

# TSH stimulates leptin secretion by a direct effect on adipocytes

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## Abstract

Leptin is a circulating hormone secreted by adipose tissue which acts as a signal to the central nervous system where it regulates energy homeostasis and neuroendocrine processes. Although leptin modulates the secretion of several pituitary hormones, no information is available regarding a direct action of pituitary products on leptin release. However, it has been pointed out that leptin and TSH have a coordinated pulsatility in plasma. In order to test a direct action of TSH on *in vitro* leptin secretion, a systematic study of organ cultures of human omental adipose tissue

was performed in samples obtained at surgery from 34 patients of both sexes during elective abdominal surgery. TSH powerfully stimulated leptin secretion by human adipose tissue *in vitro*. In contrast, prolactin, ACTH, FSH and LH were devoid of action. These results suggest that leptin and the thyroid axis maintain a complex and dual relationship and open the possibility that plasmatic changes in TSH may contribute to the regulation of leptin pulses.

*Journal of Endocrinology* (2003) **176**, 7–12

## Introduction

Leptin, the adipocyte-derived hormone, participates in the regulation of energy homeostasis by providing the central nervous system with information on the state of the adipose tissue reserves (Zhang *et al.* 1994, Casanueva & Dieguez 1999). In turn, leptin receptors at the hypothalamus activate the efferent loop leading to control of food intake and energy expenditure as a form of regulating energy stores (Campfield *et al.* 1995, Ahima *et al.* 1996, Casanueva & Dieguez 1999). In the currently accepted paradigm, leptin should be viewed as a message that is mostly operative when plasma levels are reduced due to shortage of food; thus, plasma leptin reduction activates the complex neuroendocrine and behavioural response to fasting (Ahima *et al.* 1996). In addition, leptin modulates the function of several endocrine axes, such as the somatotroph (Carro *et al.* 1999, 2000), gonadal (Mantzoros 2000, Caprio *et al.* 2001), corticotroph (Heiman *et al.* 1997) and thyroid (Legradi *et al.* 1997, 1998) axes. Through leptin, the nutritional situation of a given individual is communicated to and modulates the other endocrine activities (Casanueva & Dieguez 1999).

One of the most surprising points of leptin physiology is its pulsatility in plasma (Licinio *et al.* 1997). In fact, it is not easily understandable how a hormone produced by millions of adipocytes widely distributed throughout the body

may be synchronized to show pulses. In a comprehensive study Mantzoros *et al.* (2001) have shown that leptin and plasma thyrotrophin (TSH) levels are both highly organized and pulsatile, with similar circadian rhythms, and using a cosinor analysis they showed near superimposable peak values. This, and other studies analysing leptin and the thyroid axes, strongly suggest that leptin regulates, at least partially, TSH secretion in man (Seoane *et al.* 2000).

Although leptin modulation of the thyroid axis is well accepted, the action of the thyroid axis components on leptin secretion has not been fully characterized. Thyroid hormones *in vitro* (Menendez *et al.* 2001) or *in vivo* (Corbetta *et al.* 1997, Sreenan *et al.* 1997, Valcavi *et al.* 1997) are not particularly relevant to the secretion of leptin in man. However, the action of TSH over leptin secretion has not, to our knowledge, been assessed. On theoretical grounds at least, such a control is feasible, as we are now well aware that plasma leptin levels do not just have a stoichiometric relationship with fat mass. On the contrary, leptin levels reflect fat mass plus the action of several circulating hormones and factors, which directly operate on the adipocytes releasing leptin (Considine *et al.* 1997, Casabiell *et al.* 1998, Menendez *et al.* 2000).

In the present work we have taken advantage of a well-characterized and validated organ culture model of human adipose tissue (Casabiell *et al.* 1998, Piñeiro *et al.* 1998), to test *in vitro* the action of TSH over leptin

secretion, taking as an internal control other pituitary hormones. The working hypothesis was that the connection between the thyroid axis and leptin may well be exerted in part through TSH.

