Leptin regulates GH secretion in the rat by acting on GHRH and somatostatinergic functions

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Abstract

Leptin is a hormonal product of adipose tissue whose expression reflects the body state of nutritional reserves. Previous experiments have demonstrated that leptin is one of the metabolic signals capable of regulating GH secretion. The aim of the present study was to evaluate whether CNS-mediated mechanisms underlie the GH-releasing activity of leptin.

Freely moving mature male rats were injected i.c.v. with leptin or isovolumetric amounts of diluent once daily for 3 days and were killed 2 h after the last administration. Central injection of leptin increased pituitary GH mRNA

levels by 53.2% and hypothalamic GHRH mRNA by 61.8%, and reduced somatostatin mRNA levels by 41.5%. To evaluate the direct effect of leptin on the pituitary, it was added alone or in combination with GHRH to primary cultures of anterior pituitary cells. Addition of leptin (10^{-11} – 10^{-7} M) did not alter basal GH release nor the GH-releasing activity of GHRH. These results demonstrate that leptin is a metabolic signal that regulates GH secretion in the rat by acting on hypothalamic GH-regulatory hormones.

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Introduction

Leptin is synthesized and secreted from the adipose tissue, and it is proposed to act as a satiety factor that limits food intake and regulates energy metabolism (Campfield *et al.* 1996). Genetic mutation of leptin, as in ob/ob mice, or of the leptin receptor, as in db/db mice, leads to obesity which is characterized by enhanced food intake and a reduced rate of metabolism (Campfield *et al.* 1996).

The concept that leptin could also regulate hypothalamo–pituitary function has rapidly emerged. Indeed, treatment of obese ob/ob mice with leptin not only corrected for their excessive food intake and body weight, but also restored fertility through an activation of the hypothalamo–pituitary gonadal axis (Barash *et al.* 1996, Chehab *et al.* 1996).

Previous experiments have also demonstrated that leptin can be added to the list of the metabolic signals capable of regulating growth hormone (GH) secretion. In the rat, central administration of leptin totally prevented the disappearance of pulsatile GH secretion occurring after 3 days of fasting (Carro *et al.* 1997, Vuagnat *et al.* 1997). Demonstration that i.c.v. administration of an antiserum to leptin abolishes GH secretion (Carro *et al.* 1997) indicates that disappearance of the leptin signal rapidly alters the neuroendocrine control of GH secretion in fed rats

also. The aim of the present study was to clarify the mechanism(s) underlying the leptin effect on GH release.

Materials and Methods

In vivo experiments

Mature (8 months of age) Sprague–Dawley male rats were purchased from Charles Rivers (Calco, Italy). They had free access to standard laboratory chow and water. Room temperature was maintained between 21 ° and 23 °C in a 12 h light:12 h darkness cycle.

A polyethylene cannula (PE-10) was implanted into the right lateral ventricle of rats, under xylazine–ketamine anaesthesia, as previously described (Altaffer *et al.* 1970). The placement of the i.c.v. cannula was carefully verified at the time of killing. One week later, at 0900 h, animals were given, through the cannula, 10 µg recombinant leptin (Cabru, Milan, Italy) in 10 µl PBS (0.01 M). Control rats received isovolumetric amounts of leptin vehicle. The same treatment was repeated on the following 2 days at the same hour.

Food eaten during 24 h was recorded at 1100 h each day.

On the third day, 2 h after the last drug administration, i.e. at 1100 h, fed rats were killed by decapitation and

trunk blood was collected in EDTA-coated tubes for hormone determination. The whole brain was rapidly removed from the skull and the hypothalamus was carefully dissected out with ophthalmic scissors. Two coronal cuts (anteriorly at the level of the optic chiasma and posteriorly at the level of the mamillary bodies) were followed by two parasagittal cuts along the hypothalamic sulci and a final cut dorsally at a depth of 3–4 mm from the ventral surface of the tissue block. The tissues were rapidly frozen in pulverized dry ice. The whole pituitary was then rapidly removed from the sella turcica, the posterior lobe was discarded, and the anterior lobe was rapidly frozen.

