Identification and expression analysis of leptin-regulated immediate early response and late target genes

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INTRODUCTION

Using PC12 cells as an in vitro model system, we have identified a series of transcripts induced through activation of the leptin receptor. On the basis of kinetic studies, two distinct gene sets could be discerned: signal transducer and activator of transcription-3 (STAT-3), suppressor of cytokine signalling-3 (SOCS-3), MT-II (metallothionein-II), the serine/threonine kinase fibroblast-growth-factor-inducible kinase (Fnk) and modulator recognition factor (MRF-I), which are immediate early response genes, and pancreatitis-associated protein I (PAP I) (PAPI), squalene epoxidase, uridine diphosphate glucuronosyltransferase and annexin VIII, which are late induced target genes. At late time points a strong co-stimulation with β-nerve growth factor or with the adenylate cyclase activator forskolin was observed. To assess the validity of the PC12-cell model system, we examined the effect of leptin administration on the gene transcription of STAT-3, MT-II, Fnk and PAP I in vivo. Leptin treatment of leptin-deficient ob/ob mice increased the STAT-3, SOCS-3, MT-II and Fnk mRNA, and MT-I protein levels in liver, whereas, in jejunum, expression of PAPI mRNA was down-regulated. Furthermore, administration of leptin to starved wild-type mice enhanced the expression of MT-II and Fnk mRNA in liver, but decreased MT-II and PAPI mRNA expression in jejunum. These findings may help to explain the obese phenotype observed in some colonies of MT-I- and MT-II-null mice and/or the observation that leptin protects against tumour-necrosis-factor toxicity in vivo.

Key words: forskolin, gene induction, metallothionein, PC12 cells, representational difference analysis.

Abbreviations used: Fnk, fibroblast-growth-factor-inducible kinase; IL-6, interleukin-6; JAK, Janus kinase; mLRsh, short isoform of the mouse leptin receptor; mLPR, short isoform of the mouse leptin receptor; MT, metallothionein; MRF-I, modulator recognition factor-I; β-NGF, β-nerve growth factor; (r)NPY, (rat) neuropeptide Y; (r)PAPI, (rat) pancreatitis-associated protein I; POMC, pro-opiomelanocortin; RDA, representational difference analysis; SOCS-3, suppressor of cytokine signalling-3; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor; UGT, uridine-diphosphate glucuronosyltransferase; RT-, reverse transcription.

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EXPERIMENTAL

Cell culture and transfection

PC12 cells were cultured as described in [27]. The cells were treated with medium alone or supplemented with 100 ng/ml mouse leptin (Pe Pro Tech, London, U.K.), with forskolin (Sigma) at a concentration of 10 μM, with rat β-NGF (R & D Systems, Abingdon, Oxon, U.K.) (1 ng/ml) or with combinations of the different factors, unless otherwise indicated.

The pMet7 vector was used as an expression vector for the long and short isoform of the mouse leptin receptor (designated pMET7-mLRLo and pMET7-mLRsh respectively). PC12 cells were transfected by electroporation. Cell-surface expression was measured by specific binding of the leptin-secreted alkaline phosphatase fusion protein as described in [27].

PC12 clones stably expressing the mLRlo were obtained after electroporation with the pMET7-mLRlo expression vector together with the pHCVM-MCS vector containing the neomycin-resistance marker (a gift from Professor C. Sanderson, The Western Australian Research Institute for Child Health, Perth, Australia). Transfected cells were selected for growth in RPMI 1640 medium containing Glutamax-I (l-alanyl-l-glutamine; Gibco BRL) and supplemented with 10% (v/v) inactivated fetal-bovine serum and gentamycin (50 μg/ml). Cells were first grown in selective medium containing 500 μg/ml G418 sulphate (Calbiochem) for 7 days and in 750 μg/ml G418 from day 8 on. After 4 weeks of growth, colonies were transferred to 48-well plates in medium containing 750 μg/ml G418. Subclones were selected for leptin-responsiveness and rat panreatitis-associated-protein-I (rPAP-I) gene activation using a one-tube reverse-transcription (RT-)PCR procedure. In brief, after cell lysis, mRNA was hybridized with bionin-labelled oligo(dT) and captured in streptavidin-coated tubes. After three washing steps the same tubes were used for RT-PCR, optimized for detection of rPAP-I gene induction (mRNA capture and Titan One Tube procedure; Boehringer Mannheim).

