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Leptin activates PI-3 kinase in C_2C_{12} myotubes via janus kinase-2 (JAK-2) and insulin receptor substrate-2 (IRS-2) dependent pathways

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Summary We have recently shown that leptin mimicks insulin effects on glucose transport and glycogen synthesis through a phosphatidylinositol-3 (PI) kinase dependent pathway in C_2C_{12} myotubes. The aim of the present study was to identify the signalling path from the leptin receptor to the PI-3 kinase. We stimulated C_2C_{12} myotubes with insulin (100 nmol/l, 5 min) or leptin (0.62 nmol/l, 10 min) and determined PI-3 kinase activity in immunoprecipitates with specific non-crossreacting antibodies against insulin-receptor substrate (IRS 1/IRS 2) and against janus kinase (JAK 1 and JAK 2). While insulin-stimulated PI-3 kinase activity is detected in IRS-1 and IRS-2 immunoprecipitates, leptin-stimulated PI-3 kinase activity is found only in IRS-2 immunoprecipitates, suggesting that the leptin signal to PI-3 kinase occurs via IRS-2 and not IRS-1. Leptin-, but not insulin-stimulated PI-3 kinase activity is also detected in immunoprecipitates with antibodies against JAK-2, but not JAK-1. The data suggest that JAK-2 and IRS-2 couple the leptin signalling pathway to the insulin signalling chain. Since we have also detected leptin-stimulated tyrosine phosphorylation of JAK-2 and IRS-2 in C_2C_{12} myotubes it can be assumed that leptin activates JAK-2 which induces tyrosine phosphorylation of IRS-2 leading to activation of PI-3 kinase. As we could not detect the long leptin receptor isoform in C_2C_{12} myotubes we conclude that this signalling pathway is activated by a short leptin receptor isoform. [Diabetologia (1997) 40: 1358–1362]

Keywords leptin, leptin receptor, insulin receptor, phosphatidylinositol kinase, janus kinase

The *ob*-gene product leptin has been defined as a regulator of food intake and energy expenditure [1]. Identification and cloning of specific leptin receptors, which exist in different isoforms, has recently provided new insights into the mechanism and physiological function of leptin signalling in different tissues [2, 3]. It was shown that effects of leptin on food intake are mediated through the long isoform of the leptin receptor in the hypothalamus. However, leptin receptors are not only found in brain but also in many other tissues including lung, kidney, ovary and skeletal muscle [2, 3]. This pattern of tissue distribution suggests that leptin might exert a wide spectrum of cellular effects in tissues other than brain. We have recently shown that leptin signalling interacts with insulin signalling in a dual fashion. Leptin is able to impair the first steps of the insulin signalling chain, i.e. autophosphorylation of the insulin receptor and tyrosine phosphorylation of insulin-receptor substrate-1 (IRS-1) in HEK293 cells, NIH3T3 cells and rat-1 fibroblasts [4]. This effect was also observed in HepG2 cells [5]. On the other hand we have recently shown that leptin is also able to mimick insulin effects, i.e. stimulation of glucose transport and glycogen synthesis in C_2C_{12} myotubes [6]. This insulin-like

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Abbreviations: IRS, Insulin-receptor substrate; PI3, phosphatidylinositol 3; FCS, fetal calf serum; JAK, janus kinase; DMEM, Dulbecco's modified Eagle's medium; HIR, human insulin receptor.
TIU, trypsin inhibiting units

effect of leptin appears to be mediated by stimulation of phosphatidylinositol-3 (PI-3) kinase [6] suggesting a crosstalk between the insulin and leptin signalling chain at the level of PI-3 kinase. Activation of PI-3 kinase by leptin might be responsible both for insulin-like signals on glucose uptake and glycogen synthesis as well as for inhibitory effects on insulin signalling elements upstream of PI-3 kinase, i.e. the insulin receptor and IRS-1. The aim of the present study was to identify the signalling elements which activate PI-3 kinase in C_2C_{12} myotubes after leptin stimulation.

