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High Leptin Levels Acutely Inhibit Insulin-Stimulated Glucose Uptake without Affecting Glucose Transporter 4 Translocation in L6 Rat Skeletal Muscle Cells

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Obesity is a major risk factor for the development of insulin resistance, characterized by impaired stimulation of glucose disposal into muscle. The mechanisms underlying insulin resistance are unknown. Here we examine the direct effect of leptin, the product of the obesity gene, on insulin-stimulated glucose uptake in cultured rat skeletal muscle cells. Preincubation of L6 myotubes with leptin (2 or 100 nM, 30 min) had no effect on basal glucose uptake but reduced insulin-stimulated glucose uptake. However, leptin had no effect on the insulin-induced gain in myc-tagged glucose transporter 4 (GLUT4) appearance at the cell surface of L6 myotubes. Preincubation of cells with leptin also had no effect on insulin-stimulated tyrosine phosphorylation of insulin receptor, IRS-1 and IRS-2, phosphatidylinositol 3-kinase activity, or Akt phosphorylation. We have previously shown that insulin regulates glucose uptake via a signaling pathway sensitive to inhibitors of p38 MAP kinase. Here, leptin pretreatment reduced the extent of insulin-stimulated p38 MAP kinase phosphorylation and phosphorylation of cAMP response element binder, a downstream effector of p38 MAP kinase. These results show that high leptin levels can directly reduce insulin-stimulated glucose uptake in L6 muscle cells despite normal GLUT4 translocation. The mechanism of this effect could involve inhibition of insulin-stimulated p38 MAP kinase and GLUT4 activation. (*Endocrinology* 142: 4806–4812, 2001)

THE PRODUCT OF the obesity (*ob*) gene, leptin, (1), is synthesized and secreted primarily by adipocytes and has been shown to play an important role in the central regulation of body weight (2). It is now apparent that leptin also has important functions as a metabolic and neuroendocrine hormone (3). One significant endocrine consequence of obesity is the development of insulin resistance (4). This can lead to type 2 diabetes, which is characterized by insulin resistance and an inability of increased pancreatic insulin secretion to compensate for this defect. Interestingly, plasma leptin levels correlate positively with body weight (5), and it has been proposed that hyperleptinemia may be important in the development of insulin resistance associated with type 2 (4) and gestational (6) diabetes.

Insulin regulates blood glucose levels principally by stimulating the uptake of glucose into muscle and fat cells and curbing hepatic glucose output (7, 8). Glucose transport occurs via a family of glucose transporter proteins (GLUTs), of which GLUT4 is the major insulin responsive isoform in muscle and fat tissues. Intracellular GLUT4 is translocated to the cell surface in response to insulin, by a mechanism requiring the activation of PI3K (9, 10). However, we have shown that a membrane permeable acetoxymethylester derivative of the PI3K product PI (3, 4, 5) P_3 caused translocation of GLUT4 (Sweeney, G., R. Somwar, L. Foster, T. Jiang, G. Prestwich, P. Nielsen, and A. Klip, submitted work) without increasing glucose uptake (11). Moreover, activation of PI3K via stimuli other than insulin does not lead to elevations in glucose uptake (12). Collectively, these results suggest that PI3K activation is necessary but not sufficient to stimulate glucose uptake.

We have also shown that several inhibitors of p38 MAP kinase reduce insulin-stimulated glucose uptake without altering GLUT4 arrival at the cell surface (13, 14). Therefore, it is possible that insulin stimulates glucose uptake by increasing both the amount of glucose transporters at the cell surface and their activity. The above studies raise the hypothesis that the pathophysiology of insulin resistance may involve alterations in the ability of insulin to stimulate p38 MAP kinase and thus activate glucose transporters. Here we examine the ability of leptin to directly reduce insulin-stimulated glucose uptake and GLUT4 translocation in skeletal muscle cells and explore the molecular mechanisms underlying this effect.

Materials and Methods

Materials

Monoclonal antibody (9E10) to the myc epitope, the insulin receptor β-subunit, leptin receptor antibody (K-20) and antiphosphotyrosine antibody (PY99) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal anti-IRS-1 and IRS-2 antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal phosphospecific antibodies to p38 MAP kinase, Akt (T308), Akt (S473) and ERK were from New England Biolabs, Inc. (Beverley, MA) and to cAMP response element binding protein (CREB) was from Calbiochem (San Diego, CA). Recombinant mouse leptin was from Calbiochem (LA Jolla, CA) and human insulin (humulin) was from Eli Lilly & Co. (Toronto, Ontario, Canada).

Determination of 2-deoxyglucose uptake

L6 myotubes transfected with myc-tagged GLUT4 (L6-GLUT4myc) were grown and differentiated as previously characterized (15). Myotubes (12-well plates) were deprived of serum for 5 h with α-MEM and

Abbreviations: CREB, cAMP response element binding protein; GLUT, glucose transporter; IRS-1 or -2, insulin receptor substrates.

25 mM glucose before experimental manipulations. 2-Deoxyglucose uptake measurements were carried out for 5 min as described previously (16).