RDA (representational difference analysis), reporter and Northern-blot analyses

RDA was used to clone cDNAs from leptin+ forskolin-stimulated PC12 cells, transiently transfected with mLRLo. This RDA procedure was essentially performed as originally described [28] and modified by Braun et al. [29]. PC12 cells were transfected with the pMET7-mLRlo expression vector and stimulated for 72 h with forskolin alone or with a combination of forskolin and leptin. mRNAs were isolated using the Fast Track method (Invitrogen). A 2 μg sample of mRNA of each cell population was used for RDA analysis. After three rounds of subtraction and amplification, transcripts were subcloned into the pCDNA3 or pCR-Blunt (Invitrogen) vector and were sequenced using the Alf Express Sequencer (Pharmacia).

Generation of the pGL3-rPAPLuc reporter construct, luciferase activity assays and Northern-blot analysis were performed as described in [27].

Animal treatment

Specific pathogen-free female C57BL/6J-Lepob mice, 9 weeks old at the beginning of the experiment, and hereafter referred to as ‘ob/ob mice’, were obtained from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). Specific pathogen-free C57BL/6NCrlBr mice, 8 weeks old at the beginning of the experiment, further referred to as ‘wild-type’ were obtained from Charles River Laboratories, Sulzdld, Germany. The animals were housed in a temperature-controlled environment with 12 h light/12 h dark cycles and received water and food ad libitum, with the exception of the starvation experiment. All experiments were performed according to the European Guidelines on Animal Care and Use. Recombinant human leptin (endotoxin level < 0.1 ng/μg; R&D Systems) was diluted in endotoxin-free PBS and administered intraperitoneally at a dose of 100 μg/mouse. In the case of co-administration of 2A5, a monoclonal antibody raised against human leptin [22,30], the dose of leptin was decreased to 50 μg/mouse. The dose of the antibody was 200 μg/mouse. The endotoxin content of the antibody was 0.07 ng/mg of protein, as assessed by a chromogenic Limulus (horseshoe crab) amoebocyte lysate assay (Coatest; Chromogenix, Mölndal, Sweden). Animals were killed by cervical dislocation. Tissues were resected immediately and frozen in liquid nitrogen. RNA extraction and Northern-blot analysis were performed as described above.

Metallothionein (MT)-I RIA

Directly after killing, mouse livers were perfused with endotoxin-free PBS to remove residual blood, resected and frozen in liquid nitrogen. The samples were then homogenized 10% (w/v) in 50 mM Tris/HCl, pH 8.0, and were diluted in gelatin assay buffer prior to radioimmunoassay of MT-I, as described elsewhere [31].

RESULTS

Synergistic effects of leptin and forskolin or β-NGF on PC12 cells

In order to study leptin-receptor signalling in a neuroendocrine-related cell type, we transiently transfected PC12 cells with expression vectors for the long or the short isoform of the mouse leptin receptor (pMET7-mLRlo and pMET7-mLRsh respectively; gifts from Dr. L. Tartaglia, Millennium Pharmaceuticals, Cambridge, MA, U.S.A.) and monitored gene induction by leptin. The PC12 cell line was established from a transplantable rat adrenal pheochromocytoma and is frequently used as a model system for differentiation of neuronal cells. Stimulation with β-NGF leads to a growth arrest and the formation of dendritic processes and expression of neuronal markers. Binding studies using a mouse leptin-secreted alkaline phosphatase fusion protein, and RT-PCR analysis showed that neither undifferentiated nor differentiated PC12 cells express leptin receptors (results not shown). To determine leptin responsiveness, different reporter gene constructs were developed on the basis of the observation that stimulation of the leptin receptor leads to changes in the expression of a variety of neuropeptides, including neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) [32,33]. A first reporter construct contains a 500 bp fragment of the rat NPY promoter sequence coupled to the luciferase gene (pGL3-rNPYIuc; a gift from Dr. Geert Plaetinck, Devgen nv, Technologiepark 9, 9052 Zwijnaarde, Belgium, and J. Van der Heyden, Flanders Interuniversity Institute for Biotechnology, Medical Protein Research, Faculty of Medicine and Health Sciences, University of Ghent, Ghent, Belgium). Figure 1(A) shows that leptin stimulation of PC12 cells co-transfected with the rNPY reporter construct and pMET7-mLRlo, but not with pMET7-TRsh, led to a moderate stimulation of luciferase activity. Since cAMP elevation leads to increased levels of prepro-NPY mRNA in PC12 cells [34], we tested the effect of forskolin, a stimulator of adenylate cyclase, on NPY reporter induction. Co-stimulation led to an up-to-14-fold-enhanced reporter activity, with an optimal effect at 100 ng/ml leptin and 10μM forskolin (Figure 1B). This effect was optimal approx. 72 h post stimulation. Leptin responsiveness in PC12 cells was further
investigated using a clone stably expressing mLRLo (PC12-LR8). After transfection with a reporter construct based on the human POMC promoter (pGL3-POMCluc; a gift from Dr. G. Plaetinck and J. Van der Heyden) (Figure 1C), or a reporter construct based on the rPAP I promoter [27] (see below) (Figure 1D), leptin-induced luciferase activity was measured. Administration of β-NGF (1 ng/ml) mimicked for both reporter constructs the co-stimulatory action of forskolin. The β-NGF and forskolin effects appeared to be additive in this clone (Figures 1C and 1D). Similar data were obtained with 10 or 100 ng/ml β-NGF (results not shown).

