# Leptin secretion and negative autocrine crosstalk with insulin in brown adipocytes

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## Abstract

Leptin is an important adipocytokine whose main regulative effects on energy metabolism are exerted via activation of signalling pathways in the central nervous system. Another important regulator of energy homeostasis is insulin. The role of direct autocrine leptin effects on adipose tissue and crosstalk with insulin, in particular in the thermogenically active brown adipose tissue, remains unclear. In the present study, we have investigated leptin secretion and interaction with insulin in highly insulin-responsive immortalised mouse brown adipocytes. Leptin was secreted in a differentiation-dependent manner, and acute leptin treatment of mature adipocytes dose- and time-dependently stimulated phosphorylation of STAT3 and MAP kinase. Interestingly, acute pretreatment of fully differentiated brown adipocytes with leptin (100 nM) significantly diminished insulin-induced glucose uptake by approximately 25%. This inhibitory effect was time-dependent and maximal after 60 min of leptin prestimulation. Furthermore, it correlated with a 35% reduction in insulin-stimulated insulin receptor kinase activity after acute leptin pretreatment. Insulin-induced insulin receptor substrate-1 tyrosine phosphorylation and binding to the regulatory subunit p85 of phosphatidylinositol 3-kinase (PI 3-kinase) were diminished by approximately 60% and 40%, respectively. Taken together, this study has demonstrated strong differentiation-dependent leptin secretion in brown adipocytes and PI 3-kinase-mediated negative autocrine effects of this hormone on insulin action. Direct peripheral leptin–insulin crosstalk may play an important role in the regulation of energy homeostasis.

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## Introduction

Adipose tissue has emerged as an endocrine organ that is central to the regulation of energy homeostasis (Ahima & Flier 2000, Shuldiner *et al.* 2001). The adipocyte-derived hormone leptin appears to be an important player in this regard (Friedman *et al.* 1998, Spiegelman & Flier 2001). Hypothalamic leptin stimulation regulates the expression of a number of orexigenic and anorexigenic neuropeptides which, in turn, results in decreased food intake and increased energy expenditure mediated via activation of the sympathetic nervous system (Spiegelman & Flier 2001).

The densely innervated brown adipose tissue is an effector tissue of the sympathetic nervous system. It contributes to the regulation of energy homeostasis by virtue of expression of the mitochondrial uncoupling protein 1, which uncouples mitochondrial respiration, thereby providing heat instead of generating ATP (Nicholls & Locke 1984, Klaus 1997, Lowell & Flier 1997). Similar to white adipose tissue, brown adipose tissue is highly insulin sensitive. Furthermore, we have recently shown that immortalised brown adipocytes provide an excellent model to investigate insulin signalling and potential insulin resistance-inducing mechanisms (Fasshauer *et al.* 2000, 2001, Klein *et al.* 1999, 2000, 2002). Immortalised brown adipocytes differentiated in culture resemble mature primary adipocytes with respect to morphological, molecular and functional characteristics including adrenergic and insulin sensitivity (Klein *et al.* 1999, 2002).

Impaired insulin action in white and brown adipose tissue has been shown to be associated with the development of diabetes and features of the insulin-resistance syndrome (Kahn & Flier 2000, Abel *et al.* 2001, Guerra *et al.* 2001). Expression of the long form of the leptin receptor has been demonstrated for both white and brown adipose tissue (Siegrist-Kaiser *et al.* 1997), but only a few and controversial reports exist about direct leptin effects on insulin action. *In vivo* and *in vitro* studies in human and rat white adipose tissue found either negative effects (Muller *et al.* 1997, Zhang *et al.* 1999) or no leptin influence


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at all (Mick *et al.* 1998, Zierath *et al.* 1998). For the thermogenically active brown adipose tissue, direct crosstalk of leptin with insulin signalling and action is completely unknown.

Here, we show a robust differentiation-associated increase in leptin secretion in brown adipocytes. Furthermore, we demonstrate autocrine negative crosstalk on the proximal level of insulin signalling which transiently induces insulin resistance and suggests a pathophysiologically important direct interaction between these two signalling systems.