Measurement of GLUT4myc translocation in L6 myotubes

The content of myc-tagged GLUT4 at the cell surface was measured by an antibody-coupled densitometric assay (15) as follows: Quiescent L6 GLUT4myc myotubes, after treatment with insulin and/or leptin, were washed once with PBS then fixed with 3% paraformaldehyde in PBS for 3 min at room temperature. The fixative was immediately neutralized by incubation with 1% (wt/vol) glycine in PBS at 4 C for 10 min. The cell monolayer was then blocked with 10% (wt/vol) goat serum and 3% (wt/vol) BSA in PBS at 4 C for at least 30 min. Primary antibody (anti-c-*myc*, 9E10) was then added into the cultures at a dilution of 1:100 and maintained for 30 min at 4 C. The cells were washed with PBS before introducing peroxidase-conjugated rabbit antimouse IgG (1:1000). After 30 min at 4 C, the cells were washed and 1 ml OPD reagent (0.4 mg/ml *O*-phenylenediamine dihydrochloride and 0.4 mg/ml urea hydrogen peroxide in 0.05 M phosphate-citrate buffer) was added to each well for 10 min at room temperature. The reaction was stopped by addition of 0.25 ml of 3 N HCl. The supernatant was collected and the optical absorbance was measured at 492 nm.

Detection of tyrosine phosphorylation of insulin receptor β-subunit, IRS-1, IRS-2, and PI3K activity associated with antiphosphotyrosine

Myotubes were incubated in the presence or absence of leptin (100 nM, 30 min) then treated with 100 nM insulin for 5 min and immunoprecipitation using antiphosphotyrosine antibody performed essentially as described previously (16). Immunoprecipitated proteins were resolved by 7.5% SDS-PAGE and then electrotransferred onto polyvinylidenedifluoride membranes and immunoblotted with antiinsulin receptor β-subunit antibody (1:500) or anti-IRS-1 or -IRS-2 antibodies (1:500). Detection was by the enhanced chemiluminescence method. To determine PI3K activity, cell extracts were prepared as above for immunoprecipitation with antiphosphotyrosine antibody (16), and PI3K activity was measured as described previously (13).

Detection of Akt, p38 MAPK, ERK, and CREB phosphorylation using phosphospecific antibodies

To detect phosphorylation of Akt, p38 MAPK, ERK, and CREB using phosphospecific antibodies, cell lysates were prepared from myotubes grown on 6-well plates using 300 μl 2× Laemmli sample buffer containing 1 mM Na_3VO_4, 100 nM okadaic acid, protease inhibitors (1 mM benzamidine, 10 μM E-64, 1 μM leupeptin, 1 μM pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride), and 7.5% β-mercaptoethanol. Samples were vortexed for 1 min, passed 5 times through a 25-gauge syringe and heated for 15 min at 65 C. Lysates were then centrifuged for 5 min at 1,000 rpm. Approximately 60 μg (30 μl) were resolved by 10% SDS-PAGE then immunoblotted with phospho-antibodies (1:500 dilution). HRP-coupled goat antirabbit secondary (1:7500) was used in each case and detected by the enhanced chemiluminescence method.

Statistical analysis

Statistical analysis was performed using either paired *t* test or ANOVA test (Fischer, multiple comparisons) where appropriate.

Results

Analysis of leptin receptor expression in skeletal muscle to date has been limited to examination of mRNA. An overview of the existing literature indicates that skeletal muscle and C2C12 muscle cells express mRNA for both the long (ob.R_b) and short (ob.R_a) forms of the receptor (17–19). In addition, two studies detected the long form but did not test for expression of the short form (20, 21), and one study failed to detect the long form (22). To the best of our knowledge, there were no studies showing expression of the leptin receptor(s) protein in muscle or muscle cells. We immunoblotted L6 muscle cell lysates and total membranes and rat skeletal muscle total membranes with an antibody recognizing the extracellular domain of the leptin receptor (Fig. 1A). The results shown confirm that leptin receptor polypeptide is expressed in these cells. Based upon observations on the apparent molecular weight of leptin receptor isoforms in other tissues (23, 24) our results suggest the presence of both long (~200 kDa) and short (100–120 kDa) leptin receptor isoforms in these cells. Furthermore, since the antibody used recognizes an epitope common to all isoforms and should therefore recognize all isoforms equally, the results also suggest that the short leptin receptor isoform(s) is the predominant form expressed in these cells. This contrasts with the ratio of leptin receptor isoforms seen in hypothalamus and macrophages (23, 25) and is suggestive of potential direct effects of leptin in muscle tissue that may not be seen in other tissues. Figure 1B shows that pretreatment of L6 myotubes for 30 min with leptin (100 nM) reduced the stimulation of 2-deoxyglucose uptake by insulin. The maximum inhibition produced was approximately 50–60% of the normal insulin-stimulated response. A leptin preincubation time of 5 min was not sufficient for the inhibitory action to develop. This observation suggests that intracellular changes induced by leptin which result in inhibition of insulin action require longer than 5 min to be established. Therefore, in subsequent experiments examining the mechanism of leptin action a preincubation time of 30 min was used. Figure 1C shows that the stimulation of 2-deoxyglucose uptake was dependent on the concentration of leptin in the preincubation period. A leptin concentration of 0.1 nM had no significant effect, while at 2 nM (32 ng/ml) and 100 nM (1600 ng/ml) significant inhibition of 40–60% of the insulin response was observed.

Translocation of GLUT4 to the cell surface is required to allow stimulation of glucose uptake by insulin. Using muscle cells stably transfected with GLUT4 harboring a myc epitope tag on the first extracellular loop of the protein allowed us to examine the effect of leptin on insulin-stimulated translocation of GLUT4 to the surface of intact cells. This system yields accurate quantitative analysis of GLUT4 translocation from measurements of myc epitope exposure on a monolayer of differentiated myotubes (15). As shown in Fig. 2, although leptin reduced insulin-stimulated glucose uptake, it had no effect on the 2-fold increase in cell surface GLUT4myc induced by insulin. This result suggests that inhibition of GLUT4 translocation is not the mechanism whereby leptin inhibits insulin-stimulated 2-deoxyglucose uptake.