## Materials and Methods

Omental adipose tissue was obtained from 34 patients during elective abdominal surgery. The tissue donor group was composed of 17 females (age  $66 \pm 4$  (S.E.) years; body mass index (BMI)  $25.44 \pm 1.15$ ) and 17 males (age  $65 \pm 4$  years; BMI  $28.45 \pm 1.57$ ). Patients were taking no drugs or antibiotics and the presence of malignancy was an exclusion criteria. The study was approved by the Hospital Ethical Committee, and each participating subject provided informed consent. Excised adipose tissue was immediately transported to the laboratory in ice-cold Krebs–Ringer–Hepes buffer (NaCl, 125 mM; KCl, 5 mM; MgSO<sub>4</sub>, 1.2 mM; CaCl<sub>2</sub>, 2 mM; KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM; glucose, 6 mM; Hepes, 25 mM; pH 7.4). After removal of blood vessels and connective tissue, adipose tissue was washed with sterile Krebs–Ringer–Hepes and cut into small pieces with sharp scissors. Tissue fragments were placed in six-well dishes (300–400 mg adipose tissue/well) containing 2.5 ml Dulbecco's modified Eagle's medium plus 0.5% fetal calf serum, supplemented with penicillin (100 U/ml) and streptomycin sulphate (100 µg/ml). After a preincubation period of 1 h at 37 °C under a humidified atmosphere of 95% air–5% CO<sub>2</sub>, the medium was aspirated and 2.5 ml fresh medium were dispensed into each well. Culture medium was then collected every 24 h and replaced with fresh medium, again with or without stimuli, to obtain the 24 h secretion and the cumulative secretion until 48 h. The adipose tissue of each donor was incubated in triplicate for each tested variable (either untreated or treated samples), either hormone or dose, and the final value of each individual was the pooled value.

In order to evaluate the possible effect of the pituitary hormones on *in vitro* leptin secretion, prolactin (PRL), adrenocorticotrophin (ACTH), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and TSH were tested at different doses ( $10^{-13}$ ,  $10^{-11}$ ,  $10^{-9}$ ,  $10^{-7}$  M) with respect to control samples with the appropriate vehicle. Although in pilot experiments it was demonstrated that this organ culture was viable for more than 7 days, the changes elicited by pituitary hormones were observed only in the first 2 days, followed by a plateau in leptin release thereafter. For such reasons leptin values were analysed in the first 48 h. Histological, toluidine blue staining, and biochemical analysis, lactate dehydrogenase (LDH) release to the medium, showed that there was no tissue damage during culture. Samples were stored at  $-20$  °C until leptin assay.

PRL was obtained from the US National Institute of Diabetes, Digestive and Kidney Diseases National

Hormone and Pituitary Program (Bethesda, MD, USA). The others compounds and reagents were obtained from Sigma.

## Leptin assay

Leptin levels were measured by RIA using commercial kits (Human Leptin RIA; Linco, St Charles, MO, USA). The limit of sensitivity was 0.5 ng/ml, the intra-assay coefficient of variation was 8.3% and the interassay coefficient of variation was 6.2%.