In vitro experiments

Pituitary GH mRNA Total RNA was isolated from each pituitary by the single-step acid guanidinium thiocyanate–phenol–chloroform extraction method. Total RNA samples (15 µg/sample) from the pituitary were run on a 1.2% formaldehyde/agarose gel and transferred to nylon membranes (Hybond N, Amersham International, Little Chalfont, Bucks, UK). Filters were hybridized with a rat GH cDNA sequence kindly provided by Dr F De Noto (University of California, San Francisco, CA, USA). The probe was labelled using the Megaprime DNA labelling system (Amersham) with [α - 32 P]dCTP to a specific activity of 1×10^9 d.p.m./µg DNA. The size of the GH mRNA transcript detected by Northern hybridization, 0.8 kb, corresponded to that reported previously (Seeburg *et al.* 1977). Control of the amount of the RNA loaded was performed by reprobating the filters with [α - 32 P]dCTP-labelled β -actin cDNA. After hybridization, autoradiography was carried out at -70°C for 12 h with intensifying screens using Hyperfilm-MP (Amersham).

Hypothalamic GH-releasing hormone (GHRH) and somatostatin (SS) mRNA Total RNA was isolated from each hypothalamus by the single-step acid guanidinium thiocyanate–phenol–chloroform extraction method. Total RNA samples (15 µg/sample) were loaded on a slot blotter and the nylon membranes were hybridized with cDNA probes specific for rat GHRH (prGRF2, kindly provided by Dr K E Mayo, Northwestern University, Evanston, IL, USA) and rat SS (pSR-1 kindly provided by Dr R H Goodman, Oregon Health Center, Portland, OR, USA), already used in other experiments (De Gennaro Colonna *et al.* 1993). The specificity of the GHRH and SS probes was assessed in preliminary Northern blots in which both GHRH and SS mRNAs appeared as single transcripts of respectively 0.75 and 0.65 kb, corresponding to the reported size of GHRH (Mayo *et al.* 1985) and SS mRNA (Goodman *et al.* 1983). Probes were labelled by random primer (Megaprime,

Amersham) with [α - 32 P]dCTP to a specific activity of 1×10^9 d.p.m./µg DNA. After exposing the blots, the 32 P-labelled specific probes were stripped off and the membranes were rehybridized with a rat β -actin cDNA probe to correct for the amount of RNA applied. Autoradiography was carried out at -70°C with intensifying screens for 2–4 days. For GHRH and SS mRNA, as well as for GH mRNA, quantification of hybridization signal was performed using a computerized image analyzer, an acquisition system driven by a Macintosh computer and the 'Image' software (written by Wayne Rasband, and kindly provided by the National Institutes of Health, Bethesda, MD, USA).

mRNA levels were normalized for RNA loading and expressed as a percentage of the control group values.

Pituitary cell culture Pituitary tissue used for cell dissociation included only the anterior lobe (see above). Briefly, pituitary glands were collected in sterile F-10 medium (Sigma, St Louis, MO, USA), cut into small fragments and incubated twice for 15 min at 37°C in 5 ml F-10 medium containing 6% fetal calf serum and collagenase (2.5 mg/ml) (Boehringer Mannheim GmbH, Mannheim, Germany). Fragments were then washed in Dulbecco's PBS and Ca^{2+} - and Mg^{2+} -free medium (Sigma, Milan, Italy) and mechanically dissociated by trituration with a siliconized glass pipette. Single-cell suspensions were plated onto 24-well culture plates (5×10^5 cells/well). The cells were incubated in 1 ml F-10 medium supplemented with 10% horse serum, 4% fetal calf serum and gentamycin (25 µg/ml) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C . After 3 days, the medium was removed and replaced with 1 ml F-10 containing 0.1% BSA only, or leptin (10^{-11} – 10^{-7} M), or 10^{-7} M GHRH (human GHRH-44, Bachem, Bubendorf, Switzerland) or 10^{-7} M leptin and 10^{-7} M GHRH. Media collected at the end of the incubation periods were immediately frozen and stored at -20°C until assayed for GH.