Materials and methods

Materials. Cell culture reagents and fetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany). Human insulin and murine recombinant leptin were kindly provided from Hoechst AG (Frankfurt, Germany). [γ^{32}P]ATP (300 Ci/mmol) was from DuPont NEN (Bad Homburg, Germany). L-α-phosphatidylinositol was from Sigma (Munich, Germany). Polyclonal antibodies against p85 α, janus kinase (JAK-1) and JAK-2 were purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y., USA); specific, non-crossreacting polyclonal IRS-1 and IRS-2 antibodies were kindly provided by M.F. White (Boston, Mass., USA), monoclonal anti-phosphotyrosine antibody (PY20) was from Leinco (Ballwin, MO, USA). Protein A Sepharose was from Pharmacia (Uppsala, Sweden). The reagents for SDS/PAGE and Western blotting were purchased from Roth (Karlsruhe, Germany) and Biorad (Munich, Germany). Nitrocellulose was from Schleicher & Schuell (Dassel, Germany) and the non-radioactive enhanced chemiluminescence detection system (ECL) was obtained from Amersham (Braunschweig, Germany).

Cell culture. Monolayers of C_2C_{12} myoblasts (American Type Culture Collection, Rockville, M.D. USA) were kept in a proliferative state by maintaining them in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS and 2 mmol/l L-glutamine. Cells were grown in 10 cm diameter dishes. Proliferative myoblasts were induced to differentiate into mytotubes by replacement of the above serum-enriched medium with DMEM supplemented with 2 % horse serum. Differentiating myoblasts were fed with fresh medium every 48 h. After 9 days, 90 % of the cells were differentiated into myotubes. Cells were serum-starved in minimum essential medium (MEM) with Earle's salts, containing 0.5 % horse serum and 2 mmol/l L-glutamine 18 h prior to experimental procedures.

Assay of PI-3 activity. After incubation with 100 nmol/l insulin for 5 min or with recombinant leptin for 10 min, cells were rinsed once with ice cold PBS and lysed at 4 °C for 5 min in 1 ml of lysis buffer (50 μmol/l HEPES pH 7.2, 150 mmol/l NaCl, 1 mmol/l EGTA, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, 100 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 100 μmol/l sodium orthovanadate, 1 mmol/l PMSF, 10 μg/ml aprotinin). Lysates were centrifuged at 13000 × *g* for 10 min and the supernatants were immunopurified with the indicated antibody after dilution of Triton X-100 to 0.4 %. The immunocomplexes were absorbed to protein-A-Sepharose for 2 h. Immunoprecipitates were washed 3 times and pellets were directly incubated with L-α-phosphatidylinositol (0.1 mg/ml for 10 min in a solution containing 50 μmol/l [γ^{32}P]ATP, 1.2 mmol/l Na-orthovanadate, 5 mmol/l $MgCl_2$, 25 mmol/l HEPES, pH 7.4, at room temperature for 10 min in a final volume of 50 μl. After addition of 20 μl 8 mol/l HCl, lipids were extracted with 160 μl chloroform/methanol (1 : 1, by vol), centrifuged at 13000 × *g* for 5 min and the lower phase containing the phospholipids was lyophylized and subsequently dissolved in 5 μl chloroform/methanol (1 : 1, by vol). The products of the reaction were separated by thin layer chromatography as described [6]. ^{32}P-labelled phospholipids were detected by autoradiography. Standard lipids were run in parallel and detected with iodine vapor.

Detection of tyrosine-phosphorylated proteins. Myoblasts differentiated into myotubes were serum depleted and stimulated with or without 1, 10 and 100 nmol/l insulin for 5 min or 0.062 and 0.62 nmol/l recombinant leptin for 10 min. After 10 min incubation, the cells were immediately washed once with ice-cold PBS and lysed in a buffer containing protease and phosphatase inhibitors (20 mmol/l HEPES, 150 mmol/l NaCl, 10 % glycerol, 1 % Triton-X-100, 1.5 mmol/l $MgCl_2$, 4 mmol/l EGTA, 1 mmol/l EDTA, 1200 TIU/L aprotinin, 10 mmol/l $Na_4P_2O_7$, 2 mmol/l Na-orthovanadate, 100 mmol/l NaF, 2 mmol/l PMSF, pH 7.4). The lysates were centrifuged at 13000 × *g* for 10 min. After addition of Laemmli buffer, samples were applied to a 7.5 % SDS/polyacrylamide gel. Proteins were transferred onto nitrocellulose by electroblotting, and probed by immunoblotting with an anti-phosphotyrosine antibody (PY20).