## Materials and Methods

### *Materials*

Recombinant mouse leptin was purchased from Calbiochem, Inc. (San Diego, CA, USA), anti-insulin receptor substrate (IRS)-1 and anti-p85 antibodies, as well as recombinant IRS-1, were from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), and phosphospecific antibodies to STAT3, p44/p42 MAP kinase and Akt (S473) were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-insulin (AB3) antibody was purchased from Oncogen (Cambourne, MA, USA), and anti-phosphotyrosine antibody (PY11230) from BD Transduction Laboratories (Heidelberg, Germany). All other chemicals were from Sigma-Aldrich Co. (St Louis, MO, USA), unless stated otherwise.

### *Cell culture*

For all experiments, we used SV40T-immortalised brown adipocytes from the FVB strain of mice generated as described elsewhere (Klein *et al.* 1999). Preadipocytes were grown in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, Strathclyde, UK) supplemented with 4·5 g/l glucose, 20 nM insulin, 1 nM T3, 20% fetal bovine serum (Sigma-Aldrich Co., St Louis, MO, USA) and penicillin/streptomycin (BioWhittaker, Vervier, Belgium) (differentiation medium). When confluence was reached, differentiation was induced by complementing differentiation medium further with 250 μM indomethacine, 500 μM isobutylmethylxanthine and 2 μg/ml dexamethasone for 24 h. After changing back to differentiation medium, cell culture was continued for 5 more days before cells were starved for 24–48 h with serum-free medium prior to carrying out the experiments.

### *Determination of leptin secretion*

From day 5 to day 13 of the differentiating process, culture medium was removed from the cells every 24 h and replaced with fresh medium. Leptin release into the medium was determined using a mouse leptin radioimmunoassay (Linco Research, Inc., St Louis, MO, USA).

### *Determination of glucose uptake*

Glucose uptake assays were carried out essentially as described previously (Klein *et al.* 1999). In brief, cells were starved in serum-free medium for 48 h and washed in Krebs–Ringer–HEPES (20 mM HEPES, 136 mM NaCl, 4·7 mM KCl, 1·25 mM $MgSO_4$, 1·25 mM $CaCl_2$). After preincubation with 100 nM leptin for varying periods of time, cells were incubated with 100 nM insulin for 30 min. $^3$H-Labelled 2-deoxyglucose ($^3$H-2-DOG; 500 nCi/ml; NEN Life Science Products, Cologne, Germany) was added for 4 min before cells were washed in ice-cold phosphate-buffered saline and lysed with 0·1% SDS. Activity of $^3$H-2-DOG was measured using a beta scintillation counter.

### *Western blotting and immunoprecipitation*

Proteins were isolated using whole-cell lysis buffer containing 2 mM vanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 mM phenylmethylsulphonyl fluoride (Fluka Chemie AG, Neu-Ulm, Germany). Protein content of lysates was determined by the method of Bradford (1976) using the dye from Bio-Rad (Hercules, CA, USA). Lysates were submitted to SDS-PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell Inc., Keane, NH, USA). Membranes were blocked overnight with rinsing buffer (10 mM Tris, 150 mM NaCl, 0·05% Tween 20, pH 7·2) containing 3% bovine serum albumin (BSA) (blocking solution). Membranes were then incubated in blocking solution for 1–2 h with the antibodies indicated. Protein bands were visualised using the chemiluminescence kit from Roche Molecular Biochemicals (Mannheim, Germany) and enhanced chemiluminescence films (Amersham Pharmacia Biotech, Freiburg, Germany). For immunoprecipitation, lysates were prepared as described above, and respective antibodies immobilised to protein G-sepharose beads (Pierce, Rockford, IL, USA) were added for 4 h. Beads were then washed three times with 10 mM NaCl, 25·5 mM KCl, 200 mM HEPES, 0·05% Igepal, 1% BSA, 8·5% glycerol and 100 μM Na-vanadate, before protein–antibody complexes were dissolved in 5 × Laemmli buffer. Subsequent immunoblotting was carried out as described above.