Assays for GH, insulin, corticosterone and glucose in culture media GH concentrations in media of dissociated pituitary cells were determined by RIA with reagents kindly provided by the NIADDK-NHPP Programme. Results were expressed in terms of the growth hormone releasing peptide-2 (GHRP-2) reference standard. The sensitivity of the assay was 0.5 ng/ml. Inter- and intra-assay variabilities were lower than 10%.

Insulin and corticosterone were assayed by RIA methods using commercial kits provided by ICN (Costa Mesa, CA, USA). The sensitivity of the insulin assay was 2.5 µU/ml, that of corticosterone 10 ng/ml.

Glucose was assayed by an enzymatic colorimetric method using a commercial kit provided by Boehringer Mannheim.

Figure 1 Northern blot analysis of pituitary GH mRNA from diluent-treated (A) and leptin-treated (B) rats. Electrophoresis was performed with 15 µg aliquots of total RNA in a 1.2% formaldehyde–agarose gel. The positions of the 28S and 18S ribosomal RNAs and of the GH mRNA are shown.

Statistics

Data are reported as means $\pm$ S.E.M. The significance of differences among groups was evaluated by Student's *t*-test for unpaired data or Dunnett's *t*-test for multiple comparisons, preceded by one-way ANOVA, where appropriate. A probability of $P < 0.05$ was taken to be statistically significant.

Results

Single i.c.v. administration of leptin did not modify daily food intake (data not shown).

As shown in Fig. 1, central administration of leptin increased pituitary GH mRNA concentrations (53.2% increase, $P < 0.001$ vs diluent-treated rats). Leptin caused a 61.8% increase of hypothalamic GHRH mRNA concentrations ($P < 0.05$ vs diluent-treated rats) and a 41.5% reduction of SS mRNA levels ($P < 0.01$ vs diluent treated rats) (Fig. 2).

Addition of increasing concentrations of leptin (10^{-11} – 10^{-7} M) to cultured pituitary cells did not alter GH release (data not shown), whereas GHRH (10^{-7} M) increased GH release into the medium ($P < 0.05$ vs controls) (Fig. 3). Leptin, at 10^{-7} M, was ineffective in modifying the GH-releasing activity of GHRH (Fig. 3).

Discussion

Broadening of initial studies on the biological effects of leptin on food intake and energy metabolism (Campfield 1996, Sinha & Caro 1998) has led to the discovery of important neuroendocrine effects of this protein. In female ob/ob mice, leptin restored to normal the impaired fertility (Farid *et al.* 1996) and in lean mice accelerated vaginal opening (Ahima *et al.* 1997).

Moreover, it was recently shown in rats that peripheral administration of leptin effectively counteracted the decreased gonadotrophic and thyroid functions and the enhanced corticosteroid function ensuing from exposure to prolonged fasting (Ahima *et al.* 1997). It has, therefore, been proposed that the decrease or lack of leptin signalling from the periphery to the brain during fasting is crucial for the development of the adaptive neuroendocrine responses to caloric restriction (Ahima *et al.* 1996).

Apparently, the involvement of leptin could also be foreseen for GH regulation, since in fasted rats central administration of the protein prevented the decline of pulsatile GH secretion and recent work has shown that the action of leptin in preventing the fasting-related GH fall is mediated by a stimulation of GHRH mRNA expression (LaPaglia *et al.* 1998). Leptin stimulates GH secretion under normal metabolic conditions also, as suggested by the ability of an anti-leptin antiserum to inhibit GH release (Carro *et al.* 1997) and by the stimulatory effect of the protein on both spontaneous pulsatile GH secretion and the GH response to GHRH (Tannenbaum *et al.* 1998). The latter study also provided evidence, albeit inferential, that the GH stimulatory effect of leptin is mediated by inhibition of SS release.

Our study in fed rats provides a more direct demonstration of the central mechanisms underlying the GH secretory activity of leptin. Short-term i.c.v. administration of the protein increased the expression of GHRH and decreased SS mRNA in the hypothalamus and increased GH synthesis.

The ability of leptin to inhibit SS mRNA was previously shown by Quintela *et al.* (1997) in cultured rat fetal neurons, while leptin added to hypothalamic slices incubated *in vitro* strikingly inhibited SS release into the incubation medium (M Heiman, quoted in Vuagnat *et al.* 1997).