In Fig. 3 we examine the effect of leptin on early insulin signaling. The earliest detectable event subsequent to insulin binding is autophosphorylation of its receptor β-subunit. The increase in tyrosine phosphorylation of the insulin receptor after 5 min of insulin stimulation was not altered after 30 min preincubation with leptin (Fig. 3A). Insulin-stimulated tyrosine phosphorylation of two immediate insulin receptor substrates, IRS-1 and IRS-2, was also unaffected by leptin (Fig. 3A). PI3K has been shown to play a necessary, albeit insufficient role, in the stimulation of glucose uptake (10, 11). We examined the activity of PI3K associated with phosphotyrosine immunoprecipitates from control and insulin-stim-

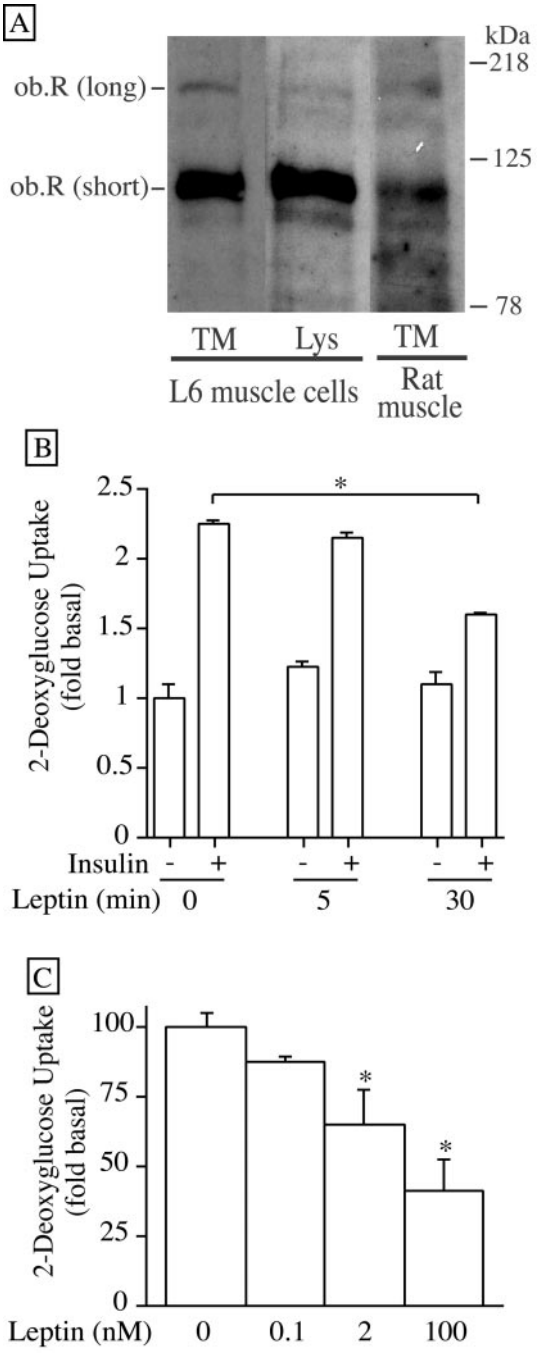

FIG. 1. Inhibition of insulin-stimulated 2-deoxyglucose uptake by leptin. A, L6 muscle cell lysates (Lys) or total membrane (TM) preparations (40 μg) and rat skeletal muscle total membranes were analyzed by Western blotting with an antibody to the extracellular domain of leptin receptor. B, Effect of either 5 min or 30 min preincubation with leptin (100 nM) on basal or insulin-stimulated 2-deoxyglucose uptake. Basal glucose uptake (average = 8.02 ± 0.13 pmol/min·mg) is expressed as 1 and the effect of insulin as fold above this value. C, Effect of 30 min preincubation of L6 myotubes with increasing concentrations of leptin (0.1 nM, 2 nM, and 100 nM) on insulin-stimulated glucose uptake (time-course). Insulin-stimulated glucose uptake in the absence of leptin is expressed as 100% and other values expressed relative to it. Results are mean of at least three experiments where each condition was assayed in triplicate. *, Significant difference ($P < 0.05$) compared with the insulin response in cells not pretreated with leptin.

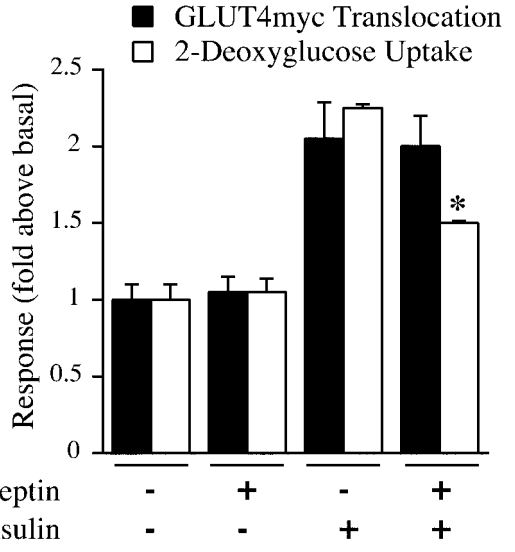

FIG. 2. Leptin has no effect on insulin-stimulated GLUT4myc translocation. Cells stably transfected with GLUT4 harboring a myc epitope tag on the first extracellular loop of the protein allow accurate quantitative analysis of GLUT4 translocation by measurement of myc epitope exposure on a monolayer of cells. A comparison of the ability of leptin (100 nM, 30 min) to affect basal and insulin-stimulated (100 nM, 20 min) GLUT4myc translocation (*filled bars*) and 2-deoxyglucose uptake (*open bars*) is shown here. Basal glucose uptake (average = 8.02 ± 0.13 pmol/min·mg or basal GLUT4 levels at the cell surface (arbitrary units) are expressed as 1 and the effect of insulin as fold above this value. Results are mean of at least three experiments where each condition was assayed in triplicate. *, Significant difference ($P < 0.05$) compared with the insulin response in cells not pretreated with leptin.

