

*Rapid communications***Leptin stimulates glucose transport and glycogen synthesis in C₂C₁₂ myotubes: evidence for a PI3-kinase mediated effect****L. Berti, M. Kellerer, E. Capp, H. U. Häring**

Eberhard-Karls-Universität Tübingen, Medizinische Klinik und Poliklinik, Innere Medizin IV, Tübingen, Germany

Summary It was recently shown that leptin impairs insulin signalling, i. e. insulin receptor autophosphorylation and insulin-receptor substrate (IRS)-1 phosphorylation in rat-1 fibroblasts, NIH3T3 cells and HepG2 cells. To evaluate whether leptin might impair the effects of insulin in muscle tissue we studied the interaction of insulin and leptin in a muscle cell system, i. e. C₂C₁₂ myotubes. Preincubation of C₂C₁₂ cells with leptin (1–500 ng/ml) did not significantly affect insulin stimulated glucose transport and glycogen synthesis (1.8 to 2 fold stimulation); however, leptin by itself (1 ng/ml) was able to mimic approximately 80–90 % of the insulin effect on glucose transport and glycogen synthesis. Both glucose transport as well as glycogen synthesis were inhibited by the phosphatidylinositol-3 (PI3)-kinase inhibitor wortmannin and the protein kinase C inhibitor H7 while no effect was

observed with the S6-kinase inhibitor rapamycin. We determined whether the effect of leptin occurs through activation of IRS-1 and PI3-kinase. Leptin did not stimulate PI3-kinase activity in IRS-1 immunoprecipitates; however, PI3-kinase activation could be demonstrated in p85 α immunoprecipitates (3.04 ± 1.5 fold of basal). In summary the data provide the first evidence for a positive crosstalk between the signalling chain of the insulin receptor and the leptin receptor. Leptin mimics in C₂C₁₂ myotubes insulin effects on glucose transport and glycogen synthesis most likely through activation of PI3-kinase. This effect of leptin occurs independently of IRS-1 activation in C₂C₁₂ cells. [Diabetologia (1997) 40: 606–609]

Keywords Leptin, phosphatidylinositol-3 kinase, insulin signalling.

The *ob* gene product leptin has been defined as a regulator of food intake and energy expenditure [1]. The identification and cloning of specific leptin receptors has recently provided the basis with which to study the mechanism and physiological function of leptin signalling in different tissues. Leptin receptors are

expressed in different isoforms [2, 3]. It was shown that effects of leptin on food intake are mediated through leptin receptors in the hypothalamus [2]. 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 earlier demonstrated that 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 (IRS)-1 in different cell types. This effect was demonstrated in rat-1 fibroblasts overexpressing human insulin receptor as well as NIH3T3 cells [4]. Very recently the same effect of leptin was demonstrated in HepG2 cells [5]. Muscle tissue plays a central role in insulin action and in the pathogenesis of insulin resistance. To discuss a potential role of leptin in the

Received: 24 January 1997 and in revised form: 3 March 1997

Corresponding author: Professor H. U. Häring, Eberhard-Karls-Universität Tübingen, Medizinische Klinik und Poliklinik, Innere Medizin IV, Otfried-Müller-Straße 10, D-72076 Tübingen, Germany

Abbreviations: 2DG, 2-deoxy-[³H]D-glucose; PI3K, phosphatidylinositol-3 kinase; SDS/PAGE, sodiumdodecylsulphate/polyacrylamide-gel electrophoresis; PMSF, phenylmethylsulfonylfluoride; PBS, phosphate buffered saline; p85 α , PI3K regulatory 85 kDa subunit; ECL, enhanced chemiluminescence; PIP, phosphatidylinositol; IRS, insulin-receptor substrate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

pathogenesis of insulin resistance it is therefore of interest to know whether in muscle tissue a crosstalk of insulin and leptin receptor signalling occurs. In the present study we used C₂C₁₂ myotubes as a model for potential leptin effects on the insulin signalling chain in muscle tissue. The data suggest a crosstalk between the insulin and the leptin signalling chain in C₂C₁₂ myotubes which leads to an activation of signalling elements downstream of IRS-1 causing insulin-like effects on glucose transport and glycogen synthesis.