## Statistical analysis

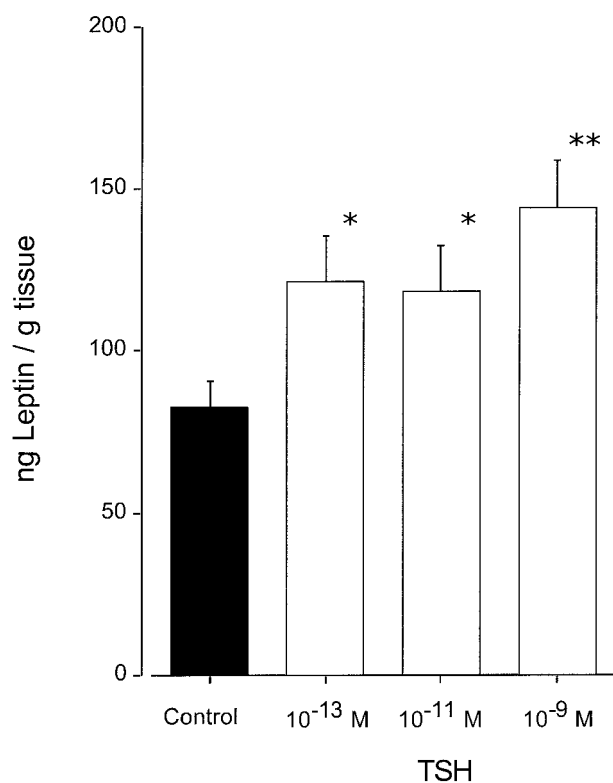
Leptin secretion is expressed as the total amount of leptin secreted into the well by a given sample (in ng/ml) with respect to total volume and divided by the amount of fat tissue in grams, i.e. ng leptin/g tissue. Unless otherwise specified, all data are presented as means  $\pm$  S.E. The *t*-test for paired data was used to evaluate leptin secretion over control samples, and  $P < 0.05$  was considered significant. Statistical analyses were made using Statview 5 software for Windows (SAS Institute Inc., Cary, NC, USA, 1999).

## Results

Spontaneous leptin secretion by omental adipose tissue to the incubation medium was progressive in control samples ( $n=34$ ) of both sexes from 0 to 48 h (data not shown). Histological and biochemical analysis showed that there was no tissue damage throughout the incubation period, tested by toluidine blue staining and LDH release to the medium, in accord with previous studies (Casabiell *et al.* 1998, Piñeiro *et al.* 1998, 1999, Menendez *et al.* 2000, 2001, Peino *et al.* 2000). As both treated and untreated samples exhibited significant leptin release in the first 48 h, plateauing thereafter, it was decided to analyse only the first 48 h period. As no gender-based differences were observed in any test, individual values were pooled.

A clear-cut stimulation of leptin secretion was observed when the adipose tissue fragments from seven donors ( $n=7$ ) were incubated in the presence of TSH ( $10^{-13}$ ,  $10^{-11}$ ,  $10^{-9}$  M); in particular, the leptin secretion rate was potently stimulated at the 48 h period at the concentration of  $10^{-9}$  ( $P < 0.0005$ ),  $10^{-11}$  ( $P < 0.05$ ) and  $10^{-13}$  M ( $P < 0.005$ ) (Fig. 1).

When the adipose tissue fragments from eight donors were incubated in the presence of PRL ( $10^{-11}$ ,  $10^{-9}$ ,  $10^{-7}$  M), a slight inhibition in the leptin secretion rate was observed at  $10^{-11}$  M ( $P < 0.05$ ) but not at other doses. The incubation with ACTH ( $10^{-11}$ ,  $10^{-9}$ ,  $10^{-7}$  M) ( $n=8$ ), FSH ( $10^{-13}$ ,  $10^{-11}$ ,  $10^{-9}$  M) ( $n=9$ ) and LH ( $10^{-13}$ ,  $10^{-11}$ ,  $10^{-9}$  M) ( $n=8$ ) did not induce significant



**Figure 1** Effect of TSH administration on *in vitro* leptin secretion by human omental adipose tissue cultures ( $n=7$ ). Means  $\pm$  S.E. \* $P<0.05$  vs control, \*\* $P<0.005$  vs control.