Reverse transcriptase-polymerase chain reaction. Total RNA from C_2C_{12} myotubes was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany). First strand reverse transcription was performed on 5 μg total RNA with random hexadeoxynucleotides and murine reverse transcriptase. PCR primer sequences were 5′-ccttcctgactccgtagta-3′ as upstream primer and 5′-ggataagcactgagtgact-3′ as downstream primer for the extracellular domain of leptin receptor. In addition, cDNA of HEK293 cells was amplified with

upstream primer: 5′-agatgttccgaaccccaaga-3′ and downstream primer 5′-ctgagaatgtgaggtgtggt-3′ yielding a 550 bp product of a part of the intracellular domain of the leptin receptor. Another primer was used for C_2C_{12} myotubes coding for the transmembranous region and part of the intracellular domain including the JAK box 1 region (upstream primer: 5′-ggacagaaccagcgcaca-3′, downstream primer: 5′-aattcagtccttgtgcccag-3′). The PCR products were sequenced and correspond to the expected leptin receptor sequences. Cycle conditions were 5 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 51 °C and 1 min at 72 °C.

Results

C_2C_{12} myotubes were tested for leptin receptor expression by RT-PCR using oligonucleotides against the extracellular domain of the mouse leptin receptor sequence. Figure 1 A (lane 2) shows the RT-PCR product at 657 bp which was identified as OB-R DNA of the extracellular domain by partial sequencing. RT-PCR using primers amplifying the intracellular domains of the long form of the leptin receptor did not reveal a product in C_2C_{12} myotubes (lane 3). As a control we screened the intracellular domains of the leptin receptor in HEK293 cells for the long receptor isoform (obR_L). In this cell type a RT-PCR product of the intracellular domain of obR_L was found (Fig. 1 A, lane 4). Primers amplifying the intracellular JAK box 1 region were also used in C_2C_{12} myotubes and revealed a PCR product at 552 bp (Fig. 1 B, lane 2). The presence of the JAK box was also confirmed by sequencing of the PCR product.

Figure 2 shows the effects of insulin and leptin on PI-3 kinase activation. PI-3 kinase activity is clearly stimulated by 100 nmol/l insulin (8.0 ± 1.4 times basal, $n = 3$) as well as 0.62 nmol/l leptin (6.2 ± 1.3 times basal, $n = 3$) which is shown in immunoprecipitates with antibodies against the regulatory subunit αp85 (Fig. 2). Signal transduction from the insulin receptor to PI-3 kinase occurs through the docking proteins IRS-1 and IRS-2. In immunoprecipitates using IRS-1 antibody PI-3 kinase is stimulated by insulin (4.2 ± 0.94 times basal, $n = 3$) however, not by leptin, suggesting that leptin activates PI-3 kinase not via IRS-1. When antibodies against IRS-2 were used, increased PI-3 kinase activity both in cells stimulated with leptin (4.6 ± 1.05 times basal, $n = 3$) as well as insulin (2.8 ± 1.5 times basal, $n = 3$) was found (Fig. 2). It is known that the leptin receptor contains in its long form two binding domains for the tyrosine kinase JAK-2, and in its short isoform one JAK-2 binding domain [3]. In addition, earlier studies have shown that the leptin receptor activates the JAK/STAT signalling cascade [7, 8]. Thus, we tested whether JAK kinases might be involved in leptin