ulated cells and found an increase of approximately 7-fold. Neither this response to insulin nor basal PI3K activity were affected by preincubation with leptin for 30 min (Fig. 3B). An important downstream target of PI3K products contributing to GLUT4 translocation is the serine/threonine kinase Akt/PKB (9). The mechanism of activation of Akt involves phosphorylation of the kinase on two residues, Thr308 and Ser473. Insulin caused a robust phosphorylation of Akt on both Thr308 and Ser473 after 10 min that was not altered by preincubation with leptin (Fig. 3C). These results are consistent with the observation that similar conditions had no effect on the ability of insulin to induce GLUT4myc translocation (Fig. 2).

We recently proposed that insulin might regulate the activity of GLUT4 via a p38 MAP kinase-dependent signaling pathway (13, 14). The p38 MAP kinases are activated by dual phosphorylation on threonine and tyrosine residues in a TGY motif. Phosphorylation of p38 MAP kinase can be determined by immunoblotting cell lysates with an antibody that specifically recognizes only the dual phosphorylated form of the enzyme. Here we examined the effect of insulin (100 nM, 10 min) on p38 MAP kinase phosphorylation in cells pretreated with leptin. Figure 4 shows that insulin caused a significant increase in p38 MAP kinase phosphorylation. However, a similar increase was not detected in lysates prepared from cells which had been pretreated with leptin before stimulation with insulin. Leptin alone had no effect on p38 MAP kinase phosphorylation. We also examined the

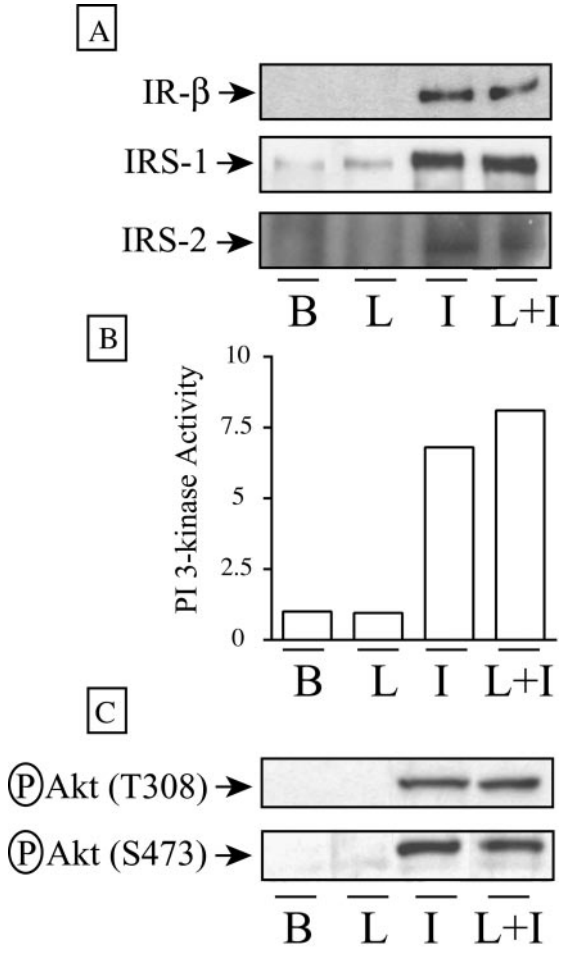

FIG. 3. Effect of leptin on insulin receptor β-subunit, IRS-1, IRS-2 and Akt phosphorylation and PI3K activity. Tyrosine phosphorylation of the insulin receptor β-subunit, IRS-1 and IRS-2 are shown in A. Cells were incubated in the absence (B: basal) or presence of leptin (L: 100 nM, 30 min) before insulin stimulation (I: 100 nM, 5 min). B, PI3K activity associated with phosphotyrosine immunoprecipitates from untreated cells (B) or cells treated with leptin (L: 100 nM, 30 min) or insulin (I: 100 nM, 10 min). C, Phosphorylation of Akt on threonine 308 (T308) and serine 473 (S473), examined by immunoblotting cell lysates prepared from basal untreated cells (B) and cells treated with leptin (L: 100 nM, 30 min) or insulin (I: 100 nM, 10 min) with phosphospecific antibodies. All results shown are representative of experiments performed three times.

activity of p38 MAP kinase in muscle cells by measuring phosphorylation of CREB, a known downstream effector of p38 MAP kinase. Treatment of cells with insulin resulted in increased phosphorylation of CREB, assessed using a phosphospecific antibody (Fig. 4). This increase in response to insulin was no longer evident when cells were pretreated with leptin, again reflecting an ability of leptin to prevent insulin-stimulated p38 MAP kinase activity. Finally, to explore any possible generalized effects of leptin we examined the effect of insulin on another member of the MAP kinase family (ERK) under similar conditions. As shown in Fig. 4, and in contrast to p38 MAPK, the increased phosphorylation of ERK in response to insulin remained intact when cells were pretreated with leptin.

