EXPERIMENTAL STUDY

Cold exposure inhibits leptin secretion *in vitro* by a direct and non-specific action on adipose tissue

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Abstract

Objective: Leptin secretion is reduced by low temperatures in experimental animals, and this effect has been explained as an adaptive mechanism to cold environments. This study investigated the *in vitro* effects of cold exposure on human white adipose tissue.

Design: To understand whether the low temperature action is a direct or a mediated effect, leptin secretion was assessed *in vitro* in human omental adipose tissue incubated at varied temperatures, from 38 donors. As an internal control, the effect of reduced temperatures on *in vitro* GH secretion by GH3 cells was assessed.

Methods: Measurement of hormones secretion was carried out with an RIA, while human ob gene mRNA expression was assessed with reverse transcription PCR.

Results: Compared with the standard temperature of 37 °C, leptin secretion by human adipose tissue was significantly ($P < 0.05$) reduced when the incubations were carried out at 34.5 °C (41% inhibition), and 32 °C (68% inhibition), with no parallel changes in the ob mRNA expression. At these reduced temperatures, glucocorticoid-mediated leptin secretion was well preserved. When the effect of reduced temperatures was assessed on *in vitro* GH secretion, a superimposable reduction was observed.

Conclusions: These results indicate: (i) that low temperatures reduce leptin secretion by acting directly on the adipose tissue and (ii) that the similar reduction in a hormone unrelated to energy metabolism, such as GH, suggests that the observed reduction is a mechanical perturbation of leptin secretion, which may be devoid of physiological implications.

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Introduction

Animals living in extreme climates show a marked seasonal variation in both food intake and energy expenditure, as a response to the changing metabolic requirements imposed by differences in environmental temperature (1–5). These two variables are under strict control by the product of the ob gene, leptin, an adipocyte-secreted hormone that acts on hypothalamic centres and regulates the energy balance by determining changes in both food intake and energy expenditure (6–8). Physiologically, leptin plasma concentrations reflect the adipose tissue deposits (9, 10) and appear to be negatively regulated by fasting (8), protein kinase C (11), androgens (12) and β -adrenergic agonists (13), whereas feeding (8, 14), oestrogens (15), glucocorticoids (15, 16) and insulin (17) act by stimulating both ob gene expression and leptin secretion.

Furthermore, leptin is implicated in physiological states that require variations in energy expenditure and energy intake like exercise (18, 19) or reproduction (20–25).

It has been shown that cold exposure reduces circulating leptin levels in rodents (26–28), suggesting that this hormone may participate in the adaptive mechanisms triggered by variations in external temperature. The inhibitory effect of low temperatures has been explained as the result of an increase in the adrenergic tone induced by exposure to cold temperatures, which would in turn act through the β -3 receptors present in the adipose tissue inducing a reduction in the expression of the leptin mRNA (26). However, the exact mechanism of leptin reduction by cold exposure is not fully understood, and it has not been elucidated whether it is a direct or indirect action upon the adipose tissue; nor is its physiological meaning known.

In this work, the action of low temperature exposure on leptin secretion has been assessed in human omental adipose tissue *in vitro*. The aims of the study were: (i) to evaluate whether the action of low temperatures was also observable for human leptin; (ii) to determine whether that action was exerted directly on the adipose tissue, or mediated by other *in vivo* factors; and (iii) to determine whether it was a physiological action or merely the result of a physical perturbation.

Materials and methods

Subjects

Omental adipose tissue was obtained from 38 non-obese patients (25 male, 13 female, aged 63.31 ± 2.61 years, and with a body mass index of 27.02 ± 0.61 kg/m²) during elective abdominal surgery. Patients were taking no drugs, nor were they under antibiotic therapy. The study was approved by the Hospital Ethical Committee and each participating subject provided informed consent.

Cell and tissue cultures

Organ adipose tissue culture was performed as described (11, 12, 15). In brief, excised adipose tissue was immediately transported to the laboratory in ice-cold Krebs–Ringer–Hepes (KRH) buffer (in mmol/l: NaCl, 125; KCl, 5; MgSO₄, 1.2; CaCl₂, 2; KH₂PO₄, 1.2; glucose, 6; Hepes, 25; pH 7.4). After removing blood vessels and conjunctive tissue, adipose tissue was washed with sterile KRH and cut into small pieces with sharp scissors. Tissue fragments were placed in six-well multiwell 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). GH3 cells were cultured essentially as described (29, 30). As their growth rate was dependent on temperature, parallel incubations were carried out for both growth hormone (GH) determination and cell counting. After a pre-incubation period of 1 h at 37 °C in a humidified atmosphere of 95% air–5% CO₂, samples were either maintained in the same incubator as internal controls, or transferred to a second incubator in parallel, pre-equilibrated at the required temperature with a similar atmosphere. Unless specifically indicated, all drugs and reagents were from Sigma, St Louis, MO, USA.

