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

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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-1), which are immediate early response genes, and pancreatitis-associated protein I (PAP I), 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 PAP I 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 PAP I 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.

INTRODUCTION

In healthy conditions, assimilation, storage and utilization of nutrient energy constitute an integrated homoeostatic system. Maintaining a relatively constant level of energy stores, and hence body weight, is based on a complex balance of food intake and energy expenditure. Hypothalamic nuclei are believed to be the sites where homoeostasis is regulated, given their important role in regulation of food intake, body weight, energy expenditure and hormone integration that steers metabolic housekeeping [1–3]. This relies in part on a signalling pathway of a hormone mainly secreted by adipocytes and acting through specific receptors in those areas of the brain that govern ingestive behaviour and metabolic activity [4]. Cloning of the mouse *ob* gene and its human homologue led to the identification of leptin [5]. Consistent with its role as the postulated adipose-derived satiety factor, administration of leptin to *ob/ob* mice caused rapid reversal of the obese phenotype by decreasing food intake and increasing energy expenditure [6–8]. Furthermore, most if not all of the obesity-associated metabolic, endocrine and immune defects were corrected [2,9].

Leptin binds to a high-affinity receptor, the product of the *db* gene. This receptor may exist in at least six isoforms [10,11]. Thus far, most attention has been focused on the isoform with long cytoplasmic domain (OB-Rb), which is predominantly expressed in certain hypothalamic nuclei [12,13] and generally believed to be the signalling competent receptor [14]. Leptin-receptor signalling potentially occurs through activation of Janus kinase 2 (JAK2) and multiple signal transducers and activators of transcription (STATs) [14]; however, only STAT-3 activation was observed in hypothalamic centres [15,16]. In addition, expression of the long receptor isoform in a wide range of peripheral tissues as well as in certain specific cell types [9,13,17–20], clearly suggests that leptin action is not restricted to hypothalamic nuclei. These findings correlate with additional functions of leptin, e.g. its angiogenic activity [19], its regulatory role in haematopoiesis [21], its modulating action in T-cell immune response [9] and involvement in the protection against tumour-necrosis-factor (TNF)-induced toxicity [22]. The relevance of a dual leptin action profile, i.e. at the central and peripheral level, cannot be underestimated, particularly in view of 'leptin resistance' in obese humans [23–26].

To gain more insight into leptin physiology, we have studied leptin-mediated gene induction in PC12 cells. Originating from a rat pheochromocytoma, these cells represent an acceptable model for studying the dual role of leptin. Besides their neural-crest origin, β-nerve-growth-factor (β-NGF)-treated PC12 cells exhibit many of the properties of mature terminally differentiated neurons, whereas undifferentiated PC12 cells may mimic peripheral leptin-responding cells. We previously demonstrated leptin-responsiveness in PC12 cells stably expressing the long isoform of the leptin receptor (mLRlo) [27]. Here we report on the identification and modulation of a series of leptin-induced genes using PC12 cells and checked representative *in vivo* modulation using both wild-type and leptin-deficient (*ob/ob*) mice.

Abbreviations used: Fnk, fibroblast-growth-factor-inducible kinase; IL-6, interleukin-6; JAK, Janus kinase; mLRlo, long isoform of the mouse leptin receptor; mLRsh, short isoform of the mouse leptin receptor; MT, metallothionein; MRF-1, modulator recognition factor-1; β-NGF, β-nerve growth factor; (r)NPY, (rat) neuropeptide Y; (r)PAP I, (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 decribed in [27].

PC12 clones stably expressing the mLRlo were obtained after electroporation with the pMET7-mLRlo expression vector together with the pHCMV-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 pancreatitis-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 biotin-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-Lep^{ob} 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, Sulzfed, 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-rNPYluc; 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-LRsh, 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

