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Ciliary neurotrophic factor influences endocrine adipocyte function: inhibition of leptin via PI 3-kinase

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Abstract

Ciliary neurotrophic factor (CNTF), originally known for its involvement in the modulation of neuronal growth, has been discovered to exert anorexigenic effects and is currently being investigated in clinical studies for the treatment of obesity and insulin resistance. This neuropeptide acts on the central nervous system. However, we have recently demonstrated direct peripheral effects on adipocyte signalling and thermogenesis. Given the emerging endocrine role of adipose tissue in the regulation of energy homeostasis and insulin resistance, we investigated potential effects of CNTF on leptin expression and secretion. Our study demonstrates a direct inhibition of leptin expression and secretion by acute and chronic CNTF treatment. Furthermore, we demonstrate a differentiation- and Janus kinase 2 (JAK2)-independent, but phosphatidylinositol 3-kinase-dependent signalling pathway mediating this negative effect. These results provide novel evidence for a role of CNTF in the selective modulation of adipocyte endocrine function which may have important implications for the regulation of energy homeostasis.

Keywords: Adipocyte; Adipocytokine; CNTF; JAK2; Leptin; PI 3-kinase

1. Introduction

Ciliary neurotrophic factor (CNTF) classically acts as a regulator of neuronal growth (Siegel et al., 2000; Sleeman et al., 2000; Weinelt et al., 2003; Xia et al., 2002; Del Bigio et al., 2001). Recently, potent anorexigenic effects of this neuropeptide in the central nervous system regulation of body weight and energy homeostasis have been described (Anderson et al., 2003; Gloaguen et al., 1997). In a previous study, we characterized potent direct CNTF effects on growth factor and metabolic signalling and thermogenesis in mouse brown adipocytes (Ott et al., 2002a). Our findings were expanded by studies in 3T3-L1 white adipocytes demonstrating CNTF-modulated adipocyte-specific gene expression and induction of the CNTF receptor complex in rodent models of obesity and diabetes (Zvonic et al., 2003).

Adipose tissue has recently emerged as an endocrine organ central to the control of energy homeostasis (Ott et al., 2002a; Havel, 2000; Fruhbeck et al., 2001; Ahima and Flier, 2000). Dysregulation of adipose tissue function is a key element in the pathogenesis of diabetes and the insulin resistance syndrome. This is illustrated by a number of studies in which a selective impairment of adipocyte function such as disruption of insulin signalling (Abel et al., 2001; Minokoshi et al., 2003; Bluher et al., 2002, 2003) or tissue-specific overexpression of 11β-hydroxysteroid dehydrogenase type 1 (Masuzaki et al., 2001) has significant systemic consequences for energy homeostasis and insulin resistance. Furthermore, due to its

Abbreviations: CNTF, ciliary neurotrophic factor; IL-6, interleukin-6; JAK, Janus kinase; LIF, leukemia inhibitory factor; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; Tyk, tyrosine kinase

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potential to expand upon selective stimulation, the thermogenic brown adipose tissue is an attractive target tissue for pharmacotherapeutic approaches in the treatment of obesity and the insulin resistance syndrome.

Leptin is the best-studied adipocytokine so far which is expressed and secreted in both white (Cammisotto et al., 2003) and brown adipose tissue (Kraus et al., 2002). Its discovery has prompted a redefinition of energy homeostasis regulation and opened a new field of research. Its main regulatory effects on energy metabolism are mediated by modulating orexigenic and anorexigenic signalling pathways in the central nervous system (Baratta, 2002; Harvey and Ashford, 2003; Jequier, 2002; Pralong et al., 2002; van Dijk, 2001) as well as energy metabolism in peripheral tissues.

In this study, we investigated direct CNTF actions on leptin expression and secretion in a recently established model of mouse brown adipocytes (Klein et al., 2002). We have extended our previous findings demonstrating direct effects on adipocyte signalling and thermogenesis (Ott et al., 2002a) and now show that this potent anorexigenic neuropeptide also directly inhibits leptin expression and secretion via a phosphatidylinositol (PI) 3-kinase-dependent signalling pathway.

2. Materials and methods

2.1. Materials

Recombinant rat CNTF was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). Recombinant murine leukaemia inhibitory factor (LIF) was obtained from Chemicon International (Temecula, CA, USA). The pharmacological inhibitors LY294002, H-89, and Ag490 were obtained from Calbiochem (La Jolla, CA, USA), PD98059 from Cell Signalling Technology, Inc. (Beverly, MA, USA). Unless stated otherwise, all other chemicals were purchased from Sigma–Aldrich Co. (St Louis, MO, USA).