Discussion

Physiological and pathophysiological actions of leptin

Obesity and its associated complications have become a health issue of epidemic proportions (26). In particular, obesity is now a well characterized major risk factor for the development of insulin resistance and type 2 diabetes (4). Despite an acute need for a detailed understanding of how increased adipose tissue mass causes insulin resistance, the underlying molecular mechanisms remain largely unknown. It has been suggested that various factors secreted by adipocytes, including leptin (4), TNF-α (27), FFA (28) and resistin (29) influence the insulin sensitivity of muscle and possibly other tissues. Here we focus on the role of leptin, the product of the obesity gene, the plasma levels of which correlate positively with body mass index (5).

Physiological control of blood glucose levels is primarily regulated by increased glucose clearance in response to insulin (7). Skeletal muscle, and to a lesser extent fat tissue, are quantitatively the most important target tissues for insulin-stimulated glucose disposal (7). Given the established association between obesity and type 2 diabetes, an important question to address is whether leptin may have a direct effect to modulate basal or insulin-stimulated glucose uptake. The detection of leptin receptor mRNAs in skeletal muscle and adipocytes suggested such a possibility (17). Here we confirmed leptin receptor protein expression (long and short forms) in L6 myotubes by Western blot analysis. It has been reported previously that leptin has no effect on basal glucose uptake in human (30), rat (31), or mouse (31, 32) skeletal muscle nor in rat (30, 32, 33) and mouse (30) adipocytes. In accordance with these reports, we show here that leptin does not alter basal glucose uptake in L6 myotubes. However, long-term leptin treatment of rodents increased 2-deoxyglucose uptake in EDL and soleus muscles, albeit to a smaller extent than that seen with insulin (34).

Importantly, we show that acute preincubation of L6 myotubes with 2 nM or 100 nM leptin reduces the ability of insulin to increase glucose uptake by up to 60%. This direct inhibitory effect of leptin required preincubation of cells with leptin for 30 min. Consistent with our observations, Friedman and colleagues (17) have shown that 1 h preincubation with leptin, again in the nanomolar range, directly suppressed insulin-stimulated glucose uptake (by 50%) in rat skeletal muscle *in vitro*. Interestingly, that study also reported that leptin could not affect insulin action in muscle from leptin-resistant obese Koletsky rats (17). Similar results to ours have also been reported for cultured rat adipocytes where 16 h preincubation with 2 nM leptin reduced insulin-stimulated glucose uptake, whereas lower leptin concentrations reduced insulin sensitivity (35). The effect of leptin is reversible as the normal response to insulin returns after leptin removal. Leptin also has inhibitory effects on other insulin-stimulated responses *in vitro*, such as conversion of fatty acids to triglycerides (36). Therefore, our studies and others suggest that leptin has a potential capacity to reduce insulin action. The concentrations of hormones used in *in vitro* studies preclude direct correlation to physiologically relevant responses, yet it is tantalizing to propose that under

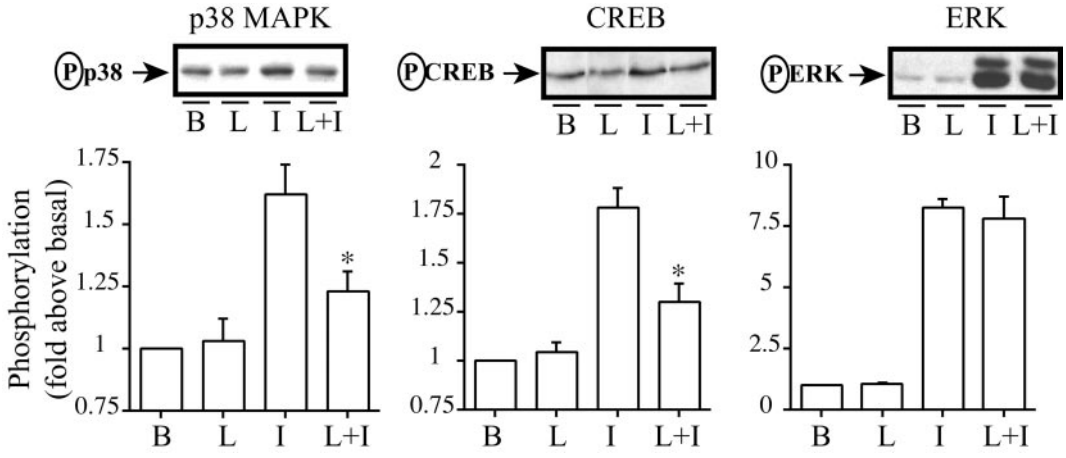

FIG. 4. Insulin-stimulated p38 MAPK and CREB, but not ERK, phosphorylation are inhibited by leptin. Phosphorylation of p38 MAP kinase, CREB, and ERK were examined by immunoblotting cell lysates prepared from basal untreated cells (B) and cells treated with leptin (L: 100 nM, 30 min) or insulin (I: 100 nM, 10 min) with phosphospecific antibodies for each respective protein. Immunoblots shown are representative of an experiment performed at least 3 times. The summary of all experiments performed (at least 3) are presented in the respective graphs as mean ± SEM. *, Significant difference ($P < 0.05$) compared with the insulin response in cells not pretreated with leptin.

certain circumstances there is potential for leptin to be an insulin resistance-inducing agent in obese individuals.