### *Insulin receptor kinase activity assay*

After preincubation with 100 nM leptin for varying periods of time, cells were incubated with 100 nM insulin for 5 min and whole-cell lysates were prepared. Insulin receptor kinase activity was determined as described previously (Klein *et al.* 1993, Krutzfeldt *et al.* 2000). In brief, lysates were added to microwell plates coated

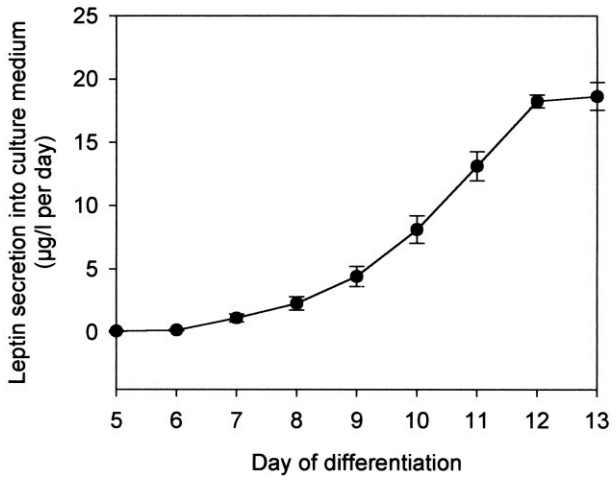


**Figure 1** Leptin secretion in brown adipocytes. Culture medium was taken every 24 h and analysed for leptin content. Results depicted represent the average ± S.E.M. of six independent experiments.

with anti-insulin receptor antibodies for 24 h at 4 °C. Incubation of plates with recombinant IRS-1 and $\gamma$-$^{32}$P-ATP (Hartmann GmbH, Braunschweig, Germany) for 2 h followed, before $^{32}$P-incorporation into IRS-1 was measured. Insulin binding to the receptor was determined with $^{125}$I-insulin (Amersham Pharmacia Biotech). Insulin receptor kinase activity is expressed as extent of IRS-1 tyrosine phosphorylation in relation to insulin binding.

### *Statistical analysis*

Data are presented as means ± S.E.M. Statistical analyses were performed with Sigma Plot (SPSS Science, Chicago, IL, USA). Statistical significance was determined using the unpaired Student's *t*-test. *P* values of $<0{\cdot}05$ were considered significant, those of $<0{\cdot}01$ highly significant.

## Results

### *Differentiation-dependent leptin secretion in brown adipocytes*

In differentiating brown adipocytes, we found a robust increase in leptin release over two orders of magnitude during a 13-day differentiation period (Fig. 1). Leptin levels were first detectable in the culture medium at day 5 at a concentration of 0·1 µg/l and exponentially increased from day 7 up to maximal concentrations of about 20 µg/l at day 12 when they reached a plateau (Fig. 1).

### *Dose- and time-dependent phosphorylation of STAT3 and MAP kinase by leptin*

To confirm leptin signalling in differentiated brown adipocytes, STAT3 and MAP kinase phosphorylation were assessed after leptin treatment. Stimulation with leptin for 5 min resulted in a significant dose-dependent increase in STAT3 phosphorylation (Fig. 2A). Furthermore, leptin-induced STAT3 phosphorylation was time-dependent with a highly significant maximal increase by about 2-fold after 2 min (Fig. 2B). Also, phosphorylation of p44/p42 MAP kinase was significantly induced approximately 2·8-fold after 5 min of leptin treatment in a time- and dose-dependent manner (Fig. 2C and D).

### *Leptin impairs insulin-induced glucose uptake*

Given the direct activation of leptin and insulin-signalling intermediates by leptin, we next investigated potential metabolic effects of leptin crosstalk with insulin. Glucose uptake is a classical biological endpoint of insulin signalling in adipose tissue. Treatment with 100 nM insulin for 30 min induced an approximately 4-fold increase in glucose uptake in differentiated brown adipocytes (Fig. 3A). Leptin treatment alone did not affect basal glucose uptake. However, leptin treatment at a concentration of 100 nM prior to incubation with insulin impaired the insulin-induced effect (Fig. 3A). This inhibitory effect was time-dependent and first detectable after 15 min of leptin prestimulation. A highly significant maximum inhibition by approximately 25% was observed after 60 min (Fig. 3A). A significant reduction in insulin-induced glucose uptake was also found after 2 h of leptin pretreatment, but prolonged periods of leptin exposure for up to 24 h did not show any significant alterations (data not shown).