2.2. Cell culture

Cells used in all experiments were SV40T-immortalized brown and white adipocytes generated as previously described (Klein et al., 2000). Pre-adipocytes were cultured to confluence in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, Scotland), supplemented with 4.5 g/l glucose, 20 nM insulin, 1 nM T3, 20% fetal bovine serum (Sigma–Aldrich Co.; St. Louis, MO, USA) and penicillin/streptomycin (BioWhittaker, Vervier, Belgium) ('differentiation medium'). Upon attaining confluence, 500 μM isobutylmethylxanthine, 250 μM indomethacine, and 2 μg/ml dexamethasone were added to the differentiation medium to induce cell differentiation ("induction"). After 24 h, cells were returned to differentiation medium and cultured for 6 more days.

2.3. Assessment of leptin secretion

Between day 1 and day 6 after induction, culture medium was removed from the cells every 24 h and replaced with fresh medium. Leptin secretion into the medium was assessed using a murine leptin radioimmunoassay (Linco Research, Inc., St Louis, MO, USA) according to the manufacturer's instructions.

2.4. Analysis of leptin gene expression

No starvation of cells in serum-free medium was done prior to total RNA isolation. Reverse transcription (RT) followed by polymerase chain reaction (PCR) was performed to analyse mRNA expression of leptin and hypoxanthine guanine phosphoribosyl transferase (HPRT) as a housekeeping gene control. Briefly, total RNA isolation was performed using the TRIzol reagent (Invitrogen; Karlsruhe, Germany) followed by a clean up with the RNeasy kit (Qiagen; Hilden, Germany). Quality of RNA was tested by visualising the RNA on an agarose gel and photometric analysis. Up to 2 μg total RNA was reverse transcribed using Superscript II (Invitrogen; Karlsruhe, Germany) and an oligo $p(DT)_{15}$ primer (Roche Molecular Biochemicals; Mannheim, Germany) in the presence of RNase inhibitor (Roche Molecular Biochemicals; Mannheim, Germany) in a 20 μl reaction. One microlitre of each RT reaction was amplified in a 25 μl PCR containing 2.5 mM $MgCl_2$, 250 nM of each primer, and 1X QuantiTect SYBR Green PCR-Mix (Qiagen; Hilden, Germany). PCR was performed on a GeneAmp 5700 Sequence Detection System (Applied Biosystems; Foster City, CA, USA). The following primers were used: leptin (accession number NM008493) 5′-GTG CCT ATC CAG AAA GTC CAG GAT G-3′ (sense) and 5′-CTG GTG AGG ACC TGT TGA TAG AC-3′ (anti-sense), and HPRT (accession number

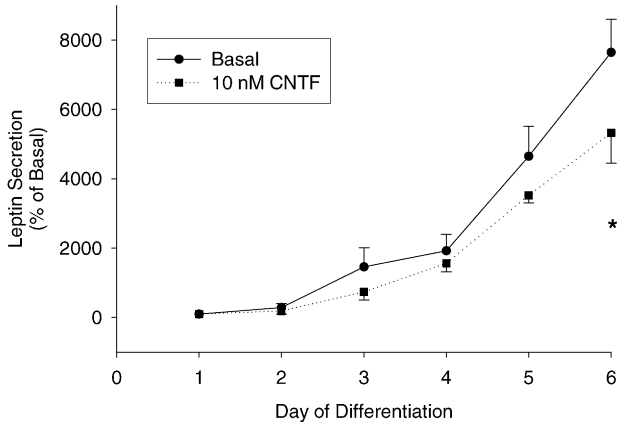

Fig. 1. CNTF inhibits leptin secretion in brown adipocytes. Cells were left untreated or were chronically exposed to 10 nM CNTF, respectively. Leptin concentration was analysed in the culture medium collected every 24 h. A scatter and line plot analysis including the S.E.M. of four independent experiments is shown. *$P < 0.05$ comparing non-stimulated to CNTF-treated cells.