It appears, therefore, that SS neurons are also a target for leptin action, although its effect on GHRH expression fits in better with the recorded stimulation of GH synthesis at the pituitary level. It is well known in fact that GHRH is the principal modulator of the synthesis of the hormone in the pituitary (Mayo *et al.* 1985).

That the effects of leptin on somatotrophic function were primarily exerted on a hypothalamic and not a pituitary target is also supported by its inability to modify basal and GHRH-stimulated GH release from dispersed rat pituitary cells. These findings are in contrast with data

Figure 2 Effect of i.c.v. administration of leptin for 3 days on hypothalamic SS mRNA (upper panel) and GHRH mRNA (lower panel) levels. Data are expressed as percentage of control values. Each value is the mean $\pm$ S.E.M. of seven or eight determinations. In each panel are inserted representative slot blot hybridization signals from diluent-treated (A) and leptin-treated (B) rats (15 μ g total RNA/slot). * $P < 0.05$ vs diluent-treated rats.

obtained in primary cultures of pig pituitary cells where high concentrations of leptin diminished the effect of GHRH stimulation (Barb *et al.* 1998).

Among hypothalamic peptides which may mediate the effect of leptin on GH-hypophysiostrophic hormones, neuropeptide Y (NPY) is a likely candidate. Co-expression of leptin receptors and prepro-NPY mRNA in the arcuate nucleus of the mouse hypothalamus (Stephens *et al.* 1995, Hakansson *et al.* 1996, Mercer *et al.* 1996, Wang *et al.* 1997) strengthens the view that leptin action on

hypothalamo-pituitary axes might be mediated, at least in rats, through an effect on NPY neurons. In this context, it is of interest that in fasting rats leptin administration inhibits the rise in hypothalamic NPY mRNA (Vuagnat *et al.* 1998).

It is well known that adaptive mechanisms to fasting in humans include a response of the GH/insulin-like growth factor-I axis which is opposite to that of rodents, i.e. stimulatory rather than inhibitory (Müller 1987). Granted that in humans also the lack of leptin signals mediates the

Figure 3 Effect of leptin (10^{-7} M) alone, GHRH (10^{-7} M) alone and a combination of leptin and GHRH on GH release from primary cultures of pituitaries from adult rats. Each value is the mean $\pm$ S.E.M. of eight to ten determinations. * $P < 0.01$ vs controls.

adaptive responses to fasting, the possibility is raised that in this species leptin may be inhibitory and not stimulatory to GH secretion, a proposition for which some evidence, albeit inferential, exists (Gills *et al.* 1997).

Resolution of this issue, by studying the effect of leptin on basal and stimulated GH secretion in humans, is mandatory; it may help our growing understanding of the role of the high leptin levels in obesity and their contribution to the hypsomatotropicism of the disease.

