Leptin activates the promoter of the interleukin-1 receptor antagonist through p42/44 mitogen-activated protein kinase and a composite nuclear factor κ B/PU.1 binding site

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We have recently shown that leptin strongly induces the expression and secretion of the interleukin-1 receptor antagonist (IL-1Ra) [Gabay, Dreyer, Pellegrinelli, Chicheportiche and Meier (2001) *J. Clin. Endocrinol. Metab.* **86**, 783–791] in monocytes. However, the intracellular signalling mechanisms involved remained unknown. We now demonstrate that the activation of the IL-1Ra promoter by leptin is strictly dependent on the presence of the long form of the leptin receptor (OB-Rb), and that it also requires the activation of the p42/44 mitogen-activated protein kinases (MAPKs) as well as the presence of a nuclear factor κ B (NF- κ B)/PU.1 composite site at position –80 of the IL-1Ra promoter. Although leptin is capable of activating a NF- κ B reporter element in transient transfection experiments, the protein

complex binding to the NF- κ B/PU.1 site of the IL-1Ra promoter is not composed of the p65/p50 subunits of NF- κ B, as is evident in electrophoretic gel mobility-shift experiments. In contrast, a protein complex which does not contain PU.1 binds to this composite element in a leptin-dependent manner. In summary, we characterize the signalling pathway for leptin and OB-Rb involved in the induction of IL-1Ra, involving p42/44 MAPK, and a yet uncharacterized complex of transcription factor(s) binding to a NF- κ B/PU.1 composite element of the IL-1Ra promoter.

Key words: cytokine, long leptin receptor isoform (OB-Rb), monocyte, signal transduction.

INTRODUCTION

Leptin is an adipocyte-derived cytokine-like peptide hormone regulating appetite and energy homeostasis at the hypothalamic level [1]. However, leptin has also been shown to exhibit direct effects on other tissues [2–6]. While we and others have demonstrated leptin receptor-dependent direct auto-/paracrine actions of leptin on adipose tissue resulting in altered gene expression and lipolysis [3,4], several observations suggested that leptin might also have actions on the immune system, particularly on lymphocytes and monocytes [2,5–9].

Leptin-deficient (*ob/ob*) mice have been shown to exhibit an increased susceptibility to lipopolysaccharide (LPS)- and tumour necrosis factor- α -induced mortality, with a protective effect of exogenously administered recombinant leptin [7,8]. *ob/ob* mice had significantly lower concentrations of interleukin-1 (IL-1) receptor antagonist (IL-1Ra) compared with their lean littermates (*ob/+*) after LPS injection, compatible with regulation of IL-1Ra secretion by leptin [10]. Since IL-1Ra is a member of the IL-1 family binding to IL-1 receptors but failing to induce any cellular response, it inhibits the effects of IL-1 on its target cells, resulting in anti-inflammatory effects *in vivo* in several experimental disease models, including septic shock, arthritis and colitis [11,12].

We have shown recently that leptin increases the secretion of IL-1Ra, but not IL-1 β , 6–10-fold from the human THP-1

monocytic cell line, as well as from human peripheral blood mononuclear cells, while the mRNA for IL-1Ra was increased within 8 h of leptin treatment [6]. We and others also demonstrated that monocytes and macrophages express the long form of the leptin receptor (OB-Rb) and that leptin induces the nuclear translocation of signal transducers and activators of transcription (STAT), suggesting that the OB-Rb is functional in these cells [6,13].

These findings not only represented a novel action of leptin unrelated to the regulation of energy balance, they also provided a novel cellular model to characterize the signalling mechanisms of leptin, as such studies are notoriously difficult to perform in the hypothalamic target cells for leptin.

Initially, the homology of the OB-Rb to the interleukin-6/gp130 receptor complex led to the demonstration of an activation of the Janus tyrosine kinase (JAK)/STAT pathway in neural cells *in vivo*, as well as in adipose cells *in vitro* [1,3,4,14]. Subsequently, OB-Rb was shown to result also in the activation of the mitogen-activated protein kinase (MAPK), through the protein tyrosine phosphatase SHP-2 [15,16]. In addition, the suppressor of cytokine signalling ('SOCS')-3 appears to be a proximal inhibitor of leptin action [17].