NM013556) 5′-GTT GGA TAC AGG CCA GAC TTT GT-3′ (sense) and 5′-CAC AGG ACT AGA ACA CCT GC-3′ (anti-sense). PCR for all targets was performed as follows: initial denaturation at 95 °C for 900 s, 40 cycles with 95 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s. Specific amplification was confirmed by producing melting curve profiles (cooling the samples to 65 °C for 10 s and heating to 95 °C in steps of 0.2 °C/s with continuous measurement of fluorescence). Optimized relative quantification was done by using the Pair Wise Fixed Reallocation Randomisation Test (Relative Expression Software Tool [REST], version 1) (Pfaffl, 2001).

2.5. Statistical analysis

"Sigma Plot" software (SPSS Science; Chicago, IL, USA) was employed for statistical analysis of all data. Results are presented as mean ± S.E.M. Unpaired Student's *t*-test and, for multiple comparisons, Bonferroni's alpha level correction were used for determination of statistical significance. *P* values <0.05 are considered significant, those <0.01 highly significant.

3. Results

3.1. CNTF inhibits leptin secretion in brown adipocytes

Leptin secretion showed a robust differentiation-dependent augmentation from basal levels to a maximal 100-fold increase on day 6 (Fig. 1). Chronic treatment with 10 nM CNTF persistently inhibited leptin secretion over the differentiation course with a significant maximal 30% reduction on day 6 as compared to untreated cells (Fig. 1). This effect appeared dose-dependent with smaller changes observed at CNTF concentrations as low as 1 nM (data not shown).

3.2. CNTF-induced suppression of leptin secretion is not caused by impaired adipocyte differentiation

Since leptin secretion is differentiation-dependent (Kraus et al., 2002), we next investigated whether the reduction of leptin secretion by CNTF treatment may result from potential effects on adipocyte differentiation. However, chronic treatment with this anorexigenic neuropeptide did not affect

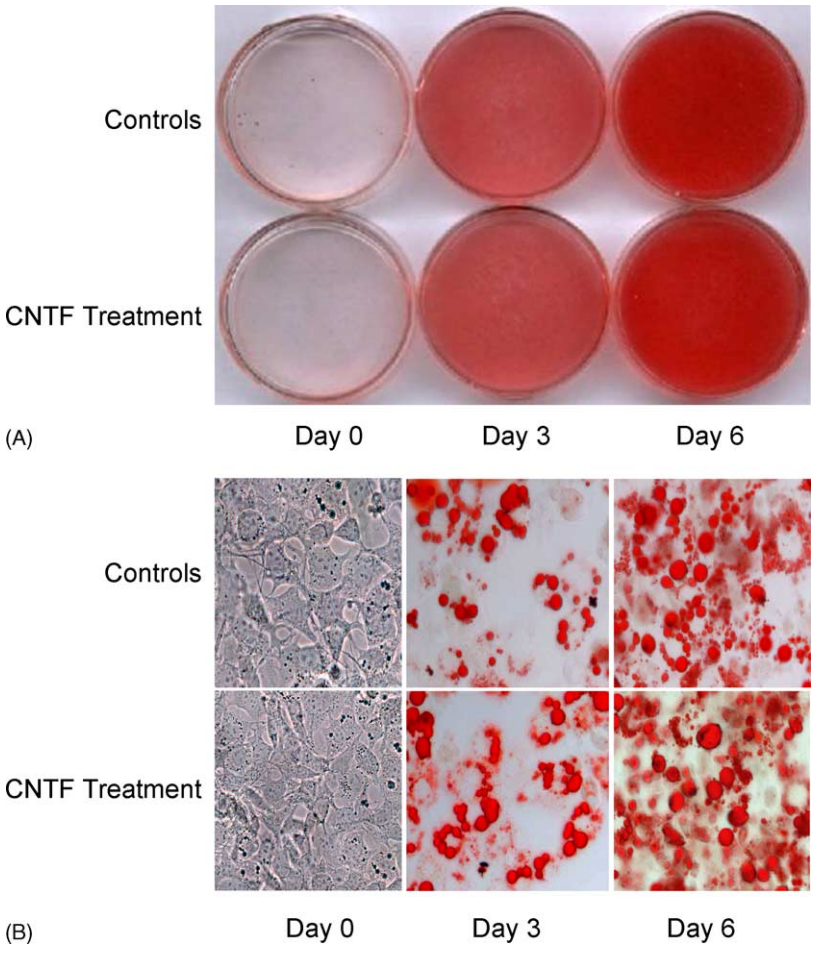

Fig. 2. CNTF does not affect adipocyte differentiation. Cells were either chronically treated with ciliary neurotrophic factor (CNTF treatment) at a concentration of 10 nM with medium changed every 24 h or non-treated (controls). Differentiating cells were stained with the fat-specific oil red O stain at the indicated times after the induction period. Microscopical images from cell culture plates were taken using a digital camera. Pictures are displayed in 20× magnification.