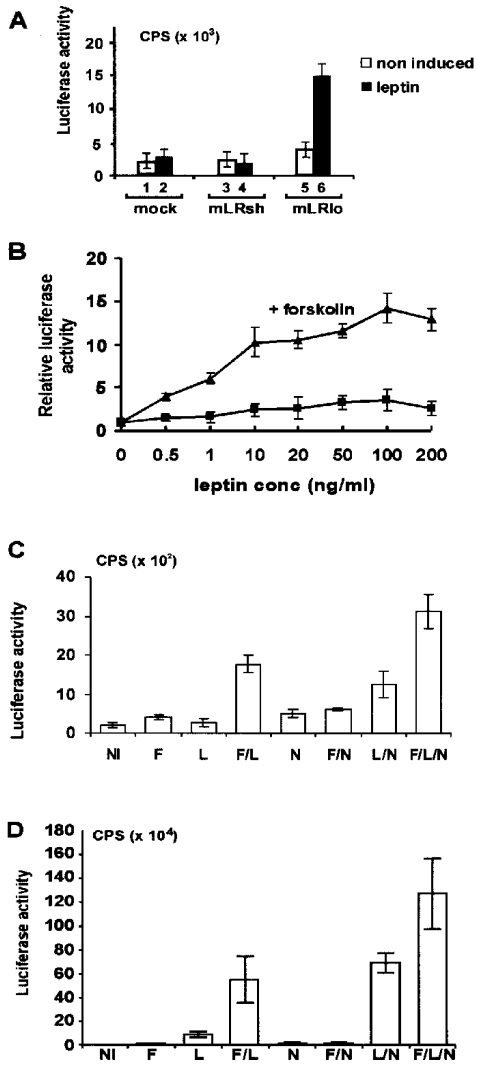

Figure 1 Synergistic signalling of leptin, forskolin and β-NGF in PC12 cells

(**A**) Leptin-induced NPY promoter activity in transiently transfected PC12 cells. PC12 cells were co-transfected with control vector (columns 1 and 2), pMET7-mLRsh (columns 3 and 4) or pMET7-mLRlo (columns 5 and 6) together with the pGL3-rNPYluc reporter. The different subcultures were mock-stimulated or treated with mouse leptin (100 ng/ml) for 72 h. Luciferase activity in cell lysates is shown; results are means ± S.D. for assays performed in triplicate. CPS, counts/s. (**B**) Forskolin co-stimulation on leptin-induced pGL3-rNPYluciferase activity. PC12 cells were transiently co-transfected with pMET7-mLRlo and the pGL3-rNPYluc reporter. Subcultures were treated with mouse leptin alone (■) or in combination with forskolin at a concentration of 10 μM (▲). After 72 h cells were lysed and assayed for luciferase activity. Average values for normalized, relative luciferase activities (*x*-fold increase) from three independent experiments are shown. (**C** and **D**) Analysis of β-NGF and forskolin co-stimulation on leptin-induced POMC luciferase (**C**) or rPAP luciferase activity (**D**). PC12-LR8 cells were transiently transfected with pGL3-rPOMCluc or pGL3-rPAPluc reporter and treated after 2 days with forskolin (F; 10 μM), leptin (L, 100 ng/ml) and rat β-NGF (N; 1 ng/ml) as indicated or were left untreated (NI). At 24 h after treatment, cells were lysed and assayed for luciferase activity. Results are means ± S.D. for assays performed five times.

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).

Table 1 Identity of the identified amplicons

Homology searches were performed using the Gapped BLAST and PSI-BLAST algorithms.

Amplicon	Homology/identity	GenBank® accession no.
1	Annexin VIII	AJ002390
2	Fnk	U21392
3	MT-II	M11794
4	MRF-1	M62324
5	PAP I	M98049
6	STAT-3	X91810
7	Squalene epoxidase	D42048
8	SOCS-3	AF075383
9	UGT	U75903

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 glucuronyltransferase (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