We have now elucidated the intracellular signalling cascade linking the activation of the OB-Rb by leptin to the activation of the IL-1Ra promoter, which involves the activation of MAPK and the binding of an as yet uncharacterized factor to the nuclear

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility-shift assay; ERK, extracellular signal-regulated kinase; GABP, GA-binding protein; IL-1, interleukin-1; IL-1Ra, IL-1 receptor antagonist; JAK, Janus tyrosine kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEM, minimal Eagle's medium; NF- κ B, nuclear factor κ B; OB-Rb, long form of the leptin receptor; PI 3-kinase, phosphoinositide 3-kinase; STAT, signal transducers and activators of transcription.

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factor κ B (NF- κ B)/PU.1 (originally described as Spi-1, or spleen focus-forming virus proviral integration 1) binding site of the IL-1Ra promoter.

MATERIALS AND METHODS

Reagents

LPS, DMSO, dithiothreitol, polymyxin B sulphate and sodium orthovanadate were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). RPMI-1640, L-glutamine and penicillin/streptomycin were obtained from Gibco-BRL (Life Technologies, Paisley, Scotland, U.K.). EDTA and EGTA were obtained from Fluka (Buchs, Switzerland). CompleteTM, a protease inhibitor cocktail, and Nonidet P-40 were purchased from Boehringer Mannheim (Mannheim, Germany). The PD98059 and U-0126 inhibitors of p42/44 MAPK were obtained from Alexis Corp. (Lausen, Switzerland) and from BioMol (Palo Alto, CA, U.S.A.), respectively. Inhibitors of tyrosine kinases (genistein) and phosphoinositide 3-kinase (PI 3-kinase; Ly 294002) were obtained from BioMol, and the PI 3-kinase inhibitor wortmannin was from Calbiochem (Juro, Lucerne, Switzerland). SB203580 (an inhibitor of p38 MAPK), PMA and okadaic acid were purchased from Alexis (Braunschweig, Germany). Oligonucleotides for electrophoretic mobility-shift assay (EMSA) experiments were synthesized by Microsynth (Balgach, Switzerland). Recombinant murine leptin was a kind gift of the Eli Lilly Company (Indianapolis, IN, U.S.A.).

Plasmids

The pcDNA3-OB-Rb expression plasmid containing the full-length murine cDNA was kindly provided by Dr R. Skoda (University of Basel, Basel, Switzerland) [18]. The pRc/CMV plasmid expression vector for PU.1 was a kind gift of Dr P. Auron (Harvard Medical School, Boston, MA, U.S.A.). The luciferase constructs containing the -1680/+1 region of the promoter for the secreted form of the human IL-1Ra (pIL-1Ra -1680-Luc) and derivatives thereof with mutations of the sites for NF- κ B (-93/-84) and/or CCAAT/enhancer binding protein (C/EBP; -158/-153), pIL-1Ra -1680 Δ NF κ B-Luc, pIL-1Ra -1680 Δ C/EBP-Luc, respectively, were reported previously [19]. To assess functionally the NF- κ B pathway, an artificial [NF- κ B]₄-Tk-Luc reporter plasmid from Clontech (Palo Alto, CA, U.S.A.) was utilized, which contains four consensus binding sites for NF- κ B (5'-CGGGAATTTC-3') upstream of the viral thymidine kinase (Tk) promoter.

Cell culture

The human HepG2 hepatoma cell line (ATCC HB-8065) was grown in minimal Eagle's medium (MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 0.25 μ g/ml fungizone.

The THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 i.u./ml penicillin/streptomycin and 2 mM L-glutamine in a 5% CO₂/95% air humidified atmosphere at 37 °C as described in [6].

Transfection experiments

HepG2 cells were seeded into six-well plates with complete MEM and 5% fetal bovine serum stripped with AG1-8X resin from Bio-Rad (Hercules, CA, U.S.A.) as described in [20]. The cells were transiently transfected with the appropriate expression and

luciferase reporter constructs using the calcium phosphate method, and luciferase reporter activity was determined and normalized for protein concentrations as described in [21]. 24 h after transfection, the cells were stimulated for 24 h with leptin in MEM supplemented with 2.5% stripped fetal bovine serum.