Measurement of hormone secretion

For each tested temperature, the adipose tissue from a given subject was independently incubated in triplicate and the medium was collected and analysed at 24 and 48 h to obtain the integrated secretion (area under the curve, AUC) at 48 h (15, 31). Samples were

stored at –20 °C until assayed for leptin secretion. Leptin levels were measured in duplicate by RIA using commercial kits (Human Leptin RIA; Linco Research Inc., St Charles, MO, USA). The limit of sensitivity was 0.5 µg/l, the intra-assay coefficient of variation was 8.3%, and the inter-assay coefficient of variation 6.2%. GH levels were determined by RIA using reagents provided by the National Hormone and Pituitary Program of NIDDK (Bethesda, MD, USA), as previously described (29, 30).

RNA preparation and reverse transcription PCR (RT-PCR)

Total RNA was isolated from frozen adipose tissues after 48 h incubation by the acidic phenol/chloroform method of Chomczynski & Sacchi (32). Tissues (about 100 mg) were homogenised using a Polytron homogeniser (Kinematica AG, Littau, CH) and recovery of total RNA was measured with a spectrophotometer (Beckman DU62; Beckman Instruments, Inc., Fullerton, CA, USA) at 260 nm. One microgram total RNA was used to perform RT-PCR. cDNAs were synthesised using 200 units of Moloney murine leukaemia reverse transcriptase (Gibco-BRL, Eggenstein, Germany) and random hexamers (Promega, Madison, WI, USA). The reaction, in a final volume of 30 µl, was carried out at 37 °C for 50 min, 42 °C for 15 min and 95 °C for 5 min in first-strand buffer (Gibco Y00146) containing 10 U ribonuclease inhibitor. The cDNA was amplified using human specific primers for the *ob* gene (upstream primer: 5'-ATGCATTGGGGAACCCTGTGCGG-3'; downstream primer: 5'-TGAGGTCCAGCTGCCACAGCATG-3'). The cDNAs were also amplified using human HPRT (hypoxanthine-guanine phosphoribosyl transferase) primers (downstream primer: 5'-AGCAAGACGTTTCAGTCCTGTC-3'; upstream primer: 5'-CAGTCCCAGCGTCGTGATTA-3'), as a quality and quantity control. PCR was performed using 0.2 mmol/l dNTPs (Promega), 1.25 U Taq-DNA polymerase (Gibco-BRL), 5 µl 10×PCR buffer (18.6 mmol/l Tris–HCl, 45.9 mmol/l KCl, 3 mmol/l MgCl₂) in a total volume of 50 µl. PCR was carried out on an Eppendorf Master Cycler gradient thermal cycler (Eppendorf GmbH, Hamburg, Germany) under the following conditions: 94 °C for 1 min for denaturation, 60 °C for 1 min for annealing and 72 °C for 1 min for extension (35 cycles), with an additional elongation cycle at 72 °C for 10 min. PCR products were separated on a 2% agarose gel and stained with ethidium bromide to visualise the resulting bands. Gels were examined under UV light and quantitated with a GelDoc 1000 Documentation System (BioRad Laboratories, Mississauga, Ontario, Canada).

Statistic analysis

Leptin (ng/g tissue) and GH secretion values (ng/10⁶ cells) were expressed as the AUC at 48 h (trapezoidal

method) and plotted as the percentage over controls. Comparisons were made by the unpaired *t*-test. Values of $P < 0.05$ were considered significant.

Results

The effect of different incubation temperatures on leptin secretion was assessed *in vitro* using an adipose tissue organ culture system recently described by our group (11, 12, 15). As no gender-based differences were observed for the effect of temperature on leptin secretion, values from both male and female donors were pooled for the analysis. The integrated *in vitro* leptin secretion (expressed as AUC after 48 h incubation in ng/g of tissue per 24 h) by human omental adipose tissue showed a marked dependence on the incubation temperature (Fig. 1A). Leptin secretion was significantly reduced at 32 °C (68% inhibition, $P = 0.0050$, $n = 10$) and 34.5 °C (41% inhibition, $P = 0.0257$, $n = 15$), while no statistically significant differences in the spontaneous leptin secretion were found between cells incubated at 39.5 °C and controls ($P = 0.5201$, $n = 13$). In samples derived from women, the ability of the adipose cells to respond to a challenge with 1 $\mu\text{mol/l}$ dexamethasone with a clear-cut increase of leptin secretion (15) was well preserved at 32 and 34.5 °C (data not shown), suggesting that the tissue remained viable and that the mechanisms that regulate leptin secretion were still acting at the reduced temperatures. On the contrary, the response to dexamethasone was suppressed at 39.5 °C, a temperature that was probably high enough to induce a certain degree of metabolic perturbation in cells on prolonged incubations (data not shown).