**Identification of genes regulated by leptin in PC12 cells**

To search for genes regulated by leptin in the PC12 cell line, an RDA experiment was performed (see the Experimental section). This procedure allowed us to clone amplicons corresponding to transcripts from leptin + forskolin-co-stimulated PC12 cells, transiently transfected with pMET7-mLRlo. After three rounds of subtraction/amplification, selectively amplified bands were purified and subcloned in the pCDNA3 or pCR-Blunt vector (Invitrogen).

Subsequent DNA sequencing revealed that a strongly induced transcript encoded rPAP I. On the basis of this observation, a simple one-tube RT-PCR procedure was set up to select for PC12 subclones stably expressing mLRLo (see the Experimental section). One stable clone, PC12-LR8, was chosen for further experiments. Individual inserts from the cloned amplicon collection were radiolabelled, and leptin-dependent gene regulation was verified and studied in more detail by Northern-blot analysis on the PC12-LR8 cell line. A total of nine leptin-regulated genes were identified (Table 1). Only up-regulated genes were observed.

**Kinetics of induction identifies immediate early response genes and late target genes**

We analysed the kinetics of induction of the above-mentioned transcripts upon leptin treatment. Interestingly, two types of gene sets could be distinguished: a group of immediate early response genes, including those encoding fibroblast-growth-factor-inducible kinase (Fnk), MT-II, modulator recognition factor-1 (MRF-1), STAT-3 and suppressor of cytokine signalling-3 (SOCS-3), in which case induction occurs within 6 h (Figure 2A), and a series of late activated target genes, including those encoding PAP I, uridine-diphosphate glucuronosyltransferase (UGT), annexin VIII and squalene epoxidase, with induction not before 6 h after stimulus (Figure 2C). We next investigated the induction of the immediate early response genes in more detail (Figure 2B). Optimal stimulation varied between 30 min (SOCS-3) and 8 h (STAT-3) post induction. The rate of SOCS-3 mRNA synthesis already showed a rapid decline only 2 h post stimulation. In the case of the late target gene set, maximum mRNA levels were observed between 22 h (PAP I and UGT) and over 96 h (annexin VIII, squalene epoxidase) post induction.

As is apparent from Figure 2, forskolin co-stimulation also distinguished both gene sets. In the case of the immediate early response genes, their induction was further increased by forskolin co-stimulation (Figure 2C). Conversely, for the late target genes, forskolin co-stimulation diminished induction (Figure 2D).
response genes (Figure 2B), some co-stimulation is apparent for MT-II and MRF-1, but only at later time points, and not in the early induction phase. In the case of SOCS-3, forskolin co-treatment even leads to a reduced induction. In contrast, a strong co-stimulatory effect is seen in the case of PAP I, UGT, annexin VIII and squalene epoxidase from 22 h post stimulation (Figure 2C). It is noteworthy that forskolin treatment alone induces a limited differentiation of the PC12 cells, yet does not affect expression of the identified genes (Figure 2C, panel F).

To address the mechanism of induction of the late gene set, the effect of the protein-synthesis inhibitor cycloheximide on rPAP I and annexin VIII mRNA expression was measured. Treatment with 50 μM cycloheximide (starting at 30 min before induction and continuing for 8.5 h) showed a strongly reduced expression of these genes 24 h post induction, implying that de novo protein synthesis is required for induction of the late target gene set (results not shown).