Preparation of nuclear extracts and EMSA

Nuclear extracts from THP-1 cells were prepared as described previously [3,6]. Briefly, THP-1 cells were cultured as described above. Prior to stimulation (24 h), the cells were transferred into medium containing 0.1% heat-inactivated fetal bovine serum. Then, 15 or 30 min after the addition of vehicle, PMA (25 nM) or leptin (625 nM), the cells were centrifuged and the pellet was resuspended in hypotonic buffer [10 mM KCl, 10 mM Hepes (pH 7.9), 0.1 mM EDTA, 0.1 mM EGTA plus freshly added 1 mM dithiothreitol and CompleteTM protease inhibitor cocktail at the concentration recommended by the manufacturer]. The cells were left to swell on ice for 15 min, after which 25 μ l of 1% Nonidet P-40 was added and the tubes were vortexed vigorously. The suspension was centrifuged for 30 s at full speed in a microfuge. The pelleted nuclei were resuspended in a cold hypertonic buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20% glycerol and Complete protease inhibitor cocktail. After 15 min of incubation the tubes were centrifuged and the supernatants were stored at -80 °C. Double-stranded oligonucleotides containing the binding site for NF- κ B/PU.1 from the IL-1Ra promoter (NF κ B/PU.1_{IL-1Ra} -101/-77, 5'-ATGCGAGGAGGGTATTTCGCTTCT-3'; LRE3, 5'-CAATGAAGGGGAAATAGAATCT-3'; LRE3-mut, 5'-CAATGAAGGATCCATAGAATCT-3') and the GA-binding protein (GABP)-binding site from the α 27 gene of herpes virus simplex (GABP, 5'-CCCCGGGAGCGGAACGGT-3') were labelled by fill-in with Klenow DNA polymerase in the presence of [α -³²P]dCTP as described in [3,6]. Competition experiments were performed with NF- κ B/PU.1 composite site of the IL-1Ra promoter (-101/-77) as a probe and unlabelled oligonucleotides corresponding to the NF- κ B (-92/-83; 5'-GGGTATTTCC-3') and PU.1 (-89/-81; 5'-TATTTCCGC-3') binding sites. Protein (3 μ g) from THP-1 cell nuclear extracts was then incubated with 30000 c.p.m. of the labelled probe, and 2 μ g of poly[d(I-C)] in EMSA binding buffer (50 mM KCl, 20 mM Hepes, 20% glycerol, 0.05% Nonidet P-40 and 10 mM β -mercaptoethanol, pH 7.5) in a final volume of 25 μ l. Incubation was performed at room temperature for 20 min and the samples were analysed on a 5% polyacrylamide gel in 0.5 $\times$ TBE (44.5 mM Tris/44.5 mM boric acid/1 mM EDTA, pH 8) for 75 min at 300 V. Gels were dried and autoradiographed. *In vitro*-translated PU.1 was obtained from the pRc-PU.1 plasmid (a kind gift of Dr P. Auron) using the TnT T7 Quick coupled transcription/translation system (Promega, Catalys AG, Wallisellen, Switzerland).

For the supershift experiments, a p65, p50 or PU.1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added to the nuclear extract or to the *in vitro*-translated protein before addition of the labelled oligonucleotides.

Western blotting

THP-1 cells were treated as appropriate and whole-cell extracts were prepared as described [22]. Proteins (20 μ g) were suspended in loading buffer (60 mM Tris/HCl, pH 6.8, 2% SDS, 5% β -mercaptoethanol, 10% glycerol and 0.01% Bromophenol Blue) and, after denaturation, loaded on to 10% acrylamide mini-gels

(Bio-Rad), run at 150 V for 1 h, and subsequently transferred on to Parablot 0.45 μ m nitrocellulose membranes (Machery-Nagel, Düren, Germany) at 100 V for 2 h at 4 °C. Thereafter, the membranes were incubated in a blocking buffer (50 mM Tris/HCl, 200 mM NaCl, 0.2% Tween 20 and 5% non-fat dried milk) for 1 h at room temperature. The membranes were then incubated for 2 h in the same buffer containing 1% non-fat dried milk with a polyclonal antibody raised against phosphorylated p42/44 (New England BioLabs, Beverly, MA, U.S.A.), phosphorylated p38 (Cell Signalling, Bioconcept, Allschwil, Switzerland), total p42/44 or total p38 MAPK (Santa Cruz Biotechnology, LabForce, Nunningen, Switzerland). The membranes were washed in the same buffer without milk and incubated for 1 h with horseradish peroxidase-labelled goat anti-rabbit antibodies (CovalAb, Oullins, France). After washing six times for 10 min, the immunoreactive bands were visualized by the enhanced chemiluminescence (ECL) detection reagent (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. The non-parametric Mann-Whitney *U* test statistic was used for comparisons between the specified conditions with SYSTAT 10 (SPSS, Chicago, IL, U.S.A.).