The specificity of this temperature effect was evaluated using as a parallel control experiments in cultures of GH3 cells, a line of pituitary-derived cells that constitutively secretes GH *in vitro*. As Fig. 1B shows, the spontaneous GH secretion by GH3 cells also showed a decrease when the temperature was reduced during the 24 h incubation, the overall picture being quite similar to the one obtained for leptin secretion, with a 64% reduction in GH secretion at 32 °C ($P = 0.0489$, $n = 4$). The regression lines showed that the effects of temperature on the secretion rate for both adipose tissue and GH3 cells were superimposable (Fig. 1C).

RT-PCR of human adipose tissue cDNA yielded a band with the expected size of 487 bp (Fig. 2). Densitometric analysis of detected amplicons showed no variation in *ob* gene expression when the samples were incubated at temperatures outside the physiological range. In particular, no significant differences were observed in leptin gene expression by samples incubated at 39.5 °C (lanes 2 and 4) or 34.5 °C (lanes 6 and 8) in comparison with their respective controls at 37 °C (lanes 1, 3, 5 and 7). These results suggest that the temperature-related differences observed in leptin secretion levels are primarily related to a modification in the

Figure 1 (A) Effect of different incubation temperatures on leptin secretion by human omental adipose tissue *in vitro*. (B) Effect of different incubation temperatures on GH secretion by GH3 cells *in vitro*. (C) Regression analysis of secretion vs incubation temperature for both adipose tissue and GH3 cells. Data (mean $\pm$ S.E.M. of the AUC at 48 h) are expressed as the percentage over the control (37 °C) values. * $P < 0.05$; ** $P < 0.005$.

secretion rate of mature protein and not to a change in the expression of the *ob* gene.

Discussion

Exposure of experimental animals to cold environments results in a decrease of circulating leptin (26–28). For animals living in zones with large seasonal temperature variations this may represent an adaptive mechanism for maximising the size of fat deposits when the

Figure 2 Agarose gel (2%) stained with ethidium bromide showing PCR products obtained using specific primers for the ob gene. No differences were observed in leptin gene expression from human adipose tissue after 48 h incubation at 39.5°C (lanes 2 and 4) and 34.5°C (lanes 6 and 8) vs their respective controls at 37°C (lanes 1, 3, 5 and 7). Quality and quantity controls were obtained by performing parallel PCR of the housekeeping gene HPRT (139 bp product).

environment temperature begins to decline, in order to survive the cold season. As the adrenergic tone increases *in vivo* when the temperature is lowered, it is possible that an increased stimulation of the adipocyte-specific β_3 -adrenergic receptors, with the ensuing increase of the intracellular cAMP levels, which would in turn inhibit the ob mRNA expression, may constitute the physiological basis for this leptin reduction. In fact, it has been reported that this is the case for mice (33) and rats (26), although not for other rodents, such as the Djungarian hamster (34). Despite the fact that in homeotherm animals the core temperature of the body is maintained during exposure to low environmental temperatures, a large fraction of the total mass of adipose tissue is localised directly under the skin, acting as insulation to avoid excessive heat loss. This anatomical localisation, together with the inherent low vascularisation of adipose tissue, causes the temperature in the subcutaneous fat deposits to fall several degrees below the core temperature upon exposure to cold. However, the possibility of a direct effect of low temperature on the adipose tissue itself has not been addressed experimentally.

In an attempt to clarify this issue, a previously validated *in vitro* organ culture system was used to test directly the effect of different temperatures on the rate of leptin secretion. As a control, we checked GH secretion by GH3 cells maintained under the same temperature conditions. The results show that the secretion of leptin by human omental adipose tissue *in vitro* is strongly temperature dependent, being reduced almost to a half when the temperature of the adipose tissue is lowered by as little as 2.5°C. This is the first demonstration that cold also reduces leptin secretion in humans and that the effect is a direct action on the adipose tissue. However, as no temperature-dependent modification on the ob mRNA levels was detected by RT-PCR, the effect of temperature does not appear to be part of a long-term physiological regulatory system

of leptin secretion, but rather a non-specific effect of temperature on the secretory pathway. Both the intracellular transport of proteins to the cell surface and the fusion of secretory granules with the plasma membrane are temperature dependent (35, 36). In fact, the vesicular forward transport events that take place along all the steps of the secretory pathway can be completely blocked at 20°C. In agreement with this interpretation, the secretion of GH by GH3 cells was also found to be temperature dependent, and was reduced by a similar extent when they were incubated at the same temperatures as the adipose tissue samples. These results point towards the interpretation that the low temperature effect on leptin secretion is a physical or mechanical effect, casting doubts on its physiological meaning. In fact, from a teleological explanation, the reduction of plasma leptin at the onset of the cold season would have a positive effect, increasing food intake, but also a negative one, reducing thermogenesis. In the animal under hibernation, a low leptin level may help to reduce energy expenditure, but the increase in appetite is useless.

In conclusion, leptin secretion by human omental adipose tissue *in vitro* was inhibited by reducing the temperature of the adipose tissue itself, probably by a reduction in the overall efficiency of the secretory pathway. Whether this represents a physiological regulatory mechanism or merely a non-specific physical or mechanical change remains to be ascertained.

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