Materials and methods

Materials. Cell culture reagents and fetal calf serum (FCS) were purchased from Life Technologies, Inc. (Eggenstein, Germany). Human insulin and murine recombinant leptin were kindly provided from Hoechst AG (Frankfurt, Germany). [γ -³²P]ATP (3000 Ci/mmol) and 2-[1,2-³H (N)]deoxy-D-glucose (2 DG) (26.2 Ci/mmol) were from DuPont NEN (Bad Homburg, Germany), while D-[U-¹⁴C]glucose (297 mCi/mmol) was from Amersham (Buckinghamshire, UK). Wortmannin, rapamycin and H7, were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Polyclonal antibodies against p85 α and IRS-1 were purchased from Upstate Biotechnology Inc. (Lake Placid, N. Y., USA); monoclonal anti-phosphotyrosine antibody was from Calbiochem (Bad Soden, Germany). Protein A Sepharose was from Pharmacia (Uppsala, Sweden). The reagents for SDS/PAGE and Western blotting were purchased from Roth (Karlsruhe, Germany) and Biorad (München, Germany). Nitrocellulose was from Schleicher & Schuell (Dassel, Germany) and the non-radioactive enhanced chemiluminescence system for ECL detection was obtained from Amersham.

Methods

Cell culture and incubations. Monolayers of C₂C₁₂ myoblasts (American Type Culture Collection, Rockville, Md., 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 12-well plates for hexose transport, and glycogen synthesis and in 10-cm diameter dishes for PI3-kinase activity assays. Proliferative myoblasts were induced to differentiate into myotubes 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. 18 h prior to experimental procedures cells were serum-starved in minimum essential medium (MEM) with Earle's salts containing 0.5% horse serum and 5 mmol/l glucose.

Deoxyglucose uptake. 2-Deoxy-[³H]glucose uptake was measured as previously described. Cell monolayers were washed once in HEPES-buffered saline solution (140 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l MgCl₂, 1 mmol/l CaCl₂, 20 mmol/l HEPES, pH 7.4) and then incubated in the same solution with different concentrations of insulin or recombinant leptin, respectively for 40 min or 1 or 18 h at 37°C. After incubation, cells were rinsed twice with HEPES-buffered saline and glucose uptake was quantitated by exposing the cells to 0.5 μ mol/l 2DG (0.5 μ Ci/ml) for 10 min at room temperature. Non-specific uptake was determined by quantitating cell-associated radioactivity in the presence of 10 μ mol/l cytochalasin B,

which blocks transporter-mediated uptake. At the end of the 10 min period, the supernatant was aspirated rapidly and the cells were washed twice with ice-cold phosphate buffered saline (PBS). The cells were lysed in 0.05 N NaOH and the associated radioactivity was determined by liquid scintillation counting. Each experiment was assayed in duplicate.

Glycogen synthesis assay. C₂C₁₂ muscle cells were incubated with different concentrations of insulin or with recombinant leptin, respectively for 40 min or for 30 min to 18 h at 37°C. Then 50 μ l of MEM containing 5 mmol/l glucose and 0.5 μ Ci of D-[U-¹⁴C]glucose was added and all incubations were continued for an additional 90 min at 37°C. Cells were then washed twice with ice-cold PBS and solubilized in 100 μ l of 30% (v/v) KOH. The extracts were heated for 30 min at 95°C and subsequently spotted onto a chromatography paper (3MM; Whatman, Schleicher and Schüll, Dassel, Germany), dried, dropped into ice cold 66% ethanol, and washed in five changes of cold ethanol (30 min each). The paper was dried and radioactivity determined by liquid scintillation counting. Each experiment was assayed in duplicate.

Assay of phosphatidylinositol-3 kinase (PI3) activity. After incubation with 1 nmol/l insulin for 10 min or with recombinant leptin for 10 min to 3 h, cells were rinsed once with ice cold PBS and lysed at 4°C for 5 min in 1 ml of lysis buffer as described previously [6]. Lysates were centrifuged at 13000 \times g for 10 min and the supernatants were incubated 3 h with anti-p85 α or anti-IRS-1 antibodies (3 μ g/ml lysate), then for an additional 2 h with protein-A-Sepharose. Immunoprecipitates were washed and pellets were directly incubated with phosphatidylinositol (0.1 mg/ml) for 10 min in medium containing 50 μ mol/l [γ -³²P]ATP, 1.2 mmol/l Na-orthovanadate, 5 mmol/l MgCl₂, 25 mmol/l HEPES, pH 7.4, at 37°C for 10 min in a final volume of 50 μ l. After addition of 20 μ l 8 mol/l HCl, lipids were extracted with 160 μ l chloroform/methanol (1 : 1, by volume), centrifuged at 13000 \times g for 5 min and the lower phase containing the phospholipids was lyophilized and subsequently dissolved in 5 μ l chloroform/methanol (1 : 1, by volume). The products of the reaction were separated by thin-layer chromatography as described [6]. ³²P-labelled spots were detected by autoradiography. Standard lipids were run in parallel and detected with iodine vapor.