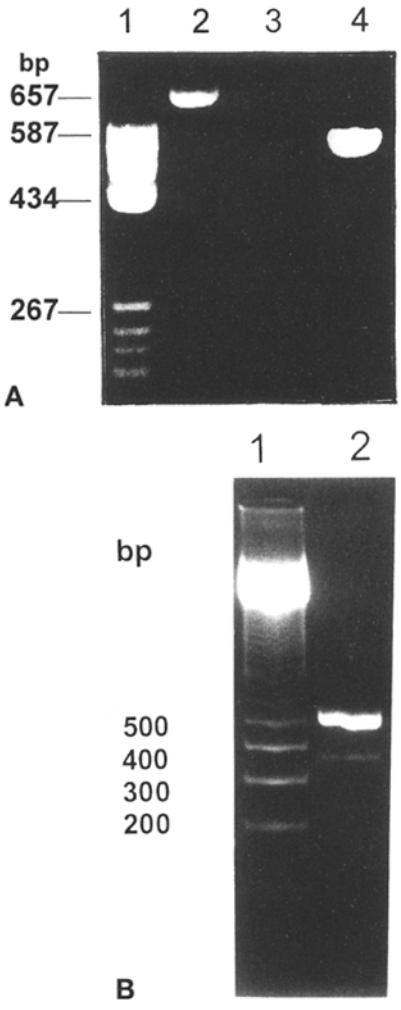

Fig. 1 A. RT-PCR product of leptin receptor mRNA from C_2C_{12} myotubes and HEK293 cells. Reverse transcription was done with an oligo-dT-primer. cDNA of 293 cells and C_2C_{12} myotubes was amplified as described in the methods section. Lane 1: Mol. weight marker. Lane 2: 657 bp product corresponding to the transcript coding for the extracellular domain of the leptin receptor. Lane 3: RT-PCR with primer amplifying a part of the intracellular domain of the leptin receptor in C_2C_{12} myotube. Lane 4: RT-PCR revealing a 550 bp product of a part of the intracellular domain of the leptin receptor in HEK293 cells. **B** A 552 bp product from cDNA of mouse C_2C_{12}-myotubes is shown, coding for the transmembraneous region and part of the intracellular domain including the jak box 1 region (lane 2). Molecular weight marker, lane 1

signalling to IRS-2 and PI-3 kinase. To address this question we immunoprecipitated proteins from leptin-stimulated C_2C_{12} myotubes with antibodies against JAK-1 and JAK-2 and determined PI-3 kinase activity in the immunoprecipitates. Figure 2 shows that leptin-stimulated PI-3 kinase activity is found in immunoprecipitates with antibodies against JAK-2 (4.8 ± 0.64 times basal), while no JAK-1 immunoprecipitated activity could be found (Fig. 2, bottom). To elucidate whether leptin induces tyrosine phosphorylation of JAK-2 and IRS-2, immunoprecipitates were