### *Leptin acutely reduces insulin receptor kinase activity*

Insulin treatment with 100 nM for 5 min strongly increased receptor kinase activity (Fig. 3B). Consistent with the inhibitory leptin effect on insulin-induced glucose uptake, prestimulation of cells with 100 nM leptin for 5 min significantly decreased insulin receptor kinase activity by 33% (Fig. 3B). Leptin-mediated inhibition of receptor kinase activity decreased somewhat after longer pretreatment periods but was still detectable after 60 min of leptin preincubation (Fig. 3B).

### *Leptin inhibits insulin-induced IRS-1 tyrosine phosphorylation and binding to phosphatidylinositol 3-kinase but not activation of Akt*

IRS-1 is a main proximal insulin-signalling intermediate that is tyrosine phosphorylated by the activated insulin receptor kinase and subsequently enables binding and activation of phosphatidylinositol 3-kinase (PI 3-kinase) which, in turn, is pivotal in mediating insulin's metabolic effects (Kahn 1994, Cheatham & Kahn 1995). IRS-1 tyrosine phosphorylation was strongly induced by insulin stimulation for 5 min (Fig. 4A). However, similar to

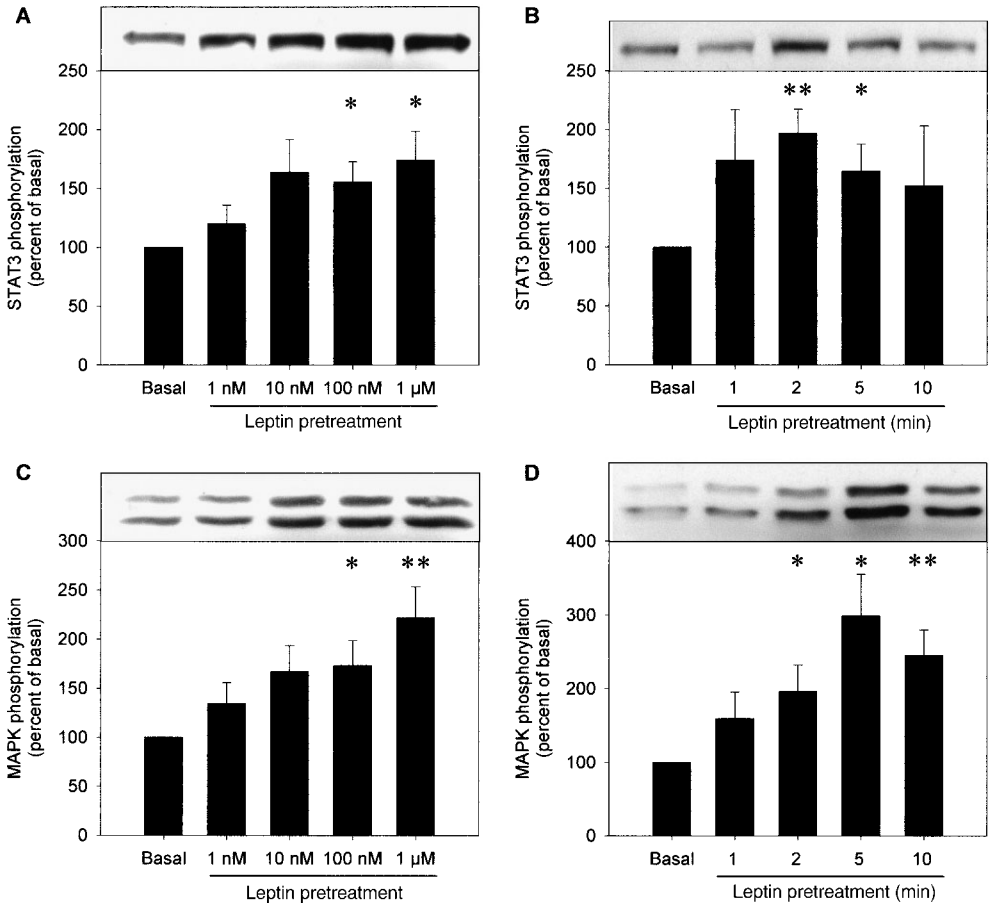


**Figure 2** Leptin induces STAT3 and MAP kinase (MAPK) phosphorylation. (A and B) Cells were starved in serum-free medium for 48 h. Subsequently, cells were treated with leptin at the concentrations indicated for 5 min or for the indicated periods of time (1 μM leptin) respectively, and immunoblots of whole-cell lysates were performed using phosphospecific antibodies to STAT3. (C and D) MAP kinase phosphorylation was measured by immunoblots using phophospecific p44/p42 MAP kinase antibodies after 10 min of leptin treatment at the concentrations