RESULTS

Leptin activates the promoter of soluble IL-1Ra in HepG2 cells in an OB-Rb- and p42/44 MAPK-dependent manner

We have shown previously that leptin rapidly and strongly induces the expression of the mRNA for IL-1Ra in THP-1 cells, and that it activates the pIL-1Ra – 1680-Luc reporter in the NIH-3T3 cell line [6]. Since hepatocytes express IL-1Ra, we chose the HepG2 cell line for transient transfection experiments. Similarly, leptin stimulated the pIL-1Ra – 1680-Luc reporter in this cell line in an OB-Rb-dependent manner, and no effect of leptin was observed without the co-transfection of an expression vector for OB-Rb (Figure 1A).

Since the OB-Rb was previously shown in other cell types to activate p42/44, p38 MAPK or PI 3-kinase [4,16,23–25], we examined the effect of various protein kinase inhibitors on the capacity of leptin and OB-Rb to activate the –1680/+1 fragment of the IL-1Ra promoter. HepG2 cells were pretreated for 1 h with inhibitors of the p42/44 MAPK (PD98059, 30 μ M, or U-0126, 5 μ M), p38 MAPK (SB 203580, 10 μ M), PI 3-kinase (wortmannin, 100 nM) and tyrosine kinase (genistein, 100 ng/ml) pathways. As shown on Figure 1(A), U-0126 as well as PD98059 completely abolished the OB-Rb-dependent activation of the IL-1Ra promoter by leptin. In contrast, leptin still significantly activated the IL-1Ra reporter in the presence of the tyrosine kinase inhibitor (genistein), p38 MAPK (SB 203580) or PI 3-kinase inhibitor (wortmannin), although to a somewhat smaller degree. Since PI 3-kinase has been implicated in mediating the effects of leptin in human monocytes [13], experiments with various concentrations of wortmannin and Ly 294002 were performed (Figure 1B). In these experiments, leptin significantly stimulated the IL-1Ra promoter, also to a smaller degree than in Figure 1(A), which may be attributable to a lower biological activity of this batch of leptin. Wortmannin and Ly 294002 did not inhibit this stimulatory effect of leptin at concentrations of 500 nM and 5 μ M, respectively, while higher concentrations of Ly 294002 (10 and 20 μ M) resulted in an increase in the stimulatory effect of leptin, suggesting that PI 3-kinase might

Figure 1 Activation of the IL-1Ra – 1680/+1 promoter by leptin requires p42/44 MAPK activity

(A) HepG2 cells were transiently transfected with 0.2 μ g of OB-Rb expression vector together with 3 μ g of IL-1Ra – 1680-Luc reporter. After transfection, the cells were pretreated for 1 h with U-0126 (5 μ M), SB203580 (10 μ M), wortmannin (100 nM) or genistein (100 ng/ml) for 30 min. Subsequently, the cells were exposed for 24 h to either vehicle or 625 nM leptin. The results are means $\pm$ S.E.M. from three independent experiments performed in triplicate (**P* < 0.05 for the stimulation by leptin; ns, non-significant; #*P* < 0.05 for the effect of different inhibitors on the stimulation by leptin). (B) As above, transiently transfected HepG2 cells were pretreated for 1 h with LY 294002 (5, 10, 20 μ M) or wortmannin (100, 500 nM) before the addition of 625 nM leptin for 24 h (for statistical details see A).

exert an inhibitory effect on the regulation of the IL-1Ra promoter under certain conditions.

Taken together these data implicate the MAPK p42/44 pathway in the activation of the IL-1Ra promoter by leptin.

Leptin induces the phosphorylation of p42/44 MAPK

To demonstrate directly that leptin increased the activity of the p42/44, rather than the p38 MAPK pathway, Western blot analysis was performed with extracts from THP-1 cells treated with vehicle (NaCl for leptin and LPS; DMSO for PMA), leptin, LPS or PMA. As shown on the autoradiograph in Figure 2(A), LPS, PMA and leptin enhanced the phosphorylation of extracellular-signal-regulated kinase (ERK) p42 and p44 after