The physiological role of leptin in controlling insulin-stimulated glucose uptake and metabolism remains controversial as other studies have shown that leptin may also improve insulin sensitivity (4). Thus, there are paradoxical reports that sustained excess or lack of leptin can both cause insulin resistance (37–40). In C_2C_{12} muscle cells leptin (30 min, 100 ng/ml) caused a small increase in glucose uptake and movement of GLUT4 to the plasma membrane (19, 41). Increased glucose metabolism and insulin sensitivity were observed in transgenic skinny mice overexpressing leptin to levels similar to those observed in obese individuals (37). In addition, leptin infusion into rats for 48 h resulted in a 30% increase in insulin sensitivity as determined by whole body glucose utilization during hyperinsulinemic glucose clamps (38). Conversely, lipodystrophy and leptin deficiency cause hyperinsulinemia and insulin resistance in mice (40), and it has been proposed that leptin could act as an antidiabetic hormone in these animals (39). Furthermore, treatment of streptozotocin-diabetic rats with leptin for 12–14 d normalized blood glucose levels and improve insulin sensitivity (42). Clearly, the effect of leptin *in vivo* is complex, and it has been proposed that the effects of leptin on glucose metabolism *in vivo* are heavily dependent on a central nervous system-regulated (43, 44). Indeed, microinjection of leptin into the ventromedial hypothalamus increased glucose uptake in peripheral tissues in rats (45). Subcutaneous infusion of leptin (4 mg/kg/d) for 7 d caused tissue-specific effects with a stimulation of glucose uptake in rat skeletal muscle and brown adipose tissue and a decrease in white adipose tissue (34).

Experimental conditions, including route of administration and duration of leptin treatment, age of animals/humans and the use of *ex vivo* tissue or cell lines may partly explain the conflicting reports on the effect of leptin on basal and insulin-stimulated glucose uptake (46, 47). In particular, the time of leptin treatment before analysis of insulin action is likely to be of great importance. Indeed, one study that reports no effect of leptin on insulin-stimulated glucose transport added both hormones together with no preincubation period (32). We have shown in this study that a short (5 min) preincubation period with leptin is not sufficient to impinge upon insulin action. Thus, the conclusion of that study is in accordance with the findings shown here that 5 min preincubation with leptin has no significant effect on insulin-stimulated glucose uptake. A slightly longer preincubation (30 min) was needed to observe the reduction in insulin action. Therefore, the variable temporal parameters employed in published literature may explain the discrepancies regarding the effect of leptin on glucose transport. However, the acute inhibitory effect of high leptin levels on insulin action seen by us and others (17, 36) in muscle cells, in fat cells (35), and liver (48, 49) or liver cells (50) uncovers an intriguing action of leptin directly on peripheral tissues, which may have been overlooked by the whole body *in vivo* studies quoted above.

Signals engaged by leptin

Our results show that leptin has no direct effect on GLUT4myc content at the surface of unstimulated L6 myotubes nor on the insulin-stimulated increase in GLUT4myc translocation. In keeping with this observation, insulin-stimulated signaling events thought to participate in mediating GLUT4 translocation were also unaffected. These included tyrosine phosphorylation of the insulin receptor, IRS-1 and IRS-2, PI3K activity and phosphorylation of Akt on Thr308 and Ser473. Thus, leptin reduced insulin-stimulated glucose uptake without any effect on incorporation of GLUT4 to the plasma membrane or in the signals mediating this event.

Until recently, translocation of GLUT4 to the cell surface was thought to be sufficient for insulin-stimulated glucose uptake (51). However, several studies have now suggested that full stimulation of glucose uptake by insulin requires both translocation and activation of GLUT4 (11, 13, 14). We have shown that inhibitors of p38 MAP kinase reduce insulin-stimulated glucose uptake in muscle and fat cells without

any effect on GLUT4 translocation (13, 14). Because the effects of leptin on L6 muscle cells resembled those found upon inhibition of p38 MAP kinase activation, we examined the possibility that leptin may prevent the increase in insulin-stimulated p38 MAP kinase and GLUT4 activity in response to insulin. Insulin-stimulated phosphorylation (activation) of p38 MAP kinase and of a known effector of p38 MAP kinase, CREB, were inhibited by leptin pretreatment. Importantly, whereas insulin-stimulated p38 MAP kinase phosphorylation was inhibited, leptin had no effect on the phosphorylation of another member of the MAP kinase family, ERK, in response to insulin.

The above results suggest that the reduction in insulin-stimulated glucose uptake by leptin may be caused by preventing p38 MAP kinase activation and subsequently GLUT4 activation in response to insulin. The precise mechanism whereby leptin can cross-talk with selective insulin-stimulated signaling events is unknown. Leptin can stimulate a diverse range of intracellular signaling pathways and recent studies have begun to demonstrate an ability of leptin to affect selective insulin signaling pathways. For example, in Fao cells insulin-stimulated IRS-1 tyrosine phosphorylation and associated PI3K activity were enhanced by leptin pretreatment, whereas signaling via IRS-2 was inhibited (24). Together with our results, these reports suggest that the intracellular cross-talk between leptin and insulin is cell type-specific and requires further investigation.

In summary, preincubation of cultured rat skeletal muscle cells with leptin can reduce the ability of insulin to increase glucose uptake. However, insulin-stimulated GLUT4 translocation, insulin receptor, IRS-1 and IRS-2 tyrosine phosphorylation, PI3-kianase activity and phosphorylation of Akt on Thr308 and Ser473 were unaffected by leptin. Instead, the molecular mechanism of reduced glucose uptake may involve decreased insulin-stimulated p38 MAP kinase activation and consequently reduced activation of GLUT4. These results suggest that insulin-stimulated GLUT4 activity can be regulated by another hormone, leptin. While other factors undoubtedly contribute to the development of insulin-resistance associated with obesity *in vivo*, this study highlights a potentially important direct effect of leptin to reduce insulin-stimulated glucose uptake in muscle cells.