Regulation of MT-II, Fnk, SOCS-3, STAT-3 and PAP I mRNA expression by leptin in ob/ob mice

In order to assess the value of our in vitro model system for obesity, we investigated the regulation by leptin of a subset of the identified genes in vivo. Recombinant human leptin (R & D Systems) was administered intraperitoneally to leptin-deficient ob/ob mice in a single dose of 100 μg of leptin/mouse. Mice were killed by cervical dislocation 5 h after treatment and total RNA was isolated from liver and jejunum. Northern-blot analysis was performed using MT-II, Fnk, SOCS-3, STAT-3 and PAP I as probes (Figure 3). Leptin treatment of ob/ob mice caused a clear induction of MT-II, SOCS-3, STAT-3 and Fnk mRNA expression in liver, while expression of PAP I in jejunum was downregulated by leptin. In a separate experiment, three out of four ob/ob mice showed clear induction of MT-II and Fnk mRNA in liver 2 h after stimulation with leptin (100 μg/mouse) in combination with the 2A5 antibody (200 μg/mouse). 2A5 has been shown before to potentiate leptin activity in vivo [22,30]. At 12 h after injection, expression levels were decreased to control values (results not shown).
Effects of starvation on MT-II, Fnk and PAP I mRNA expression in wild-type mice

Plasma concentration of leptin is lowered rapidly by fasting [33]. We therefore hypothesized that administration of exogenous leptin to wild-type mice 24 h after the start of food deprivation would result in a more prominent gene induction in comparison with \( \text{ob/ob} \) mice. We first investigated the effect of starvation on MT-II and Fnk expression in the liver of wild-type mice (Figure 4A). Mice, starved for 24 h received a single-dose injection of human leptin intraperitoneally (R & D Systems; 50 \( \mu \)g/mouse) in combination with the 2A5 anti-(human leptin) antibody (200 \( \mu \)g/mouse). As a control, additional mice were given a single injection of endotoxin-free PBS. The leptin effect was evaluated by Northern-blot analysis after 2, 6 and 12 h under these prolonged starvation conditions.

Figure 4 MT-II and Fnk gene expression in starved wild-type mice

Wild-type mice were starved for 36 h. After 24 h mice were treated with PBS (−) or leptin (50 \( \mu \)g, supplemented with 200 \( \mu \)g of 2A5 anti-(human leptin) antibody; + ). At different time points (−24, 0, 2, 6, 12 h, indicated at the top) mice were killed. RNA was extracted from liver tissue (A) or jejunum (B) and subjected to Northern-blot analysis using MT-II and Fnk as probes. Hybridization with the mouse \( \beta \)-actin was used as a control and is shown below. Assays were performed and are illustrated in duplicate. Exposure times to BioMax MS films shown in (A) were 2 h, 2 days and 3 h for MT-II, Fnk and \( \beta \)-actin respectively. In (B), exposure times were 2 h for MT-II and 3 h for \( \beta \)-actin.

Expression analysis of leptin-regulated genes

Induction of MT-I protein by leptin in \( \text{ob/ob} \) mice

Since it has been shown previously that MT-I and MT-II are co-ordinately expressed [35], and given the availability of a sensitive assay for MT-I, we tested whether leptin could induce MT-I protein in \( \text{ob/ob} \) mice. Either human leptin (100 \( \mu \)g/mouse) was administered once and mice were killed 5 h after injection, or, in a similar experiment, \( \text{ob/ob} \) mice were treated with leptin twice daily for 2 days and once on the third day, 1 h before killing. Again, as a control, mice were treated with endotoxin-free PBS. Liver samples were taken and analysed by RIA for MT-I measurement, as previously described [31]. Whereas a single leptin injection only produced a slight increase in hepatic MT-I protein, a strong and significant induction of MT-I protein could be observed after repeated leptin injections (Figure 5).

DISCUSSION

In the present study we have explored the use of the PC12 cell line as a model system to study gene induction via the leptin receptor. RDA analysis was performed on undifferentiated cells expressing mLRlo. Leptin treatment itself did not influence the PC12 differentiation status (results not shown). Two distinct functional groups of leptin-induced genes were identified: immediate early response genes, which encode proteins primarily involved in signalling and transcription regulation, and a late induced gene set, which may represent functional target genes.