adipocyte differentiation as assessed by the fat-specific oil red O staining (Fig. 2A). Moreover, as judged microscopically, cell morphology at early and late differentiation stages did not change under the CNTF treatment (Fig. 2B). Furthermore, total protein amounts were similar in cells chronically stimulated with CNTF as compared to control cells.

3.3. CNTF inhibits leptin gene expression

To further assess the kinetics and signalling pathways for this decrease in leptin secretion, we next studied the time course of leptin mRNA expression in differentiated adipocytes treated with CNTF. Acute treatment of brown adipocytes with 10 nM CNTF resulted in a time-dependent reduction of leptin mRNA synthesis with a significant decrease first seen after 1 h and a maximal 34% reduction after 2 h (Fig. 3A). In newly generated white adipocytes obtained by the identical SV40T immortalisation procedure, acute treatment of differentiated cells with 10 nM CNTF for 2 h induced a reduction of leptin expression by approximately 60% (Fig. 3B). Longer CNTF treatment periods did not produce stronger inhibitory effects in both brown and white adipocytes (data not shown).

Other members of the CNTF cytokine family were also assessed for their effects on leptin mRNA expression. Whereas LIF (30 ng/ml) did not influence leptin gene expression, stimulation of differentiated adipocytes with interleukin-6 (IL-6) (30 ng/ml) for 8 h and 24 h reduced leptin expression by about 40% as compared to basal levels (data not shown).

3.4. Inhibition of leptin expression by CNTF is dependent on PI 3-kinase but independent of JAK2, MAP kinase, and PKA

Interestingly, treatment of brown adipocytes with the PI 3-kinase inhibitor LY294002 (10 μM) completely abrogated the CNTF-induced impairment of leptin expression (Fig. 4A) suggesting an involvement of PI 3-kinase in this inhibitory

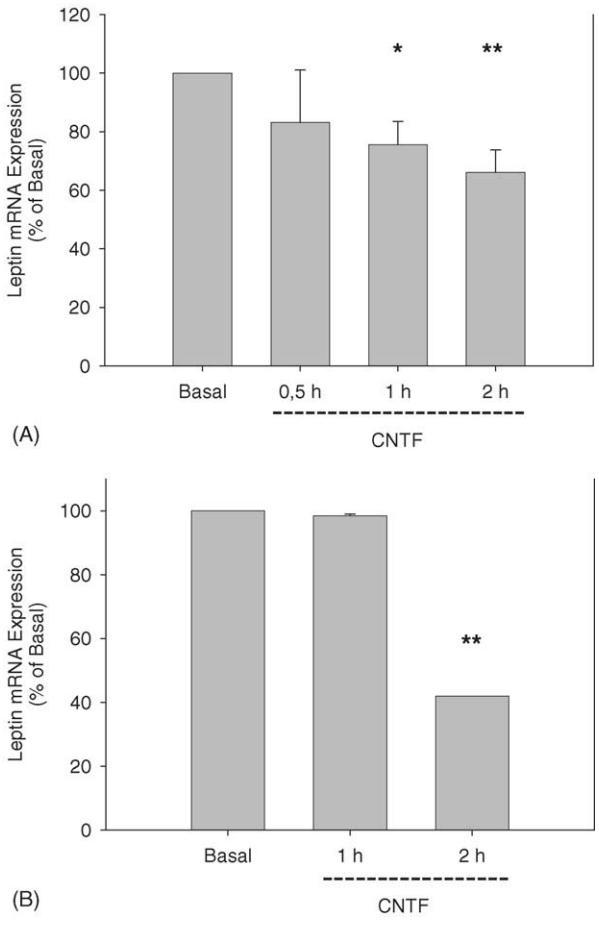

Fig. 3. CNTF inhibits leptin mRNA expression in a time-dependent manner. Differentiated brown (A) or white (B) adipocytes were incubated with 10 nM CNTF for the indicated periods of time. Leptin mRNA quantification was analysed as described in Section 2. A bar graph analysis including the S.E.M. of two–four independent experiments is shown. *$P < 0.05$ and **$P < 0.01$ comparing non-stimulated to CNTF-treated cells.