indicated or for the indicated times at the concentration of 1 μM respectively. Bar graph analyses with the S.E.M. of at least three independent experiments and representative immunoblots are shown in each panel. *$P$<0·05 significant and **$P$<0·01 highly significant differences as compared with basal phosphorylation.

inhibition of insulin receptor kinase, leptin pretreatment (100 nM) significantly reduced insulin-induced IRS-1 tyrosine phosphorylation. Again, the effect was time-dependent (Fig. 4A). Yet, by contrast to insulin receptor kinase activity, significant inhibition of IRS-1 tyrosine phosphorylation was somewhat delayed and appeared to be biphasic with a maximal 60% inhibition after 60 min of leptin pretreatment.

Insulin induced an approximately 3-fold increase in IRS-1 binding to the regulatory subunit p85 of PI 3-kinase (Fig. 4B). Consistent with the kinetics for IRS-1 tyrosine phosphorylation, binding of IRS-1 to p85 was diminished by leptin stimulation in a time-dependent biphasic manner with a maximal 50% reduction occurring after 60 min of leptin preincubation (Fig. 4B).

Akt is an important signalling element downstream of PI 3-kinase. Data regarding its involvement in mediating glucose uptake are controversial (Kitamura *et al.* 1998, Summers *et al.* 2000, Cho *et al.* 2001, Hernandez *et al.* 2001). Insulin stimulation for 5 min strongly induced Akt activation as assessed using a phosphospecific Akt antibody (Fig. 4C). Interestingly, this insulin-induced increase was not altered by leptin pretreatment.

## Discussion

This study has demonstrated strong differentiation-dependent leptin secretion in brown adipocytes and autocrine negative effects of this adipocytokine on proximal