Among the products of the immediate early response genes, we identified STAT-3 and SOCS-3, which have both been implicated...
in leptin receptor signalling in vitro and in vivo both in hypothalamic nuclei and in peripheral tissues [15,36,37]. In line with these observations, we could also demonstrate increased STAT-3 and SOCS-3 mRNA expression in liver of leptin-treated ob/ob mice. The induction of immediate early response genes typically peaks around 6–8 h post stimulation. Intriguingly, the kinetics of induction of SOCS-3 are different, showing a strong initial induction pulse peaking around 0.5–1 h post induction. Prolonged expression of SOCS-3, coinciding with the other immediate early response genes, is also observed, allowing identification using the RDA procedure. Adams and co-workers have recently reported a similar rapid induction phase of SOCS-3 gene expression by growth hormone in 3T3-F442A fibroblasts. Only early time points were shown, leaving the later induction phase undetected [38]. Forskolin treatment inhibits the very early induction of SOCS-3 gene expression (Figure 2B). This is an interesting observation, since reduction of SOCS-3 synthesis may help to explain the co-stimulatory effects of forskolin seen at later time points.

The identification of MT-I and -II as leptin-induced genes is of special interest, since it was recently shown that mice with targeted disruption of both MT-I and MT-II genes become obese, with elevated plasma leptin levels [39]. In agreement with a potential role for MT-II in obesity control by leptin is our observation that MT-II expression is strongly modulated in liver and jejunum upon leptin treatment in both ob/ob mice and starved wild-type mice, which is in line with direct effects of leptin on liver and intestine [40,41]. Very recently, functional STAT-binding elements have been described in the MT promoter [42], and modulation of MT-I and II expression by other cytokines has been demonstrated before [43]. The precise role of MT induction by leptin is unclear, but recently zinc exchange between MT and zinc-finger proteins (e.g. the transcription factor Gal4) has been demonstrated [44]. Also, transactivation of nuclear factor κB by MT has recently been reported [45]. This suggests that MT proteins could alter transcription-factor activity, which would be in line with our observation that MT-II belongs to the immediate early response gene set. Modulation of MT-I and MT-II expression by leptin may also help to explain the observed protective effect of leptin against TNF toxicity [22], since MTs can protect against oxidative stress in the acute-phase response [46].

We also report on the in vitro and in vivo modulation of Fnk mRNA expression by leptin. This gene belongs to the polo family of serine/threonine protein kinases and is the murine orthologue of Cnk (cytokine-inducible kinase). Fnk can also be induced by fibroblast growth factor, a potent vascular cell mitogen and angiogenic factor. In analogy, leptin is also reported to be an angiogenic factor [19], suggesting that Fnk might play a role in angiogenesis. Fnk is also expressed in the brain, and was recently shown to be targeted to dendrites of activated neurons [47].

The most pronounced induction of a late target gene is seen for PAP I, especially in the presence of forskolin or β2-NGF co-stimulation. The physiological role of the late-induced PAP I and annexin VIII gene products remains unknown at present. PAP I gene transcription has been shown to occur in the pancreas and intestine, two organs where expression of mRNAI has also been demonstrated. The observed transcriptional activation of the UGT and squalene epoxidase [48] genes may suggest interference of leptin in steroid ‘housekeeping’. Interestingly, a strongly enhanced potency of leptin was observed in adenalecotomized rats. This effect could be inhibited in a dose-dependent manner by glucocorticoid supplementation [49]. These observations are in line with a critical peripheral function of leptin.

It is intriguing that differential regulatory effects are observed in different organs: leptin treatment causes up-regulation of MT transcripts in the liver, but suppresses starvation-induced expression in jejunum. Such down-modulation in jejunum is also observed for PAP I. Apparently, the cellular context determines the outcome of the signalling event. This may help explain why, in PC12 cells, NPY promoter activity is up-regulated upon leptin treatment, in contrast with the down-modulation observed in hypothalamic centres in vivo [50].

In conclusion, the PC12 cell line offers a novel interesting tool to study leptin signalling and concomitant gene induction. Underscoring the validity of the PC12 model system is the observation that three out of nine leptin-induced genes identified via a RDA procedure (STAT-3, SOCS-3 and MT-II) have been correlated with obesity or leptin resistance. Furthermore, expression of two more transcripts, PAP I and Fnk, is also modulated by leptin in vivo, suggesting a role in leptin physiology. Undifferentiated PC12 cells may be particularly useful for the analysis of peripheral leptin functions. In addition, work is in progress to analyse gene-sets induced by leptin in the differentiated neuronal phenotype of the PC12 cell.

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