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References

- Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier & Flier JS 1996 Role of leptin in the neuroendocrine response to fasting. *Nature* **382** 250–252.
- Ahima RS, Dushay J, Flier SN, Prabakaran D & Flier JS 1997 Leptin accelerates the onset of puberty in normal female mice. *Journal of Clinical Investigation* **99** 391–395.
- Altavater FB, De Balbian Vester F, Hall S, Long CS & D'Eencarnacao P 1970 A simple and inexpensive cannula technique for chemical stimulation of the brain. *Physiology and Behavior* **5** 119–121.
- Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kujper JL, Clifton DK & Steiner RA 1996 Leptin is a metabolic signal to the reproductive system. *Endocrinology* **137** 3144–3147.
- Barb CR, Yan X, Azain MJ, Kraeling RR, Ramoacek GB & Ramsay TG 1998 Recombinant porcine leptin reduces food-intake and stimulates growth hormone secretion in swine. *Domestic Animal Endocrinology* **15** 77–86.
- Campfield LA, Smith FJ & Burn P 1996 The OB protein (leptin) pathway – a link between adipose tissue mass and central neural networks. *Hormone and Metabolic Research* **28** 619–632.
- Carro E, Senaris R, Considine RV, Casanueva FF & Dieguez C 1997 Regulation of *in vivo* growth hormone secretion by leptin. *Endocrinology* **138** 2203–2206.
- Chehab FF, Lim ME & Lu R 1996 Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nature Genetics* **12** 318–320.
- De Gennaro Colonna V, Fidone F, Cocchi D & Müller EE 1993 Feedback effects of growth hormone on growth hormone-releasing hormone and somatostatin are not evident in aged rats. *Neurobiology of Aging* **14** 503–507.
- Farid A, Guisez Y, Fache I, Campfield LA, Plaetinck G, Van der Heyen J, Tavernier J, Fiers W, Burn W & Devos R 1996 Efficient secretion of biologically active recombinant OB protein (leptin) in *Escherichia coli*, purification from the periplasm and characterization. *Protein Expression and Purification* **12** 249–258.
- Gills MS, Oogood AA, O'Neill PA, Adams JE, Thorner MO, Shalet SM & Clayton PE 1997 Relationship between growth hormone (GH) status, leptin and body composition in healthy and GH-deficient elderly subjects. *Clinical Endocrinology* **47** 161–167.
- Goodman RH, Aron DC & Ross BA 1983 Rat pre-pro-somatostatin: structure and processing by microsomal membranes. *Journal of Biological Chemistry* **258** 5571–5573.
- Hakansson ML, Hulting AL & Meister B 1996 Expression of leptin receptor mRNA in the hypothalamic arcuate nucleus – relationship with NPY neurones. *Neuroreport* **7** 3087–3092.
- LaPaglia N, Steiner J, Kirteins L, Emanuele M & Emanuele N 1998 Leptin alters the response of the growth hormone releasing factor–growth hormone–insulin-like growth factor-I axis to fasting. *Journal of Endocrinology* **159** 79–83.
- Mayo KE, Cerelli GM, Rosenfeld MG & Evans RM 1985 Characterization of cDNA and genomic clones encoding the precursor to rat hypothalamic growth hormone releasing factor. *Nature* **314** 464–469.
- Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Morgan PJ & Trayhurn P 1996 Coexpression of leptin receptor and prepro-neuropeptide Y mRNA in arcuate nucleus of mouse hypothalamus. *Journal of Neuroendocrinology* **8** 733–735.
- Müller EE 1987 Neural control of somatotrophic function. *Physiological Reviews* **67** 962–1053.
- Quintela M, Senaris R, Heiman ML, Casanueva FF & Dieguez C 1997 Leptin inhibits *in vitro* hypothalamic somatostatin secretion and somatostatin mRNA levels. *Endocrinology* **138** 5641–5644.
- Seeburg PH, Shine J, Martial JA, Baxter JD & Goodman HM 1977 Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. *Nature* **270** 486–494.
- Sinha MK & Caro JF 1998 Clinical aspects of leptin. *Vitamins and Hormones* **54** 1–30.
- Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hale J, Hofmann J, Hsiung HM, Kriauciunas A, MacKellar FC, Zhang XY & Heimna M 1995 The role of neuropeptide Y in the antiobesity action of the obese gene product. *Nature* **377** 530–532.
- Tannenbaum GS, Gurd W & Lapointe M 1998 Leptin is a potent stimulator or spontaneous pulsatile growth hormone (GH) secretion and the GH response to GH-releasing hormone. *Endocrinology* **139** 3871–3875.
- Vuagnat BAM, Pierroz DD, Lalaoui M, Englaro P, Pralong FP, Blum WF & Aubert ML 1998 Evidence for a leptin–neuropeptide Y axis for the regulation of growth hormone secretion in the rat. *Neuroendocrinology* **67** 291–300.
- Wang Q, Bing C, Al-Barazani K, Mossakowaka DE, Wang XM, McBay DL, Neville WA, Taddayon M, Picavance L, Dryden S, Thomas MEA, McHale MT, Gloyer IS, Wilson S, Buckingham R, Arch JRS, Trayhurn P & Williams G 1997 Interactions between leptin and hypothalamic neuropeptide Y neurones in the control of food intake and energy homeostasis in the rat. *Diabetes* **46** 335–341.

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