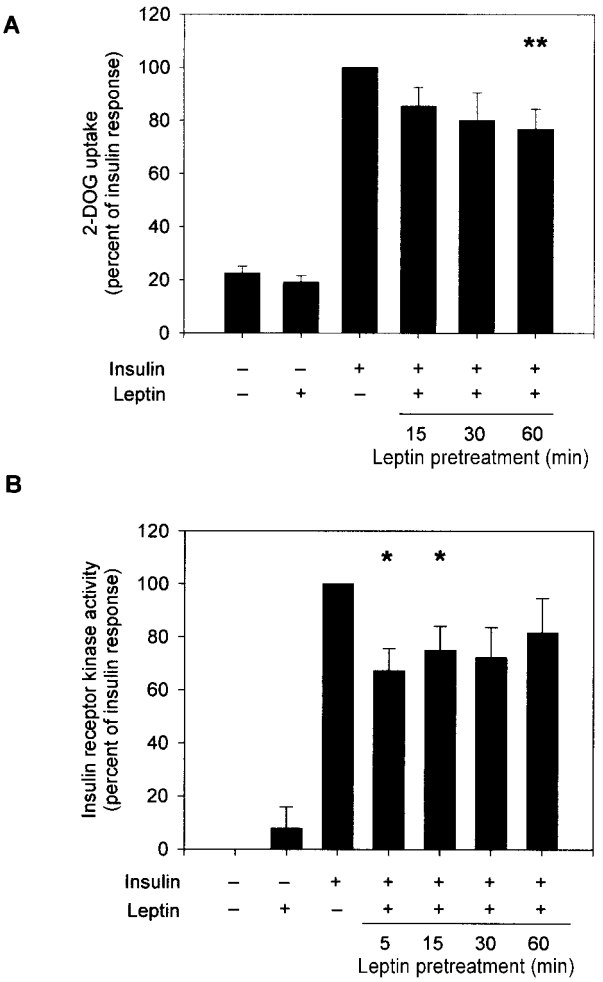


**Figure 3** Acute leptin pretreatment inhibits insulin-induced glucose uptake and acutely diminishes insulin-induced insulin receptor kinase activity. (A) Cells were starved in serum-free medium for 48 h and subsequently exposed to leptin (100 nM) for 15, 30 and 60 min prior to insulin stimulation (100 nM) for 30 min. At the end of the insulin stimulation period, cells were incubated with $^{3}$H-2-DOG (2-DOG) for 4 min, and uptake was measured in a beta scintillation counter. Bars represent the mean and S.E.M. of ten independent experiments with triplicate determination of each. $^{**}P<0{\cdot}01$ highly significant differences as compared with insulin stimulation alone. (B) Cells were starved in serum-free medium for 24 h and treated with 100 nM leptin for 5, 15, 30 and 60 min prior to 5 min stimulation with insulin (100 nM). Insulin receptor kinase activity was determined using $^{32}$P-labelled IRS-1 as described in the Methods section. The bar graph analysis represents the mean of three independent experiments with duplicate determination. $^{*}P<0{\cdot}05$ significant differences as compared with the insulin-induced response.

insulin-signalling components resulting in acute and transient induction of insulin resistance.

Studies of leptin expression and secretion in brown adipocytes have been inconsistent. While one study failed to detect leptin (Cinti *et al.* 1997), others reported either diminished (Moinat *et al.* 1995) or normal leptin expression and secretion in both human and rodent brown adipocytes (Deng *et al.* 1997, Zilberfarb *et al.* 1997, Buyse *et al.* 2001) as compared with white adipocytes. In the present study, we used a well-characterised adipocyte model of SV40T-immortalised brown fat cells that are similar to primary brown adipocytes in morphological, molecular and functional aspects (Klein *et al.* 1999, 2000, 2002). We found an increase in leptin release over two orders of magnitude. Thus, our study clearly argues for a robust and differentiation-dependent leptin expression and secretion in brown adipose tissue.

Adipose tissue has emerged as an endocrine organ that is central to the regulation of energy homeostasis and insulin resistance (Ahima & Flier 2000, Kahn & Flier 2000, Shuldiner *et al.* 2001, Spiegelman & Flier 2001). Recent data suggest that selective impairment of insulin signalling in the thermogenic brown adipose tissue by a tissue-specific disruption of the insulin receptor gene induces a diabetic state (Guerra *et al.* 2001). Interestingly, our study has demonstrated significant acute negative effects of leptin on insulin-induced glucose uptake. To our knowledge, this is the first report on direct leptin interactions with insulin signalling and action in brown adipocytes. In white adipocytes, few and conflicting studies have been published. While some authors did not find any effects of leptin on insulin-stimulated glucose uptake in isolated rat white adipocytes (Mick *et al.* 1998, Zierath *et al.* 1998), others suggest a negative leptin influence on insulin-regulated metabolic actions including glucose uptake in both rat and human adipocytes (Muller *et al.* 1997, Zhang *et al.* 1999). One possible explanation for the inconsistency of previous studies in white adipocytes may be the small and transient character of changes induced by leptin stimulation. However, in a physiological and pathophysiological context, these alterations could entail important chronic metabolic consequences by inducing long-lasting changes, e.g. in gene expression patterns or secretion of other adipocytokines implicated in energy balance regulation.