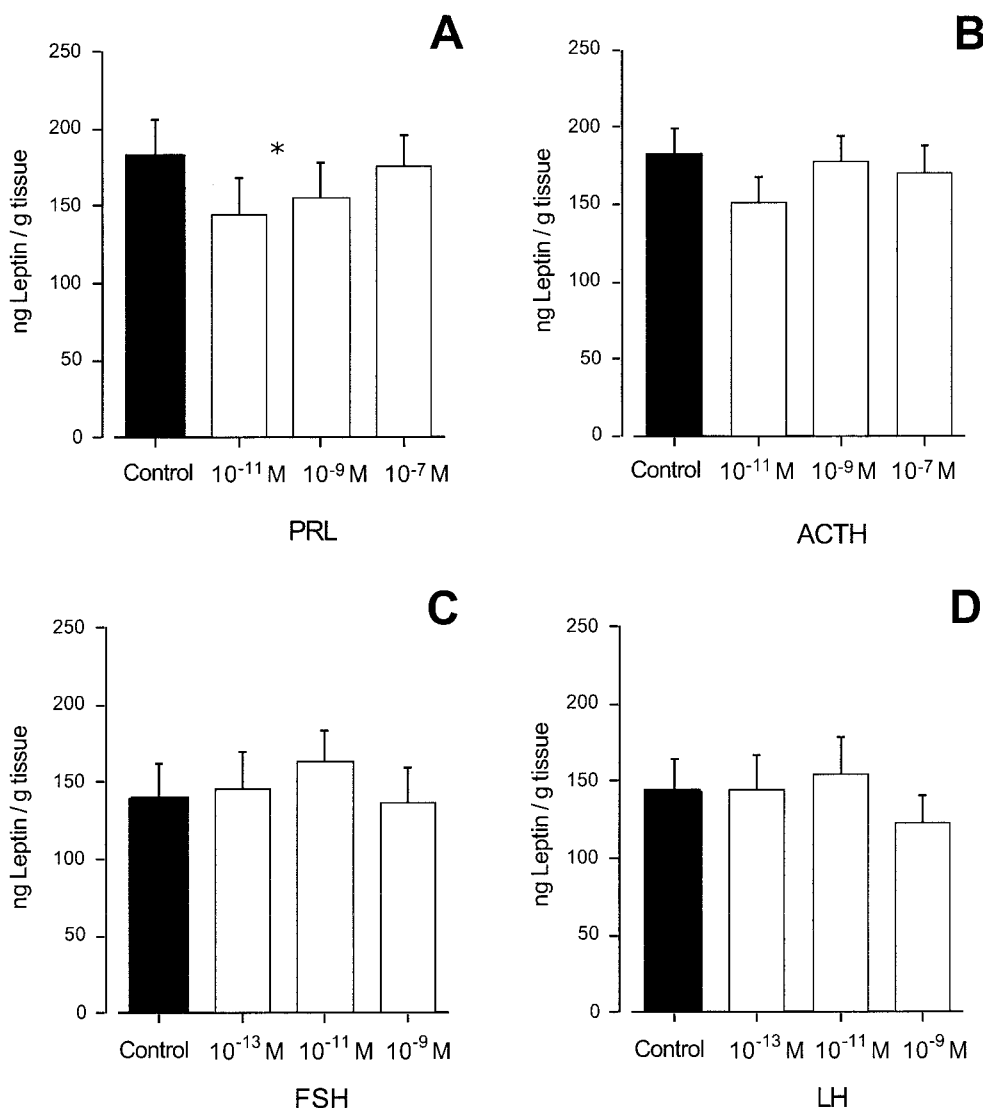
changes in leptin secretion rates from adipose tissue culture (Fig. 2).

## Discussion

In the present work it has been unambiguously demonstrated, and to the best of our knowledge for the first time, that TSH releases leptin by a direct action at the adipocytes in human adipose tissue. This result fits with the presence of TSH receptors in adipose tissue, frequently reported, but until now devoid of physiological action (Sorisky *et al.* 2000). In support of the specificity of the action observed, other pituitary hormones, such as PRL and ACTH, were devoid of action, and the same lack of direct activity was observed for pituitary hormones with similar structures to TSH, such as LH and FSH. These data supply information for understanding the variables controlling leptin levels in plasma and support the view that net adipose mass is the main determinant of these levels, but that in addition the action of steroid (Casabiell *et al.* 1998, Piñeiro *et al.* 1999) and non-steroid hormones (Menendez *et al.* 2001) plus other non-hormonal factors (Piñeiro *et al.* 1998, Peino *et al.* 2000), determine the final level of leptin release into