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References

1. **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 372:425–432
2. **Friedman JM, Halaas JL** 1998 Leptin and the regulation of body weight in mammals. Nature 395:763–770
3. **Wauters M, Considine RV, Van Gaal LF** 2000 Human leptin: from an adipocyte hormone to an endocrine mediator. Eur J Endocrinol 143:293–311
4. **Kahn BB, Flier JS** 2000 Obesity and insulin resistance. J Clin Invest 106:473–481
5. **Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF** 1996 Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334:292–295
6. **Kautzy-Willer A, Pacini G, Tura A, Bieglmayer C, Schneider B, Ludvik B, Prager R, Waldhausl W** 2001 Increased plasma leptin in gestational diabetes. Diabetologia 44:164–172
7. **Kahn BB** 1996 Lilly lecture 1995. Glucose transport: pivotal step in insulin action. Diabetes 45:1644–1654
8. **Shulman GI** 2000 Cellular mechanisms of insulin resistance. J Clin Invest 106:171–176
9. **Wang Q, Somwar R, Bilan PJ, Liu Z, Jin J, Woodgett JR, Klip A** 1999 Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. Mol Cell Biol 19:4008–4018
10. **Tanti JF, Gremeaux T, Grillo S, Calleja V, Klippel A, Williams LT, Van Obberghen E, Le Marchand-Brustel Y** 1996 Overexpression of a constitutively active form of phosphatidylinositol 3-kinase is sufficient to promote Glut 4 translocation in adipocytes. J Biol Chem 271:25227–25232
11. **Jiang T, Sweeney G, Rudolf MT, Klip A, Traynor-Kaplan A, Tsien RY** 1998 Membrane-permeant esters of phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 273:11017–11024
12. **Isakoff SJ, Taha C, Rose E, Marcusohn J, Klip A, Skolnik EY** 1995 The inability of phosphatidylinositol 3-kinase activation to stimulate GLUT4 translocation indicates additional signaling pathways are required for insulin-stimulated glucose uptake. Proc Natl Acad Sci USA 92:10247–10251
13. **Sweeney G, Somwar R, Ramlal T, Volchuk A, Ueyama A, Klip A** 1999 An inhibitor of p38 mitogen-activated protein kinase prevents insulin- stimulated glucose transport but not glucose transporter translocation in 3T3–L1 adipocytes and L6 myotubes. J Biol Chem 274:10071–10078
14. **Somwar R, Perreault M, Kapur S, Taha C, Sweeney G, Ramlal T, Kim DY, Keen J, Cote CH, Klip A, Marette A** 2000 Activation of p38 mitogen-activated protein kinase alpha and beta by insulin and contraction in rat skeletal muscle: potential role in the stimulation of glucose transport. Diabetes 49:1794–1800
15. **Wang Q, Khayat Z, Kishi K, Ebina Y, Klip A** 1998 GLUT4 translocation by insulin in intact muscle cells: detection by a fast and quantitative assay. FEBS Lett 427:193–197
16. **Somwar R, Sweeney G, Ramlal T, Klip A** 1998 Stimulation of glucose and amino acid transport and activation of the insulin signaling pathways by insulin lispro in L6 skeletal muscle cells. Clin Ther 20:125–410
17. **Ishizuka T, Ernsberger P, Liu S, Bedol D, Lehman TM, Koletsky RJ, Friedman JE** 1998 Phenotypic consequences of a nonsense mutation in the leptin receptor gene (fak) in obese spontaneously hypertensive Koletsky rats (SHROB). J Nutr 128:2299–2306
18. **Fruhbeck G, Gomez-Ambrosi J, Martinez JA** 1999 Pre- and postprandial expression of the leptin receptor splice variants OB-Ra and OB-Rb in murine peripheral tissues. Physiol Res 48:189–195
19. **Berti L, Gammeltoft S** 1999 Leptin stimulates glucose uptake in C2C12 muscle cells by activation of ERK2. Mol Cell Endocrinol 157:121–130
20. **Lin J, Barb CR, Matteri RL, Kraeling RR, Chen X, Meinersmann RJ, Rampacek GB** 2000 Long form leptin receptor mRNA expression in the brain, pituitary, and other tissues in the pig. Domest Anim Endocrinol 19:53–61
21. **Liu YL, Emilsson V, Cawthorne MA** 1997 Leptin inhibits glycogen synthesis in the isolated soleus muscle of obese (*ob/ob*) mice. FEBS Lett 411:351–355
22. **Hoggard N, Mercer JG, Rayner DV, Moar K, Trayhurn P, Williams LM** 1997 Localization of leptin receptor mRNA splice variants in murine peripheral tissues by RT-PCR and in situ hybridization. Biochem Biophys Res Commun 232:383–387
23. **O'Rourke L, Yeaman SJ, Shepherd PR** 2001 Insulin and leptin acutely regulate cholesterol ester metabolism in macrophages by novel signaling pathways. Diabetes 50:955–961
24. **Szanto I, Kahn CR** 2000 Selective interaction between leptin and insulin signaling pathways in a hepatic cell line. Proc Natl Acad Sci USA 97:2355–2360
25. **Zhao AZ, Shinohara MM, Huang D, Shimizu M, Eldar-Finkelman H, Krebs EG, Beavo JA, Bornfeldt KE** 2000 Leptin induces insulin-like signaling that antagonizes cAMP elevation by glucagon in hepatocytes. J Biol Chem 275: 11348–11354
26. **Pi-Sunyer FX, Laferrere B, Aronne LJ, Bray GA** 1999 Therapeutic controversy: Obesity—a modern-day epidemic. J Clin Endocrinol Metab 84:3–12
27. **Peraldi P, Spiegelman B** 1998 TNF-α and insulin resistance: summary and future prospects. Mol Cell Biochem 182:169–175
28. **Bergman RN, Ader M** 2000 Free fatty acids and pathogenesis of type 2 diabetes mellitus. Trends Endocrinol Metab 11:351–356
29. **Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA** 2001 The hormone resistin links obesity to diabetes. Nature 409:307–312
30. **Ranganathan S, Ciaraldi TP, Henry RR, Mudaliar S, Kern PA** 1998 Lack of