Figure 2 Leptin increases the phosphorylation of p42/44 ERK in THP-1 cells

(A) THP-1 cells were pretreated for 30 min with either polymyxin B (Poly; 10 μ g/ml) or U-0126 (5 μ M). Thereafter, the cells were incubated with LPS (100 ng/ml for 30 min), leptin (625 nM for 15 or 30 min) or NaCl (0.9%) as control vehicle. Additionally, cells were incubated with PMA (25 nM) or DMSO as control vehicle for 30 min. A Western blot was performed using an antibody against the phosphorylated (upper panel) or total (lower panel) form of p42/44 ERK. Quantification of the leptin-induced phosphorylation of ERKs normalized for total ERK content is represented in the right-hand panel. (B) THP-1 cells were incubated for 15 and 30 min with PMA (25 nM in DMSO), DMSO alone, leptin (625 nM in 0.9% NaCl) or NaCl (0.9%) alone as control vehicle. Western blots were performed using an antibody against the phosphorylated (upper panel) or total (lower panel) form of p38 MAPK. Quantification of the leptin effect on the phosphorylation of p38 after normalization for total ERK content is represented in the right-hand panel.

30 min as compared with their respective vehicle (NaCl for leptin). The quantification shown in the right-hand panel of Figure 2(A) shows that leptin stimulates the phosphorylation of ERK after just 15 min (2.6- and 3.1-fold after 15 min, and 2.7- and 1.7-fold after 30 min for p42 and p44, respectively, after correction for total ERK content). While the effect of LPS was virtually abolished by 10 μ g/ml polymyxin B and p42/44 phosphorylation returned to their basal level (as compared with NaCl), this LPS antagonist had no effect on the response to leptin, demonstrating that leptin is not contaminated by bacterial endotoxin. However, as expected, U-0126 annihilated the basal and stimulated activation of the ERKs by leptin. In keeping with the results obtained in transfection experiments, leptin did not significantly induce p38 phosphorylation after 15 and 30 min of stimulation, as shown on the autoradiograph in Figure 2(B) (upper left-hand panel, phosphorylated p38; lower left-hand panel, total p38). The quantification and correction for total p38 content resulted in a 1.1- and 1.5-fold stimulation by leptin after 15 and 30 min, respectively (Figure 2B, right-hand panel). These data demonstrate that leptin rapidly activates the p42/44 ERK pathway in THP-1 monocytic cells.

A proximal NF- κ B site in the promoter of IL-1Ra is required for activation by leptin

Several binding sites for transcription factors have been identified in the promoter for the soluble form of human IL-1Ra, among

them elements for NF- κ B (−93/−84), C/EBP (−158/−153) and STAT (−416/−408). Since leptin stimulates the phosphorylation of the p42/44 MAPK and its effect on the IL-1Ra promoter is abolished by U-0126, but not the tyrosine kinase inhibitor genistein, we speculated that NF- κ B or C/EBP, rather than STATs, might be the downstream mediators in this OB-Rb-dependent signalling cascade. Indeed, since ERKs are known activators of the NF- κ B pathway [26], we tested whether the NF- κ B or C/EBP sites in the IL-1Ra promoter were involved in this activation. Hence, we transfected mutated variants of the IL-1Ra −1680-Luc reporter into HepG2 cells in the presence or absence of co-transfected OB-Rb. Figure 3(A) shows that the IL-1Ra −1680 Δ NF- κ B-Luc construct cannot be activated by leptin, while the IL-1Ra-1680 Δ C/EBP-Luc reporter is still stimulated 1.6 $\pm$ 0.2-fold by leptin in the presence of OB-Rb (Figure 3B). Hence, the NF- κ B-binding site at position −93/−84 of the promoter of the secreted form of IL-1Ra is required for the activation by leptin.

Leptin activates the NF- κ B pathway

We then tested whether leptin is capable of directly activating a reporter construct containing NF- κ B consensus sites upstream of an heterologous promoter ([NF- κ B]₄-Tk-Luc). Leptin was able to activate significantly this reporter 1.7 $\pm$ 0.08-fold, and this response was again entirely dependent on the presence of OB-Rb

Figure 3 A NF- κ B-binding site is required for the OB-Rb-dependent activation of the IL-1Ra promoter by leptin

(A) HepG2 cells were transiently transfected with 0.2 μ g of pcDNA3 or OB-Rb expression vector together with 3 μ g of IL-1Ra -1680 Δ NF κ B-Luc reporter construct. After transfection, the cells were treated for 24 h with either vehicle or leptin (625 nM; the results are the means $\pm$ S.E.M. of three independent experiments performed in triplicate). (B) HepG2 were transiently transfected with 0.2 μ g of pcDNA3 or OB-Rb expression vector together with 3 μ g of IL-1Ra -1680 Δ C/EBP-Luc reporter construct. After transfection, the cells were treated for 24 h with either vehicle or leptin (625 nM; the results are the means $\pm$ S.E.M. of two independent experiments performed in triplicate; * P < 0.05).