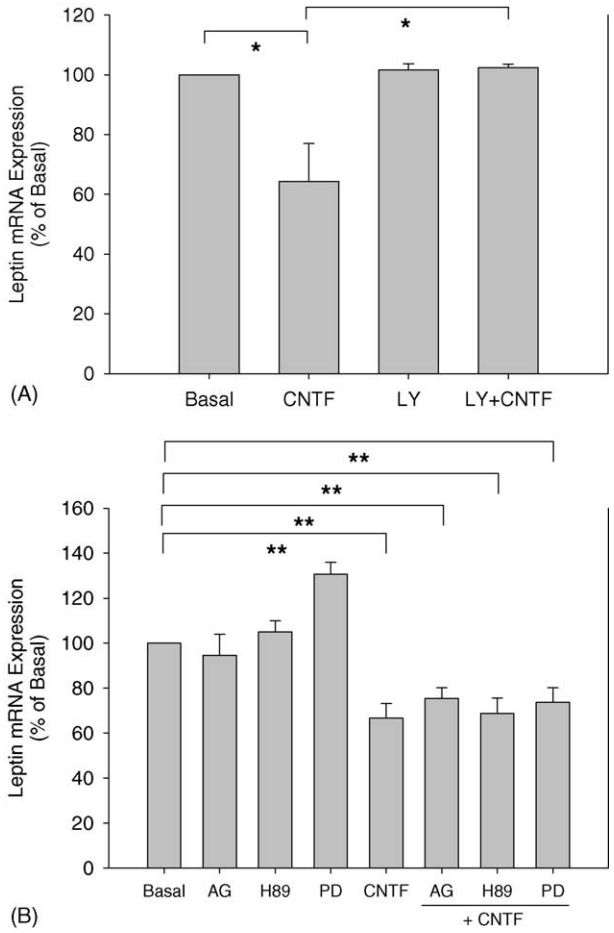

Fig. 4. CNTF-induced inhibition of leptin mRNA expression is dependent on PI 3-kinase, but not JAK2, p44/42 MAP kinase, or PKA. The quantitative analysis of leptin mRNA expression was done as described in Section 2. Pretreatment with LY294002 (10 μM, A), Ag490 (100 μM, B), H89 (10 μM, B), and PD98059 (50 μM, B) was for 1h, followed by the addition of 10 nM CNTF for 2 h. A bar graph analysis including the S.E.M. of three–seven independent experiments is shown. *$P < 0.05$, **$P < 0.01$ comparing CNTF-treated to non-treated cells, respectively.

effect. By contrast, the negative effect of CNTF on leptin expression was not altered by inhibition of Janus kinase 2 (JAK2) (Ag490, 100 μM), protein kinase A (PKA) (H89, 10 μM), and mitogen-activated protein (MAP) kinase (PD98059, 50 μM; Fig. 4B). Treatment of cells with the pharmacological inhibitors AG490 and H89 alone did not significantly affect basal leptin mRNA levels (Fig. 4B). PD98059 alone exerted a small but significant positive effect on basal leptin levels. Yet, PD98095 did not significantly alter the inhibitory CNTF effect (Fig. 4B).

4. Discussion

In this study, we show an acute and chronic inhibition of leptin expression and secretion by the anorexigenic neuropeptide CNTF. This effect appears to be differentiation-independent and mediated by a PI 3-kinase-dependent signalling pathway.