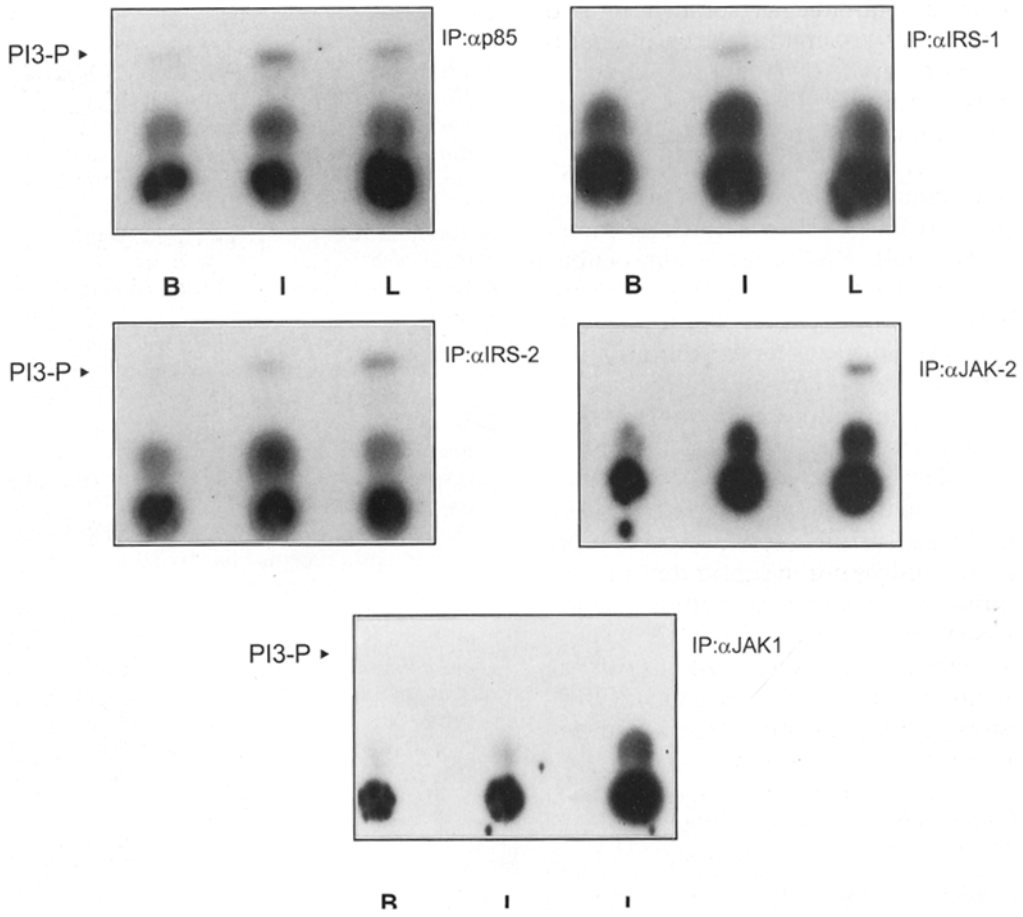

Fig. 2. Effect of leptin on PI3-kinase activity. C_2C_{12} myotubes were deprived of serum for 18 h prior to incubation either with insulin (I) (100 nmol/l) for 5 min or with leptin (L) (0.62 nmol/l) for 10 min. Cell lysates were immunoprecipitated with the antibodies as indicated and in vitro kinase assay was performed using L-α-phosphatidylinositol as a substrate. A representative autoradiography is shown. The same results were obtained in three independent experiments B, basal

blotted against phosphotyrosine antibodies. Tyrosine phosphorylation of JAK-2 and IRS-2 (data not shown) is stimulated at the same leptin concentrations (0.062–0.62 nmol/l) as necessary for maximal activation of glucose transport and glycogen synthesis [6].

Discussion

We have investigated the interaction of insulin and leptin signalling in different cell systems and our data suggest that leptin might interfere with insulin signalling in a dual way. HEK293 cells transfected with HIR and IRS-1 show an inhibition of the early insulin signalling events, i.e. insulin receptor autophosphorylation and IRS-1 phosphorylation after incubation with leptin at concentrations which are observed in obese subjects (> 3 nmol/l). On the other hand we have shown that leptin is also able to stimulate glucose transport and glycogen synthesis in C_2C_{12} myotubes [6] at low concentrations between 0.062 and 0.62 nmol/l leptin. The aim of the present study was to identify the signalling elements which transduce the leptin effect in C_2C_{12} myotubes. The data shown in Figure 1 suggest that this effect is most likely mediated by a short leptin receptor isoform. On the other hand, the PCR results cannot completely rule out the existence of the long leptin receptor isoform in these cells at very low expression levels or a new splice variant. The insulin signalling pathway from the receptor to PI-3 kinase occurs via the docking proteins IRS-1 and IRS-2 [9]. Both proteins can interact with a large number of downstream signalling elements after phosphorylation on tyrosine residues. IRS-1 and IRS-2 share common signalling pathways and biological effects; however, only IRS-2 is able to transduce leptin activation of PI-3 kinase in C_2C_{12} cells. IRS proteins are not only engaged by the insulin receptor but also by different cytokine receptors [9]. Since the obR shares common elements with the cytokine receptor family, stimulation of IRS proteins by leptin might occur through analogous mechanisms. Our data suggest that upon leptin stimulation JAK-2 and IRS-2 mediate activation of PI-3 kinase while IRS-1 is not involved. It is known [2, 3]

that the leptin receptor contains in its long form two binding domains for the tyrosine kinase JAK-2, and in its short isoform one JAK-2 binding domain. Recently it was shown that the leptin receptor activates JAK-2 kinase in a haematopoietic cell line [8]. Tyrosine phosphorylation of IRS-1/IRS-2 by JAK-kinases upon insulin and interleukin stimulation was also demonstrated earlier [9]. We observed tyrosine phosphorylation of JAK-2 and IRS-2 after leptin incubation of C_2C_{12} myotubes (data not shown). Therefore, it is conceivable that leptin-dependent phosphorylation of JAK-2 is followed by tyrosine phosphorylation of IRS-2 which couples to PI-3 kinase via binding of SH2 domains. In summary, these data suggest that PI-3 kinase activation by leptin is mediated by a JAK-2/IRS-2 dependent pathway in C_2C_{12} myotubes which is followed by activation of glucose transport and glycogen synthesis. Thus, PI-3 kinase plays a pivotal role for a crosstalk between leptin and insulin signalling by stimulation or augmentation of insulin-like effects downstream from PI-3 kinase. A role of PI-3 kinase for a negative feedback to the insulin receptor and IRS-1 by the serine kinase activity of PI-3 kinase seems possible as well and needs further investigation.

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