Detection of tyrosine-phosphorylated proteins. Myotubes grown in 12-well plates were serum depleted and treated with or without 1, 10 and 100 nmol/l insulin or 1 to 50 ng/ml recombinant leptin. After incubations were finished, 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 MgCl₂, 4 mmol/l EGTA, 1 mmol/l EDTA, 1200 Trypsin inhibiting units/l aprotinin, 15 mmol/l benzamide, 54 U/ml bacitracin, 2 mmol/l Na-orthovanadate, 2 mmol/l phenylmethylsulphonyl fluoride (PMSF), 100 mmol/l NaF, pH 7.4). The lysates were centrifuged at 13000 \times 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.

Results

In analogy to earlier experiments in rat-1 fibroblasts and NIH3T3 cells C₂C₁₂ myotubes were preincubated with leptin in concentrations between 1 and 500 ng/ml

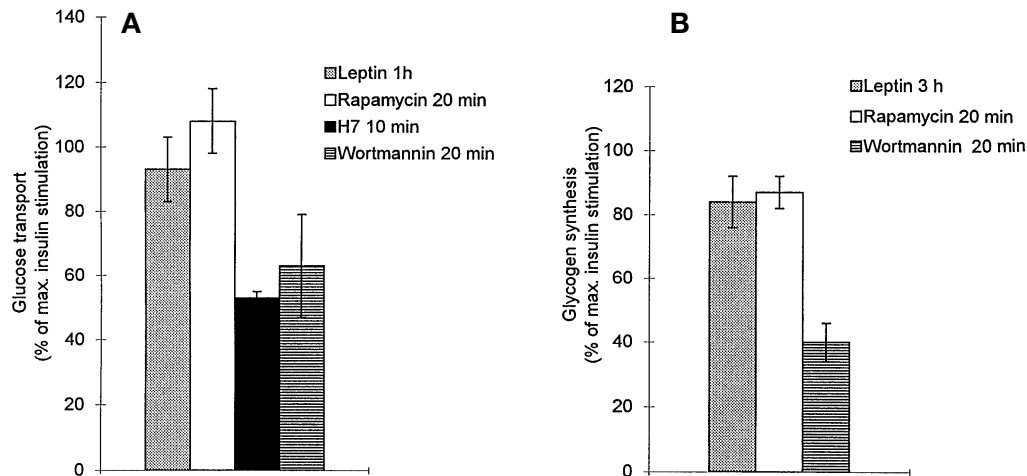


Fig 1A. Effect of rapamycin, H7 and wortmannin on leptin-stimulated glucose transport. Quiescent C_2C_{12} myotubes were pretreated in the presence of rapamycin (50 nmol/l), wortmannin (50 nmol/l) or H7 (10^{-4} mol/l) for the time indicated. Then the incubation medium was aspirated and replaced with medium containing 1 ng/ml recombinant leptin. After 1 h 2-DG uptake was measured. Data are the means \pm SEM of four independent experiments each performed in duplicate. **B** Effect of rapamycin and wortmannin on glycogen synthesis. Quiescent cells were incubated in the presence of rapamycin (50 nmol/l) and wortmannin (50 nmol/l) for the times indicated. The medium was then aspirated, cells washed once with PBS and recombinant leptin was added for 3 h. Glycogen synthesis was assessed as described in the experimental section. Results are the mean value \pm SEM of four experiments performed in duplicate

before stimulation with insulin. Insulin stimulates glucose uptake in C_2C_{12} myotubes approximately twofold. Half maximal insulin effects are obtained at 0.1 nmol/l. Preincubation of cells for 1 h, 3 h and 18 h with leptin at concentrations between 1 and 500 ng/ml did not significantly alter the insulin stimulated 2 DG uptake. However, leptin by itself was able to mimic almost quantitatively the insulin effect on glucose uptake. This effect of leptin is obtained at a concentration of 1 ng/ml. At an incubation time of leptin for 1 h $84 \pm 8\%$ ($n = 5$) of the insulin stimulated glucose uptake is observed. This leptin effect declines at longer incubation periods (3 h, $54 \pm 6\%$, 18 h, $52 \pm 12\%$, $n = 5$). Leptin mimics the insulin effect on glycogen synthesis as well, but in contrast to glucose uptake glycogen synthesis increases with prolonged leptin incubation (30 min, $32 \pm 5\%$, 1 h, $38 \pm 6\%$, 3 h, $84 \pm 7\%$, 18 h, $86 \pm 10\%$, $n = 5$).