In addition to CNTF's well-established anorexigenic effects in the central nervous system (Anderson et al., 2003; Xu et al., 1998; Lambert et al., 2001), we have previously demonstrated direct and selective effects of this neuropeptide on important growth factor and metabolic signalling pathways in brown fat (Ott et al., 2002a). Moreover, we found that CNTF enhanced β3-adrenergic stimulation of uncoupling protein-1 which mediates thermogenesis and may thus be important in this neuropeptide's anti-obesity effects. Evidence from other studies supports the notion of direct peripheral actions of CNTF on adipose tissue (Zvonic et al., 2003) and describes the activation of similar signalling cascades in a variety of tissues and cells of murine and human origin (Kobayashi and Mizisin, 2000; Kaur et al., 2002). Interestingly, CNTF receptor expression appears to be induced in mouse models of obesity and diabetes (Zvonic et al., 2003). In this study, we extend our previous findings and demonstrate that CNTF directly modulates endocrine adipocyte function. A number of excellent studies testify to the complex nature of the fat cell as an endocrine organ fulfilling an influential role in the regulation of energy homeostasis and insulin resistance (Kahn and Flier, 2000; Spiegelman and Flier, 2001). Furthermore, it now becomes evident that adipose tissue is not only an important site of hormone synthesis (Rajala and Scherer, 2003), but also serves as a relay station for central (Serradeil-Le Gal et al., 2000) and peripheral orexigenic and anorexigenic signals such as ghrelin (Ott et al., 2002b) and interleukin-6 (Fasshauer et al., 2003). This is of crucial relevance as it has been demonstrated that selective alterations in adipocyte function have important implications for whole body energy homeostasis, insulin resistance, and even life expectancy (Minokoshi et al., 2003; Bluher et al., 2002, 2003).

In elucidating the signalling cascade responsible for the inhibition of leptin secretion and expression by CNTF, we found that PI 3-kinase inhibition by LY294002 completely abrogated CNTF's inhibitory effect. Our findings are in accordance with another study also demonstrating involvement of PI 3-kinase in leptin expression and secretion in primary white rat adipocytes (Bradley and Cheatham, 1999). However, in that study, inhibition of PI 3-kinase in rat adipocytes decreased insulin- and dexamethasone-stimulated leptin secretion. Taking into account different transcriptional and secretory cell responses as well as the central position of this enzyme and its different isoforms in the complex network of growth factor and metabolic signalling pathways, many explanations for this apparent discrepancy can be envisioned. Awaiting results from further studies, our data suggest a different employment of PI 3-kinase signalling pathways by CNTF and insulin and indicate a role for this important signalling element in integrating receptor-type-specific multidirectional downstream pathways. In contrast to PI 3-kinase, inhibition of MAP kinase had no discernible effect on CNTF-induced inhibition of leptin expression. In a previous study, we described CNTF-induced activation of AKT as well as MAP kinase in a JAK2-dependent manner (Ott et al., 2002a). However, in this study, JAK2 inhibition failed to reverse CNTF's inhibitory effect on leptin secretion. This implies the existence of a second CNTF-induced, but JAK2-independent signalling pathway mediating PI 3-kinase activation. An alternate pathway may involve other members of the JAK-family. Indeed, studies have implicated JAK1- (Kaur et al., 2002), JAK3- (Dolcet et al., 2001), and tyrosine kinase (Tyk)-mediated PI 3-kinase activation by CNTF and other interleukin-6-family cytokines (Oh et al., 1998; Lelievre et al., 2001).

Although in the majority of obese humans, the anorexigenic effect of leptin is less pronounced than in mice (Yanovski and Yanovski, 2002), this adipocytokine still appears to be crucial in human pathophysiology as recently demonstrated by dramatic beneficial effects on insulin resistance in lipatrophic patients (Oral et al., 2002). Thus, from a physiological perspective, it may seem counterintuitive that CNTF downregulates leptin expression and secretion in fat, since both factors represent powerful anorexigenic mediators. However, data from several other studies show that other anorexigenic substances such as metformin downregulate leptin expression in mice and humans as well (Fruehwald-Schultes et al., 2002; Morin-Papunen et al., 1998; Pasquali et al., 2000). It is tempting to speculate that this downregulation of leptin secretion could be a mechanism to overcome leptin resistance by causing an upregulation of leptin-receptors in response to decreased leptin levels. Data by Scarpace et al. suggest that high leptin levels do indeed cause leptin resistance characterised by attenuation of the anorexigenic effect of leptin and, after a longer time-period, an attenuated thermogenic effect in brown fat (Scarpace et al., 2002). However, although plausible from a physiological point of view, data presented here are derived from cell models and thereby only indicate possible biological mechanisms awaiting an indispensable proof in physiological systems.

In conclusion, this study characterises for the first time a selective direct modulation of endocrine adipocyte function by the anorexigenic neuropeptide CNTF. The CNTF-induced

suppression of leptin expression and secretion appears to be independent of differentiation and mediated via a PI 3-kinase-dependent signalling pathway. Our findings indicate that adipose tissue is an important element in regulatory circuits mediating this neuropeptide's effects on energy homeostasis and underscore the complex role of adipose tissue in integrating homeostatic signals both from the central nervous system and the periphery.

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