plasma. The organ culture model used in this work shows a considerable advantage, such as simplicity and convenience of manipulation, facts highly relevant when dealing with adipose tissue. Dispersed adipose tissue culture presents the disadvantage of disruption of the structural framework plus elimination of the small-diameter adipocytes. In addition, isolated adipocytes float, making cultures cumbersome. The present model has been employed successfully by us (Casabiell *et al.* 1998, Piñeiro *et al.* 1998, 1999, Menendez *et al.* 2000, 2001, Peino *et al.* 2000) and other groups (Gottschling-Zeller *et al.* 1999, Williams *et al.* 2000, Tomlinson *et al.* 2001), and strict control studies showed no damage up to 7 days of incubation. Although leptin release should be detected after just a few hours of incubation, the amount released by the small pieces of tissue employed is very small. The system of collecting media every 24 h allows assay of the cumulative secretion of leptin. As both spontaneous and stimulated leptin secretion was observed in the first 48 h, released leptin being at a plateau thereafter, values were calculated in the first 48 h period. That situation is different from the leptin release elicited by nuclear hormones, such as glucocorticoids, that may be observed over several days, but it is in keeping with the prompt action of protein hormones operating on surface receptors.

The relationship between the thyrotrophin-releasing hormone (TRH)–TSH–thyroid hormone axis and leptin is complex but meaningful. In fact, it is a well known fact that food deprivation, a condition associated with low plasma leptin concentrations, leads to low thyroid hormone levels plus decreased synthesis of TRH at hypothalamic structures and decreased TSH synthesis at the pituitary (Orban *et al.* 1998). Acting as an afferent signal to the brain, leptin administration to food-deprived animals is able to restore the decreased proTRH mRNA in the neurons of the paraventricular nucleus (Legradi *et al.* 1997, 1998). This increase in TRH may explain why leptin administration raises TSH levels, and normalizes the reduced thyroid hormone levels in euthyroid food-deprived rats (Ahima *et al.* 1996, Legradi *et al.* 1997). However, studies in humans (Rosenbaum *et al.* 2000, 2002) have shown that alteration in body weight may reduce leptin, and thyroid hormone, without altering TSH values, while other reports have described a leptin action on TSH release (Seoane *et al.* 2000). As the reduction in thermogenesis, due to a reduction in thyroid hormone levels, is one of the most remarkable adaptive responses to fasting, it is easy to imagine that low energy intake leads to reduced leptin levels and that this reduction acting at the level of the hypothalamus induces a step-wise reduction in TRH, then in TSH, and finally in thyroid hormones, with the ensuing final adjustment of the general metabolism of the individual to the new situation. In support of this hypothetical mechanism, some patients with leptin deficiency due to genetic mutations exhibit hypothalamic hypothyroidism (Clement *et al.* 1998). The efferent loop of



**Figure 2** Effect of (A) PRL ( $n=8$ ), (B) ACTH ( $n=8$ ), (C) FSH ( $n=9$ ) and (D) LH ( $n=8$ ) administration on *in vitro* leptin secretion by human omental adipose tissue cultures. Means  $\pm$  S.E. \* $P<0.05$  vs control.

the leptin–thyroid axis relationship has also been studied, in part supported by the known alteration in body weight that occurs in thyroid diseases. However, both in experimental animals (Escobar–Morreale *et al.* 1997, Fain *et al.* 1997), and in humans (Corbetta *et al.* 1997, Sreenan *et al.* 1997, Valcavi *et al.* 1997, Leonhardt *et al.* 1998, Pinkney *et al.* 1998, Yoshida *et al.* 1998), no data have demonstrated in a definitive way that thyroid hormones may change circulating leptin levels, and the scarce leptin changes observed were explained by parallel alterations in body weight of the subjects under observation. Supporting that lack of action of thyroid hormones, studies with human adipose tissue showed no changes in leptin release when incubated with significant concentrations of thyroid

hormones *in vitro* (Menendez *et al.* 2001). The present report of a direct action of TSH on leptin secretion, exerted directly at the adipocyte level in human tissue, adds a new dimension to the complex relationship between the two points of the system, although it must be remembered that other factors continuously operate superimposed on the TSH actions in order to modulate the final levels of leptin in plasma.