To test which signalling elements might be involved in the effect of leptin on glucose transport and glycogen synthesis we used the protein kinase C (PKC) inhibitor H7 (10^{-4} mol/l), the S6-kinase inhibitor rapamycin (50 nmol/l) and the PI3-kinase inhibitor wortmannin (50 nmol/l). Wortmannin was able to inhibit partially the leptin effect on glucose transport as well as glycogen synthesis suggesting a potential

involvement of PI3-kinase (Fig. 1 A). H7 was able to inhibit the leptin effect on glucose uptake suggesting a role for PKC in leptin induced glucose uptake as well. As the inhibitory effect of wortmannin on leptin induced stimulation of glucose transport and glycogen synthesis suggests an involvement of PI3-kinase we studied whether leptin mimics insulin effects through activation of IRS-1 dependent PI3-kinase. The immunoblot with anti-phosphotyrosine antibodies shown in Figure 2 A demonstrates the effect of insulin on IRS-1 phosphorylation at 185 kDa in C_2C_{12} myotubes. In contrast to insulin leptin does not stimulate tyrosine phosphorylation of IRS-1.

In order to test whether leptin is able to activate PI3-kinase, phosphatidylinositol phosphorylation was measured in cells stimulated with leptin. PI3-kinase activity was determined in immunoprecipitates using either IRS-1 antibodies or p85 α antibodies against the regulatory subunit of PI3-kinase. Figure 2 B shows that insulin stimulates PI3-kinase in IRS-1 immunoprecipitates while leptin was without any effect. In contrast when p85 α antibodies were used to immunoprecipitate PI3-kinase activity both insulin (5.68 ± 3.16 -fold of basal $n = 5$) as well as leptin (3.04 ± 1.50 -fold of basal $n = 5$ at 10 ng/ml) were able to stimulate PI3-kinase activity.

Discussion

The insulin like effects of leptin on glucose transport and glycogen synthesis are inhibited by PI3-kinase inhibitors and PKC inhibitors. This observation is in good agreement with current thinking about the signalling steps involved in the insulin stimulated translocation of glucose transporter containing vesicles and the activation of glycogen synthesis. The present data suggest a crosstalk between leptin- and insulin signalling at the level of PI3-kinase. Activation of PI3-kinase by leptin might explain both the insulin-like effects on downstream signalling as well as the inhibitor effects on receptor autophosphorylation and IRS-1 phosphorylation observed in other cells [7, 8].