(Figure 4A). We then examined whether leptin was able to induce the nuclear translocation of NF- κ B in THP-1 cells. Gel shift experiments showed that leptin induces the specific binding (Figure 4B, *) of nuclear proteins from THP-1 monocytic cells to a NF- κ B consensus binding site. In contrast, this binding activity is not observed after stimulation by LPS, which slightly increased a slower-migrating binding activity, corresponding to NF- κ B binding, as evidenced by supershift experiments with a p65 antibody (Figure 4B). The relatively small effect of LPS on NF- κ B induction may be accounted for by low levels of CD34 [27] and/or high basal NF- κ B binding. Additional transfection experiments performed in the presence of U-0126 demonstrated

the requirement of ERK activity for the leptin-dependent activation of NF- κ B in HepG2 cells (Figure 4C).

Leptin induces a binding activity to the NF- κ B/PU.1 site of the IL-1Ra promoter

Since leptin is capable of enhancing the transcriptional activity from a NF- κ B-regulated reporter construction in HepG2 cells transfected with OB-Rb, and it induces a binding activity on a NF- κ B consensus binding site, we examine whether leptin also induced a binding activity on a NF- κ B site from the IL-1Ra promoter (NF κ B/PU.1_{IL-1Ra}). Shown in Figure 5(A) is an EMSA experiment on this binding site incubated alone or together with nuclear extracts from THP-1 cells treated for 15 or 30 min with vehicle (V) or leptin (L; 625 nM). The gel shift shows that leptin induces the appearance of a novel band (Figure 5A, marked by *) after 15 min, with a further increase in intensity after 30 min of leptin treatment. In order to characterize this band, PMA was used for its known activating effect on NF- κ B [28]. As is apparent on lanes 3 and 6 in Figure 5(B), PMA and leptin induced the appearance of band shifts migrating to different positions. The EMSA band observed after PMA was supershifted by an antibody against the p65 subunit of the NF- κ B complex (lane 4), while the band induced by leptin migrated more quickly (Figure 5B, marked by *) and could not be supershifted with antibodies against either p65 or p50 (Figure 5B, lanes 7 and 8). The oligonucleotides corresponding to the -101/-77 region of the IL-1Ra promoter used in these experiments have been described previously as containing a composite site for NF- κ B/PU.1 and GABP [29]. Therefore, in order to better characterize the mobility shift induced by leptin, competition experiments were performed with shorter oligonucleotides encompassing a more limited region of either the NF- κ B or PU.1 moiety of this composite site (Figure 5C). The competition experiment depicted in Figure 5(C) (left-hand panel) suggests a greater affinity of the leptin-induced band for the PU.1 oligonucleotides, since the shift almost completely disappeared with 10 $\times$ of the unlabelled PU.1 oligonucleotide, while the same amount of NF- κ B oligonucleotide induced a smaller decrease in the intensity of the shift. To test further whether PU.1 might be the protein binding to this site in a leptin-dependent manner, *in vitro*-translated PU.1 was incubated with the -101/-77 NF- κ B/PU.1 probe and run in parallel to leptin-treated THP-1 nuclear extracts (Figure 5C, middle panel). The results show that PU.1 migrates faster than the band induced by leptin (Figure 5C, *), suggesting that it is either another factor or that PU.1 forms homo- or hetero-dimers with another protein. However, supershift experiments with a polyclonal antibody against PU.1 did not modify the leptin-induced binding activity, and the p65 antibody supershifted only the basal NF- κ B binding, suggesting that none of the two proteins are involved in the shift observed (Figure 5C, right-hand panel). However, the PU.1 antibody utilized was capable of supershifting *in vitro*-translated PU.1 binding to the IL-1Ra promoter (results not shown). EMSAs were also performed with three additional probes corresponding to the selective PU.1-binding site LRE3 of the IL-1Ra promoter, its mutated version (LRE3 mut; [29]), and an oligonucleotide for GABP [30]. Nuclear extracts from leptin-treated THP-1 cells bound to neither the LRE3 (Figure 6A, lane 2), LRE3-mut (Figure 6B, lane 2) nor GABP sites (Figure 6C, lane 2), while *in vitro*-translated PU.1 is capable of binding, as expected, to the LRE3 site (Figure 6A, lanes 4 and 5), and to a lesser degree to the LRE3-mut probe (Figure 6B, lanes 4 and 5). These results strongly suggest that the leptin-induced DNA-binding activity is specific for the NF- κ B/PU.1 composite site in the IL-1Ra promoter, and that it does not consist of PU.1.

