12] Our present results therefore suggest that T_3 could play a role in the modifications of energy balance found in rats fed a high-fat diet. Our results on hyperthyroid rats are in agreement with previous observations that oxygen consumption increases from the first day of T_3 treatment, while the increase in food intake appears after about 1 week of treatment.[1] The increased ME intake thus appears not to be due to increased T_3 serum concentrations directly, but probably to a progressive depletion of fat stores, a signal known to regulate energy intake and that takes some time to be generated.

Another objective of the present study was to gain further insight into the relationship between thyroid state and leptin secretion, since to date only a few studies have been carried out and the results obtained give conflicting evidence.[14–18] Here we show that serum leptin concentrations significantly increased in hypothyroid rats and decreased in hyperthyroid rats. The existence of an inverse correlation between thyroid state and leptin concentrations has been also suggested by others.[14–18] Since serum leptin concentrations reflect body lipid stores, the variation found in the present study could be due to changed thyroid state and/or to changed fat stores. To gain insight into the effect of varied T_3 concentrations on the secretion of leptin, independent of the size of adipose tissue stores, we studied the leptin/body fat relationship existing in rats in different thyroid states. A highly significant correlation between serum leptin concentrations and body fat was always found. However, when the regression lines obtained for the various groups of rats were compared, a significant difference between the three lines was obtained ($P < 0.05$). This result indicates that the relationship between leptin and body fat changes when thyroid state is altered, ie the same amount of fat gives significantly

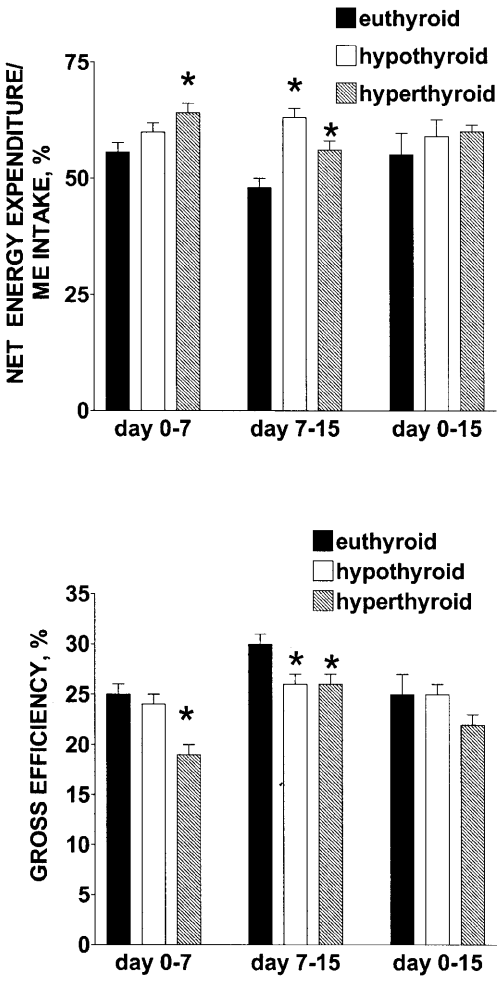

Figure 8 Net energy expenditure and body energy gain expressed as a percentage of metabolizable energy (ME) intake in rats in different thyroid states. Values are the means ± s.e.m. of eight different rats. *$P < 0.05$ compared to euthyroid rats.

higher leptin concentrations in hypothyroid rats and significantly lower concentrations in hyperthyroid ones. This result clearly indicates that an inverse relationship exists between T_3 and leptin serum concentrations, and that this relationship is not only the result of changes in body fat stores induced by changed T_3 concentrations. It has been recently shown that sympathetic nervous system inhibits leptin production in adipose tissue, and that this action is mediated by β_3-adrenergic receptors.[24–26] In addition, it is well known that T_3 regulates the density of β_3-adrenoreceptors as well as the sympathetic response of white fat.[27] It can therefore be suggested that the inhibitory action of T_3 on leptin production could be the result of the altered sensitivity of white fat to sympathetic stimuli. The above result is in agreement with some previous reports,[14–17] but it is in contrast with Syed *et al*.[18] This discrepancy could be due to higher dose of T_3 used in the above study compared to that used by us, as well as to the fact that in the above study hypothyroidism was induced by methimazole instead of PTU. Another important difference could be the age of the rats; Syed *et al*[16] used rats older than those used here (about 25 days old). Fat mass increases differently during development: in fact, from age 30 to 60 days there is an increase in fat cell size and number, while from 60 days onwards the fat cell number remains constant and fat cell size increase continues.[28] In addition, both increases are controlled by T_3, with a more marked effect on fat cell number.[28] Therefore, it can be suggested that our young rats, being in a phase of active growth of fat cell number, are more sensitive to changes in thyroid state. It is also possible that different phases of growth of the adipose tissue are associated with differences in the leptin secretion capacity, which could in turn be differently influenced by changes in thyroid state.

Linear regression analysis also shows a strong direct correlation between T_3 serum concentrations and ME intake or energy expenditure. This result is in agreement with the well-known stimulatory effect of T_3 on food intake and thermogenesis.[1–3] On the other hand, an inverse relationship between leptin serum concentrations and ME intake or energy expenditure was found. The last result is consistent with the inhibitory action of leptin on food intake, but is in contrast to the predicted thermogenic action of leptin.[4–7] However, to our knowledge, the thermogenic effect of leptin has been postulated on the basis of results obtained with pharmacological doses of leptin on leptin-deficient mice (*ob*/*ob*).[7,29,30] Therefore, the relationship between leptin and energy expenditure needs to be further clarified.

In conclusion, on the basis of the present results there is an interaction of circulating T_3 with the leptin system. In addition, the effect of thyroid hormone on leptin system seems to be inhibitory. Elucidation of this effect could be important for understanding of the mechanisms which regulate energy homeostasis.

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