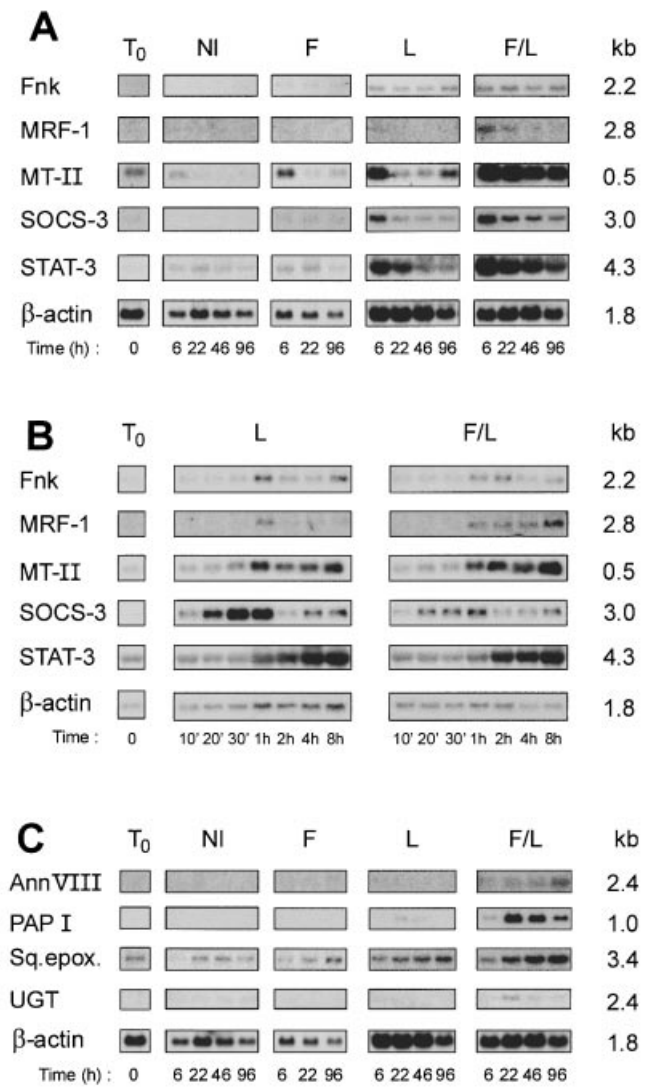

Figure 2 Kinetics of induction of leptin-responsive genes in PC12-LR8 cells

Autoradiographs of Northern-blot experiments using PC12-LR8 cells treated as shown and probed for expression of genes as indicated. T_0 gives the expression level prior to stimulation. Hybridization with the mouse β-actin probe was used as a control. Sizes of the transcripts are marked on the right. (**A**) Northern-blot analysis of the immediate early response genes. PC12-LR8 cells were left untreated (NI, non-induced), or were treated with forskolin (F), leptin (L) or a combination of both (F/L) for the indicated time points. Exposure times to BioMax MS films for the different transcripts were: for Fnk and MRF-1, 6 days; for MT-II, 5 days; for SOCS-3, 14 h; for STAT-3, 14 h; and for β-actin, 5 h. (**B**) Immediate early response gene-set: early kinetics. PC12-LR8 cells were treated with leptin alone (L) or with a combination of leptin and forskolin (F/L) for the indicated length of time. Exposure times to BioMax MS films were: Fnk and MRF-1, 3 days; MT-II, 3 days; SOCS-3, 2.5 days; STAT-3, 2.5 days; β-actin, 1 h. (**C**) Northern-blot analysis of the late target genes. PC12-LR8 cells were left untreated (NI), or were treated with forskolin (F), leptin (L) or a combination of both (F/L) for the indicated length of time. Exposure times to BioMax MS films were: annexin VIII, 6 days; PAP I, 14 h; squalene epoxidase and UGT, 5 days; β-actin, 14 h.

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).

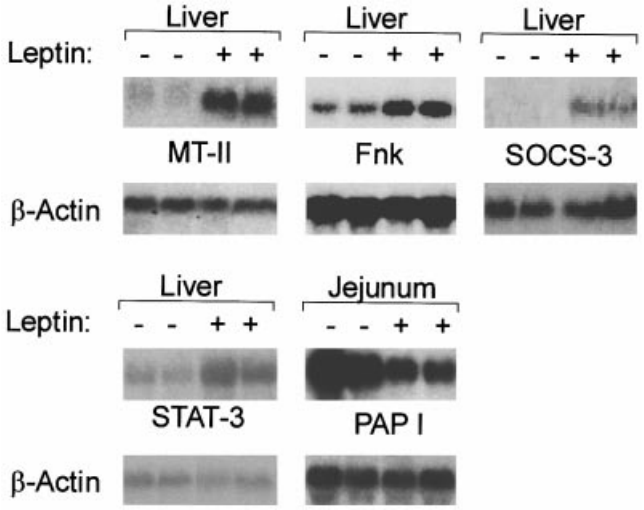

Figure 3 MT-II, Fnk, SOCS-3, STAT-3 and PAP I gene expression in leptin-treated *ob/ob* mice

Leptin-deficient *ob/ob* mice were left untreated (−) or were treated with leptin (100 μg; +) intraperitoneally. Mice were killed 5 h after treatment. Northern-blot analysis of MT-II, Fnk, SOCS-3 and STAT-3 in liver and PAP I expression in jejunum is shown. Hybridization with the mouse β-actin probe was used as a control and is shown below. Exposure times to BioMax MS films were 4 h, 3 days, 3 days, 12 h, 4 h and 8 h for MT-II, Fnk, SOCS-3, STAT-3, PAP I and β-actin respectively.

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 down-regulated 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).