The TSH-mediated leptin release may be of interest when trying to understand the leptin pulsatility in plasma. Until now no acceptable explanation has been provided for the unexpected situation of pulsatility of a hormone produced by millions of adipocytes dispersed throughout the body. Obviously, such a disperse production would

need a synchronizer or a pace-maker coming from outside the adipose tissue. One candidate could be the sympathetic nervous system that powerfully interacts with the adipose tissue, but other contributing factors should exist, and one of them may well be TSH, which, as opposed to TRH, circulates in significant concentrations and exhibits strong pulsatility. In fact, Mantzoros *et al.* (2001) have shown in a systematically and mathematically validated analysis that TSH and leptin exhibit coordinated pulsatility. As the amount of leptin released in the tissue culture model is low, the cumulative amount released was analysed at 48 h, thus it was not possible to test if short-time (minutes) TSH pulses may elicit short-term leptin discharges. Although the results given in the present study do not demonstrate that TSH participates even minimally in leptin pulsatility, such an action is possible.

In order to analyse whether the actions of TSH were specific or, on the contrary, shared by other pituitary hormones, some structurally related and unrelated pituitary hormones were also studied, with negative results. Changes in PRL levels have been associated with alterations in leptin levels suggesting a positive relationship (Gualillo *et al.* 1999), a fact that may well explain the known association of hyperprolactinaemia and obesity (Nunes *et al.* 1980, Greenman *et al.* 1998). However, there is no support either in experimental animals (Gualillo *et al.* 1999) or in the present work, for a direct PRL action on the adipocyte production of leptin. Similarly, hypercorticotid states are associated with enhanced leptin levels (Leal-Cerro *et al.* 1996, Masuzaki *et al.* 1997) and glucocorticoids are potent inducers of leptin synthesis and secretion at the adipose tissue level (Considine *et al.* 1997, Casabiell *et al.* 1998), but no data link ACTH with a direct action on the adipocyte and the present results also discard that mechanism. Finally, while the action of leptin over gonadotrophin-releasing hormone secretion by the hypothalamus is well supported (Yu *et al.* 1997, Carro *et al.* 1999, Magni *et al.* 1999), no direct interaction between LH or FSH and leptin has been reported previously or observed in this work. All these negative results with hormones, other than TSH, lend further support to the specificity of the TSH action and its relevance. As a final observation, no gender-based differences were observed in the leptin secreted by the adipose tissue fragments when exposed to the pituitary hormones. That is a relevant observation when considering that adrenal and gonadal steroids show a marked difference in their action upon *in vitro* leptin secretion depending on the sex of the tissue donor (Casabiell *et al.* 1998, Piñeiro *et al.* 1999). These results suggest that, contrary to hormone actions on nuclear receptors, hormones operating through surface receptors have no gender-based specificity.

In conclusion, TSH significantly stimulates leptin secretion by human adipose tissue *in vitro*, suggesting a new mechanism in the interrelationship between the adipose tissue organ and the thyroid axis.

## Acknowledgements

This work was supported by grants from Fondo de Investigación Sanitaria, Spanish Ministry of Health and the Xunta de Galicia and by grant 009906153187 from MURST (40%, Rome, Italy) and CISD (Rome, Italy). Jesus P Camina is a recipient of a Research Contract from the Spanish Ministry of Health, Fondo de Investigación Sanitaria, Institute of Health Carlos III at Research Area of the CHUS.

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Received 11 September 2002

Accepted 17 September 2002