effect of leptin on glucose transport, lipoprotein lipase, and insulin action in adipose and muscle cells. Endocrinology 139:2509–2513
31. **Furnsinn C, Brunmair B, Furtmuller R, Roden M, Englisch R, Waldhausl W** 1998 Failure of leptin to affect basal and insulin-stimulated glucose metabolism of rat skeletal muscle in vitro. Diabetologia 41:524–529
32. **Zierath JR, Frevert EU, Ryder JW, Berggren PO, Kahn BB** 1998 Evidence against a direct effect of leptin on glucose transport in skeletal muscle and adipocytes. Diabetes 47:1–4
33. **Mick G, Vanderbloomer T, Fu CL, McCormick K** 1998 Leptin does not affect adipocyte glucose metabolism: studies in fresh and cultured adipocytes. Metabolism 47:1360–1365
34. **Wang JL, Chinookoswong N, Scully S, Qi M, Shi ZQ** 1999 Differential effects of leptin in regulation of tissue glucose utilization *in vivo*. Endocrinology 140:2117–2124
35. **Muller G, Ertl J, Gerl M, Preibisch G** 1997 Leptin impairs metabolic actions of insulin in isolated rat adipocytes. J Biol Chem 272:10585–10593
36. **Muoio DM, Dohm GL, Tapscott EB, Coleman RA** 1999 Leptin opposes insulin's effects on fatty acid partitioning in muscles isolated from obese *ob/ob* mice. Am J Physiol 276:E913–E921
37. **Ogawa Y, Masuzaki H, Hosoda K, Aizawa-Abe M, Suga J, Suda M, Ebihara K, Iwai H, Matsuoka N, Satoh N, Odaka H, Kasuga H, Fujisawa Y, Inoue G, Nishimura H, Yoshimasa Y, Nakao K** 1999 Increased glucose metabolism and insulin sensitivity in transgenic skinny mice overexpressing leptin. Diabetes 48:1822–1829
38. **Sivitz WI, Walsh SA, Morgan DA, Thomas MJ, Haynes WG** 1997 Effects of leptin on insulin sensitivity in normal rats. Endocrinology 138:3395–3401
39. **Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL** 1999 Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. Nature 401:73–76
40. **Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, Goldstein JL** 2000 Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and *ob/ob* mice. Mol Cell 6:77–86
41. **Berti L, Kellerer M, Capp E, Haring HU** 1997 Leptin stimulates glucose transport and glycogen synthesis in C2C12 myotubes: evidence for a P13-kinase mediated effect. Diabetologia 40:606–609
42. **Chinookoswong N, Wang JL, Shi ZQ** 1999 Leptin restores euglycemia and normalizes glucose turnover in insulin- deficient diabetes in the rat. Diabetes 48:1487–1492
43. **Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ** 1997 Acute stimulation of glucose metabolism in mice by leptin treatment. Nature 389: 374–377
44. **Rouru J, Cusin I, Zakrzewska KE, Jeanrenaud B, Rohner-Jeanrenaud F** 1999 Effects of intravenously infused leptin on insulin sensitivity and on the expression of uncoupling proteins in brown adipose tissue. Endocrinology 140: 3688–3692
45. **Minokoshi Y, Haque MS, Shimazu T** 1999 Microinjection of leptin into the ventromedial hypothalamus increases glucose uptake in peripheral tissues in rats. Diabetes 48:287–291
46. **Qiu J, Ogus S, Lu R, Chehab FF** 2001 Transgenic mice overexpressing leptin accumulate adipose mass at an older, but not younger, age. Endocrinology 142:348–358
47. **Fruhbeck G, Salvador J** 2000 Relation between leptin and the regulation of glucose metabolism. Diabetologia 43:3–12
48. **Ceddia RB, Lopes G, Souza HM, Borba-Murad GR, William William Jr WN, Bazotte RB, Curi R** 1999 Acute effects of leptin on glucose metabolism of *in situ* rat perfused livers and isolated hepatocytes. Int J Obes Relat Metab Disord 23:1207–1212
49. **Cohen SM, Werrmann JG, Tota MR** 1998 13C NMR study of the effects of leptin treatment on kinetics of hepatic intermediary metabolism. Proc Natl Acad Sci USA 95:7385–7390
50. **Cohen B, Novick D, Rubinstein M** 1996 Modulation of insulin activities by leptin. Science 274:1185–1188
51. **Mueckler M** 2001 Insulin resistance and the disruption of Glut4 trafficking in skeletal muscle. J Clin Invest 107:1211–1213