Consistent with the impairment of insulin-induced glucose uptake, we have found an inhibition of important proximal insulin-signalling elements by leptin. Insulin receptor kinase activity is maximally inhibited after 5 min of leptin pretreatment and subsequently returns to normal, whereas kinetics of downstream signalling events and the final metabolic response are delayed. This may represent an example for the above-mentioned phenomenon of acute transient alterations inducing longer-lasting biological consequences. The kinetics of IRS-1 tyrosine phosphorylation and binding to PI 3-kinase appear to be biphasic. Furthermore, in contrast to PI 3-kinase activity, we did not find leptin-mediated alteration of insulin-stimulated Akt activation which is in line with recent studies suggesting a dissociation between PI 3-kinase and Akt in mediating glucose uptake (Kitamura *et al.* 1998,

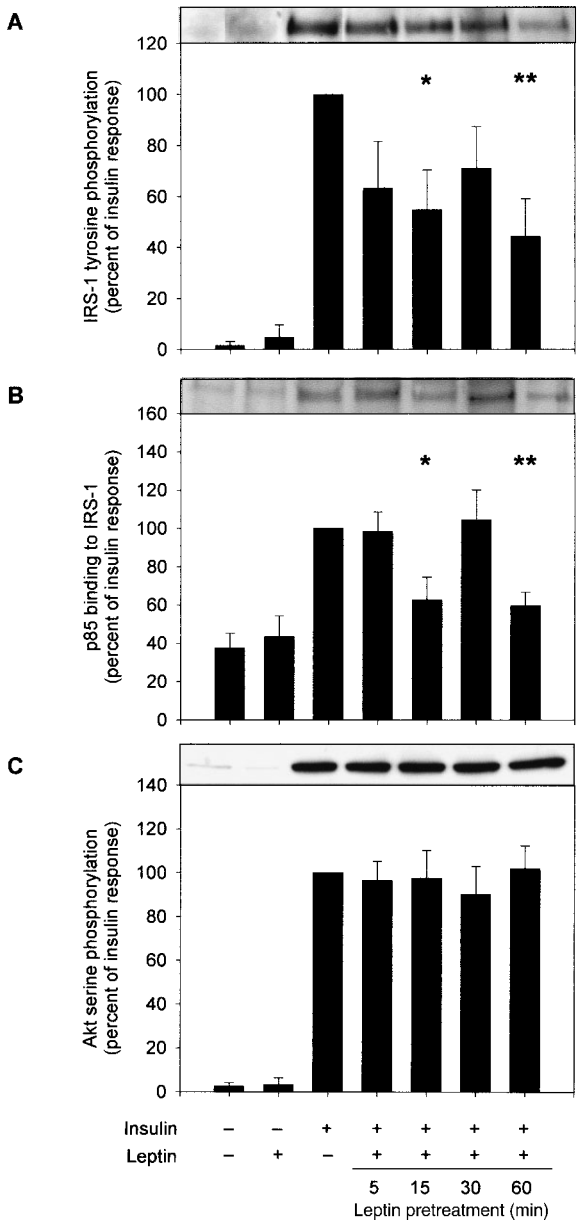


**Figure 4** Leptin acutely inhibits proximal insulin-signalling components. Cells were starved in serum-free medium for 24 h and treated with 100 nM leptin for 5, 15, 30 and 60 min prior to stimulation with insulin (100 nM, 5 min). *$P<0{\cdot}05$ significant and **$P<0{\cdot}01$ highly significant differences relative to the insulin response. (A) IRS-1 tyrosine phosphorylation was analysed by immunoprecipitating with an anti-IRS-1 antibody and immunoblotting using an anti-phosphotyrosine antibody. The bar graph analysis represents the mean of five independent experiments (with S.E.M.). (B) p85 binding to IRS-1 was analysed by immunoprecipitating with an anti-IRS-1 antibody and immunoblotting using an anti-phosphotyrosine antibody. Bars represent the mean of four independent experiments (with S.E.M.). (C) Phosphorylation of Akt was analysed by direct immunoblotting using an anti-phosphospecific antibody to Akt (S473). Bar graph analysis represents the mean of four independent experiments (with S.E.M.).

Nadler *et al.* 2001). Taken together, these findings may reflect additional signalling pathways, which interact on several post-receptor levels. Of note, negative leptin effects on insulin-induced glucose uptake independent of alterations in proximal insulin-signalling components have recently been described in skeletal muscle cells (Sweeney *et al.* 2001).

In summary, this study has demonstrated differentiation-dependent leptin secretion in brown adipocytes and elucidated autocrine insulin resistance-inducing effects of leptin on major insulin-signalling components. Direct interactions between these two hormonal systems may have important implications for the control of energy balance.

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