Figure 4 Leptin activates the NF- κ B pathway

(A) HepG2 cells were transiently transfected with 0.2 μ g of pcDNA3 or OB-Rb expression vector together with 3 μ g of [NF- κ B]₄-Tk-Luc reporter construct or pTAL empty vector. After transfection, the cells were treated for 24 h with either vehicle or leptin (625 nM; results are means $\pm$ S.E.M. of five independent experiments performed in triplicate; * P < 0.001). (B) THP-1 cells were treated for 15 min with vehicle (NaCl, 0.9%), leptin (625 nM) or LPS (100 ng/ml). Nuclear extracts (NE) were prepared and EMSAs were performed as described in the Materials and methods section using a ³²P-labelled oligonucleotide containing the NF- κ B consensus sequence (5'-AGTTGAGGGGACTTTCCAGG-3'; left-hand panel). The LPS-treated nuclear extract was preincubated with antibodies directed against the p50 subunit of NF- κ B (right-hand panel). (C) HepG2 cells were transiently transfected with 0.2 μ g of pcDNA3 or OB-Rb expression vector together with 3 μ g of [NF- κ B]₄-Tk-Luc reporter construct. After transfection, the cells were pretreated for 30 min with U-0126 (5 μ M) before the addition of vehicle or leptin (625 nM) for the subsequent 24 h (results are means $\pm$ S.E.M. of two independent experiments performed in triplicate; * P < 0.05).

DISCUSSION

It has become increasingly apparent that leptin has pleiotropic actions on various cell types beyond its regulatory effects on energy balance at the hypothalamic level. These peripheral targets of leptin include adipocytes, lymphocytes, ovarian cells, endocrine pancreatic cells, as well as monocytes [2–6,9,10,31]. We have shown recently that leptin induces the expression and secretion of IL-1Ra in monocytes, which is an anti-inflammatory cytokine currently undergoing clinical testing for the treatment of rheumatoid arthritis. In addition, measurements of IL-1Ra in the sera of hyperleptinaemic obese patients reveal markedly elevated levels of this cytokine antagonist, compatible with its regulation by leptin *in vivo* [32]. Since leptin selectively induces the expression of IL-1Ra without any significant effect on IL-1 β , it is of interest to elucidate the signalling mechanism involved in this distinct action of leptin. Moreover, since the functional resistance to leptin is a hallmark of human obesity, an under-

standing of the signalling mechanisms of the OB-Rb is of interest. Although progress has been made in elucidating the signal transduction pathways of leptin and its receptor in the hypothalamus, these studies are necessarily limited by the difficult access to cells expressing endogenous OB-Rb as well as by the limited number of genes known to be directly regulated by the OB-Rb. Hence, we used the IL-1Ra promoter as a model target gene to understand its activation by leptin.

We now demonstrate that the activation of the IL-1Ra promoter by leptin is entirely dependent on OB-Rb and that this involves the activation of the p42/44 MAPK, rather than the JAK/STAT, p38 MAPK or PI 3-kinase pathways. Although we and others have previously documented the nuclear translocation of STATs in response to leptin in the hypothalamus, adipose tissue and monocytes [3,6,14], other pathways such as the activation of MAPK are also implicated [15,16]. With regard to the IL-1Ra promoter, the STATs do not appear to play a crucial role, as the activation by leptin to a NF- κ B site at position

Figure 5 Leptin induces binding activity to the NF- κ B/PU.1_{IL-1Ra} site in THP-1 cells