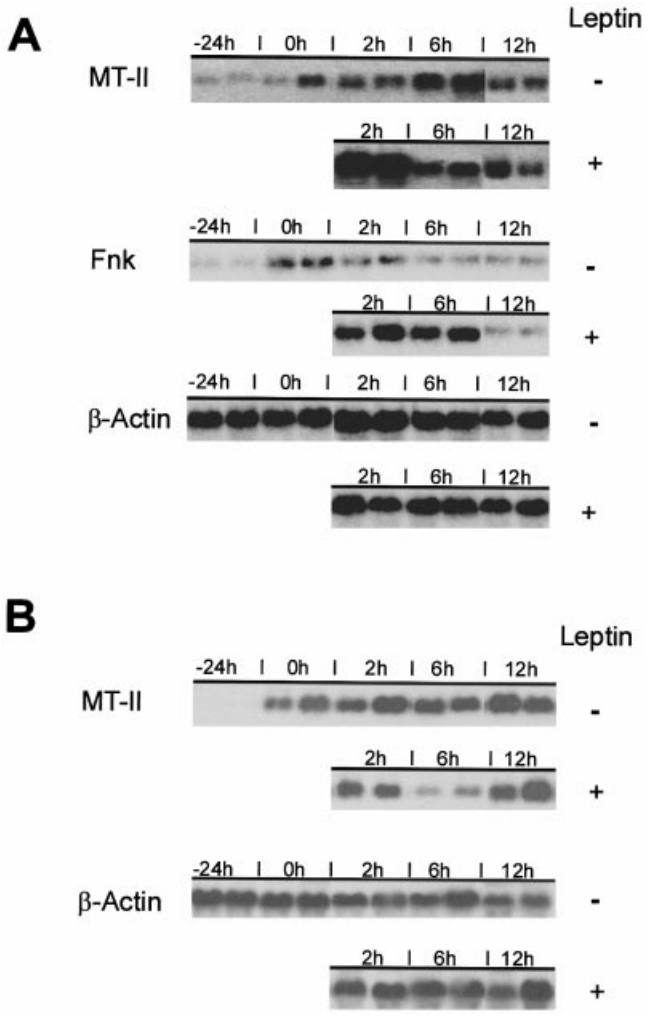

Figure 4 MT-II and Fnk gene expression in starved wild-type mice

Wild-type mice were starved for 36 h. After 24 h mice were treated with PBS (−) or leptin [50 μg, supplemented with 200 μg 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 β-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 β-actin respectively. In (**B**), exposure times were 2 h for MT-II and 3 h for β-actin.

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 *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 intraperiteonally (R & D Systems; 50 μg/mouse) in combination with the 2A5 anti-(human leptin) antibody (200 μg/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.

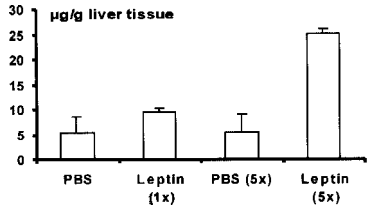

Figure 5 MT-I protein levels in leptin-treated *ob/ob* mice

ob/ob Mice were treated with a single injection of endotoxin-free PBS (PBS) or human leptin (Leptin 1 ×) and killed 5 h after administration. Similarly, mice were subjected to five repeated injections with PBS (PBS 5 ×) or leptin (Leptin 5 ×) for 2 days and were killed 1 h after the fifth injection. Liver was extracted for MT-I dosage as described in the Experimental section. Each column shows the mean value ± S.D. for three measurements. The results shown are representative of two independent experiments.

Starvation led to a moderate increase in MT-II and Fnk mRNA expression in the liver. This effect was markedly enhanced by leptin treatment, leading to a strong induction of MT-II and Fnk expression 2 h post injection. mRNA expression levels returned to those observed in the PBS-treated control mice, 6 and 12 h after leptin administration for MT-II and Fnk respectively.

Starvation also led to a spontaneous induction of MT-II mRNA in jejunum. In contrast with the observation in liver, this effect was suppressed by leptin + 2A5 treatment 6 h post injection. The expression levels of MT-II recovered to control levels 12 h post injection (Figure 4B). A similar pattern was observed for PAP I mRNA expression in jejunum, showing a reduction 24 h after leptin + 2A5 treatment in starved mice, compared with the PBS-treated controls (results not shown). Modulation of SOCS-3 and STAT-3 mRNA expression in starved wild-type mice could not be shown.

Induction of MT-I protein by leptin in *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 *ob/ob* mice. Either human leptin (100 μg/mouse) was administered once and mice were killed 5 h after injection, or, in a similar experiment, *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 β-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 mLRlo 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 adrenalectomized 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 jejenum. 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.

We thank José Van der Heyden, Dr. Geert Plaetinck, Dr. Lou Tartaglia and Dr. Xaveer Van Ostade for materials and discussions, Claudia Lins and Gabriele Holtappels for help with RT-PCR analyses and RNA purification, Professor Dr. Peter Brouckaert, Dr. Claude Libert and Dr. Nozomi Takahashi for guidance through the *in vivo* experiments, Anne Wood for assistance with the RIA and Wim Drijvers for artistic help. W. W. is a Fellow with the Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie ('IWT'). Annick Verhee was supported by a grant from the Geconcerteerde Onderzoekacties (GOA 96012). This work was funded by the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO-V G.0149.99).

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Received 31 August 1999/3 February 2000; accepted 7 March 2000