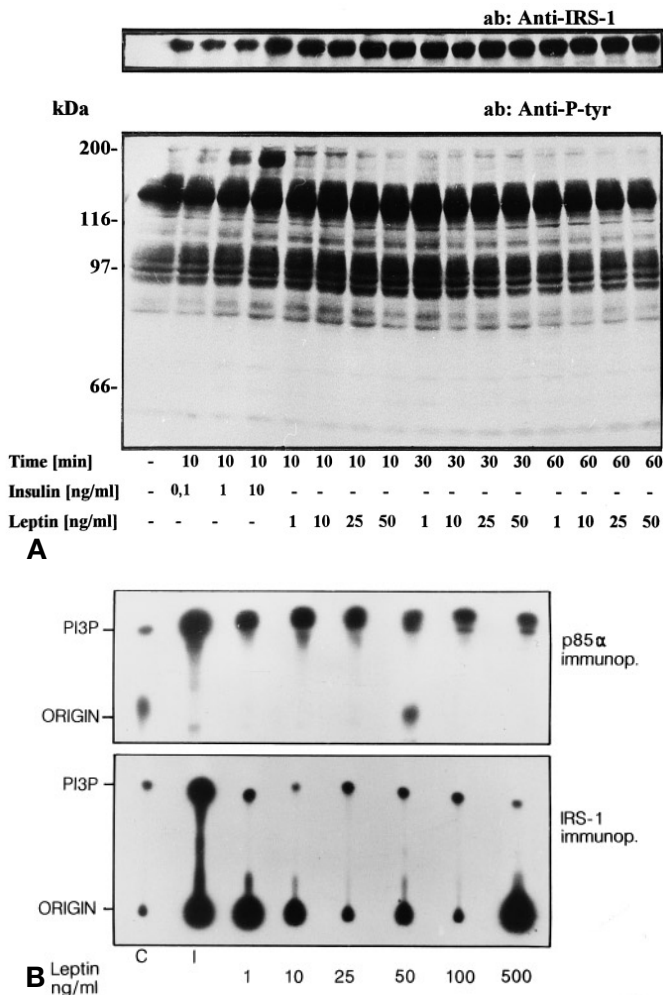


Fig. 2A. Effect of insulin and leptin on protein tyrosine phosphorylation. Serum-depleted myotubes were incubated without or with 0.1, 1 and 10 nmol/l insulin for 10 min or with recombinant leptin as indicated. The stimulation was stopped by addition of ice cold PBS and cells were lysed as described in the experimental section. Portions (40 μ l of cell lysate were subjected to SDS/PAGE and immunoblotted using antiphosphotyrosine specific antibodies. The same nitrocellulose was reprobed with anti-IRS-1 antibodies (upper panel). **B** Effect of leptin on PI-3K activity. C_2C_{12} myotubes were deprived of serum for 18 h prior to incubation either with insulin (I) (10 nmol/l) for 10 min or with leptin (1–500 ng/ml) for 1 h. Whole cell lysates were immunoprecipitated with either anti-p85 α or anti-IRS-1 antibodies and in vitro kinase reactions were performed using phosphatidylinositol as a substrate. A representative autoradiography is shown. The same results were obtained in five independent experiments

A central role of PI3-kinase both in the signalling to glucose transport and glycogen synthesis is well accepted [7, 9]. On the other hand it was earlier shown that PI3-kinase is a good candidate for inhibitory feedback effects on insulin-induced receptor- and IRS-1 phosphorylation [8]. Beside PI-3 kinase there are also other candidates for the leptin induced IRS-1 and insulin receptor inhibition. For example mitogen activated protein (MAP) kinase is activated by insulin and a negative feedback loop to IRS-1 and

the insulin receptor have been discussed as well. PKC might be another candidate for a leptin stimulated serine kinase since the PKC inhibitor H7 is able to block the leptin effects on glucose transport.

C_2C_{12} myotubes express predominantly the glucose transporter isoform GLUT1. It has to be assumed that both the insulin and the leptin effect on glucose uptake are mediated by a translocation of GLUT1. Although this glucose transporter isoform represents only a minor fraction in skeletal muscle and heart, it is conceivable that leptin could be a modulator of basal glucose uptake in skeletal muscle since GLUT1 is to a large extent responsible for basal glucose transport. It is interesting to note that for the inhibitory effects on insulin signalling in rat-1 fibroblasts we and others required high concentrations of leptin above 50 ng/ml which are only reached in obese subjects. The insulin-like effects in C_2C_{12} myotubes are, however, obtained at very low concentrations (10 ng/ml) [10]. The dual role of leptin in insulin signalling appears to be concentration dependent and this could be a result of various isoforms of leptin receptors which might couple to different signalling pathways or have different affinities for leptin.

Acknowledgements. This work was supported by a grant from the Deutsche Forschungsgemeinschaft and the Fortune program to H. U.H. and M.K. We thank Dr. J. Ertl, Hoechst Company, Germany, for the generous gift of leptin.

References

- Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature (London)* 372: 425–432
- Tartaglia LA, Dembski M, Weng X et al. (1996) Identification and expression cloning of a leptin receptor, OB-R. *Cell* 83: 1263–1271
- Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhail A, Platica D, Snodgrass HR (1996) Novel b219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nature Medicine* 2: 585–589
- Kroder G, Kellerer M, Häring HU (1996) Effect of leptin on insulin signalling in rat-1 fibroblasts overexpressing HIR. *Exp Clin Endocrinol Diabetes* 104: 66
- Cohen B, Novick D, Rubinstein M (1996) Modulation of insulin activities by leptin. *Science* 274: 1185–1188
- Sun XJ, Miralpeix M, Myers MG Jr et al. (1992) Expression and function of IRS-1 in insulin signal transmission. *J Biol Chem* 267: 22662–22672
- Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *J Biol Chem* 269: 3568–3573
- Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM (1996) IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α and obesity-induced insulin resistance. *Science* 271: 665–668
- Welsh GI, Foulstone EJ, Young SW, Tavare JM, Proud CG (1994) Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and mitogen-activated protein kinase. *Biochem J* 303: 15–20
- Kellerer M, Rett K, Renn W, Groop L, Häring HU (1996) Circulating TNF α and leptin levels in offspring of NIDDM patients do not correlate to individual insulin sensitivity. *Horm Metab Res* 28: 737–743