(A) THP-1 cells were treated for 15 or 30 min with NaCl (0.9%; V) or leptin (625 nM; L). Nuclear extracts were prepared and EMSAs were performed as described in the Materials and methods section using a 32 P-labelled oligonucleotide containing the sequence of the NF- κ B/PU.1_{IL-1Ra} site from the IL-1Ra promoter. * Represents the shift of an unknown factor. (B) THP-1 cells were treated for 15 min with either vehicle (DMSO for PMA and NaCl for leptin), PMA (25 nM) or leptin (625 nM). Supershift experiments were performed by the addition of a polyclonal antibody directed against either the p65 or the p50 subunit of NF- κ B prior to the addition of the 32 P-labelled oligonucleotide to the nuclear extracts. (C) Left-hand panel: EMSA competition experiments were performed with NF- κ B/PU.1 composite site of the IL-1Ra promoter (-101/-77) as probe and unlabelled oligonucleotides corresponding to the NF- κ B (-92/-83) and PU.1 (-89/-81) binding sites. 10 $\times$ and 100 $\times$ the amount of labelled probe added to the incubation mixture containing nuclear extract from THP-1 cells treated with 625 nM leptin for 30 min, prior to the addition of the labelled probe. Middle panel: leptin-treated THP-1 nuclear extracts and increasing amounts of *in vitro*-translated PU.1 (PU.1) or untranscribed reticulocyte lysate (U) was incubated with the NF- κ B/PU.1_{IL-1Ra} probe, and EMSA was performed as described previously. Right-hand panel: THP-1 cells were treated for 15 min with NaCl (0.9%; V) or leptin (625 nM; L). Nuclear extracts were prepared and EMSAs were performed as described in the Materials and methods section using a 32 P-labelled oligonucleotide containing the sequence of the NF- κ B/PU.1_{IL-1Ra} site from the IL-1Ra promoter. Supershift experiments were performed by the addition of a polyclonal antibody directed against either the p65 subunit of NF- κ B or PU.1 prior to the addition of the 32 P-labelled oligonucleotide to the nuclear extracts. NS, non-specific binding; *, shift of unknown factor.

Figure 6 The leptin-induced DNA-binding activity in THP-1 cells is not PU.1 alone, nor GABP

(A) Nuclear extracts (NE) of THP-1 treated with leptin or vehicle for 30 min, or 1 or 2 μ l of *in vitro*-translated PU.1 (PU.1) or untranslated reticulocyte lysate (U), were incubated with a labelled oligonucleotide LRE3, a PU.1-binding site of the IL-1Ra promoter. (B) Conditions were as in (A) with a mutated PU.1-binding-site probe, LRE3-mut. (C) Labelled oligonucleotide corresponding to the GABP-binding site of the α 27 gene of herpes simplex virus 1 was used as probe for EMSA analysis with nuclear extracts of THP-1 treated with leptin or vehicle for 30 min.

–93/–84. In addition, the tyrosine kinase inhibitor genistein did not inhibit the stimulation of IL-1Ra by leptin, as would be predicted if the JAK/STAT pathway was involved. In contrast, leptin activated p42/44 MAPK in THP-1 cells, compatible with previous observations in the hypothalamus, adipocytes and transfected cell lines [4,16]. For the first time we now demonstrate that the NF- κ B pathway is a downstream target of OB-Rb signalling. Leptin activated the transcriptional activity from a NF- κ B consensus element in an OB-Rb-dependent manner, and it induced the nuclear translocation of an activity binding to the NF- κ B_{IL-1Ra} site. Moreover, mutation of a known NF- κ B-binding site within the proximal part of the IL-1Ra promoter abolished the activating effect of leptin on this promoter. However, the PU.1 transcription factor, as well as GABP, were shown to bind to a site in the IL-1Ra promoter overlapping with the NF- κ B_{IL-1Ra} region [29]. Band-shift experiments with more specific probes for PU.1 and GABP did not show any induction of DNA binding by leptin, suggesting that neither of the two transcription factors is involved. In addition, supershift experiments with nuclear extracts from THP-1 cells failed to demonstrate the presence of PU.1 in the leptin-induced complex binding to the NF- κ B/PU.1 composite site of the IL-1Ra promoter. This suggests, together with the slower migration of this complex compared with PU.1 alone, that a novel protein (complex) is involved.

Based on our finding of an OB-Rb-dependent activation of the typically pro-inflammatory MAPK and NF- κ B pathways in monocytic cells, the prediction would be that leptin might have overall inflammatory, rather than anti-inflammatory, actions *in vivo*. Results from our laboratories indicate that this may well be the case, as *ob/ob* mice appear to have a milder form of experimental arthritis compared with wild-type animals [33], and leptin induces the secretion of other proinflammatory factors from monocytes in a MAPK-dependent manner [34]. This is in keeping with the clinical observation of local inflammatory

reactions observed after the subcutaneous administration of leptin in humans [35].

In summary, we have shown that leptin activates the human promoter for secreted IL-1Ra in an OB-Rb- and ERK-dependent manner through a proximal NF- κ B/PU.1 composite site. However, the protein(s) binding to this site are neither the p65, p50 subunits of NF- κ B, nor PU.1, but an as yet uncharacterized factor. Whether this signalling pathway is also involved in mediating the hypothalamic effects of leptin remains to be elucidated.

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