Selective interaction between leptin and insulin signaling pathways in a hepatic cell line

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Leptin is a 16-kDa hormone secreted by adipocytes and plays an important role in control of feeding behavior and energy expenditure. In obesity, circulating levels of leptin and insulin are high because of the presence of increased body fat mass and insulin resistance. Recent reports have suggested that leptin can act through some of the components of the insulin signaling cascade, such as insulin receptor substrates (IRS-1 and IRS-2), phosphatidylinositol 3-kinase (PI 3-kinase), and mitogen-activated protein kinase, and can modify insulin-induced changes in gene expression *in vitro* and *in vivo*. Well differentiated hepatoma cells (Fao) possess both the long and short forms of the leptin receptor and respond to leptin with a stimulation of *c-fos* gene expression. In Fao cells, leptin alone had no effects on the insulin signaling pathway, but leptin pretreatment transiently enhanced insulin-induced tyrosine phosphorylation and PI 3-kinase binding to IRS-1, while producing an inhibition of tyrosine phosphorylation and PI 3-kinase binding to IRS-2. Leptin alone also induced serine phosphorylation of Akt and glycogen synthase kinase 3 but to a lesser extent than insulin, and the combination of these hormones was not additive. These results suggest complex interactions between the leptin and insulin signaling pathways that can potentially lead to differential modification of the metabolic and mitotic effects of insulin exerted through IRS-1 and IRS-2 and the downstream kinases that they activate.

The product of the *ob* gene is leptin, a 16-kDa peptide hormone produced by adipocytes that acts in the hypothalamus and plays a central role in regulation of feeding behavior and energy homeostasis (1–4). The leptin receptor (OB-R) occurs in several isoforms that differ in the length of their intracellular domains because of alternative splicing of the gene (5, 6). The long form is termed OB-Rb or OB-R_L and is expressed abundantly in specific nuclei of the hypothalamus. The short forms, OB-Ra, c, d, and e (collectively referred to OB-R_S), have a wide tissue distribution. The long form of the OB-R belongs to the gp130 family of cytokine receptors that also includes the receptor for IL-6, leukocyte inhibitory factor, and granulocyte colony stimulating factor. These receptors act by activating cytoplasmic tyrosine kinases of the Janus kinase (JAK) family that in return phosphorylate specific transcription factors of the Stat (signal transducer and activator of transcription) family (7–10). On phosphorylation, the Stat proteins dimerize and translocate to the nucleus where they bind to specific nucleotide sequences and induce gene expression. Despite the abundance of the short forms of receptor, little is known about their physiological significance. Cells transfected with the short form of receptor may be capable of activating JAK kinases but fail to phosphorylate Stat proteins or activate gene expression (6, 8).

In vivo and *in vitro* evidence supports the hypothesis that leptin and insulin signaling networks may be connected at several levels. Intravenous infusion of leptin in mice increases glucose turnover, stimulates glucose uptake in skeletal muscle and brown adipose tissue, and causes a decrease in hepatic glycogen content (11). *In vivo* leptin has also been reported to enhance insulin's action to inhibit hepatic glucose output, while antagonizing insulin action on the gene expression for two key metabolic enzymes, glucokinase and phospho*enol*pyruvate carboxykinase (PEPCK; refs. 12 and 13).

Direct cross talk between the leptin and insulin signaling systems in *in vitro* systems remains unclear. In HepG2 human hepatoma cells, leptin antagonizes insulin-induced down-regulation of PEPCK expression and decreases insulin-stimulated tyrosine phosphorylation of IRS-1 but enhances IRS-1-associated phosphatidylinositol 3-kinase (PI 3-kinase) activity (14); in C_2C_{12} cells, leptin stimulates a non-IRS-1-associated PI 3-kinase and mimics insulin action on glucose transport and glycogen synthesis (15). In OB-R_L-transfected HepG2 cells, leptin treatment resulted in the recruitment of p85 to IRS-2 but did not modulate the response to insulin (9). Together, these data point toward cell- and tissue-specific interactions between leptin and insulin signaling that are quite diverse. In the present study, we further characterized leptin effects on insulin action by using well differentiated, highly insulin-responsive Fao hepatoma cells as a model system of liver metabolism. We now report a divergence of leptin effects on insulin-stimulated IRS-1- and IRS-2-mediated signaling and three downstream kinases, suggesting a complex and multidimensional interaction between these two hormonal signaling systems.

Experimental Procedures

Cell Culture. Fao hepatoma cells were maintained in RPMI medium 1640 supplemented with 10% (vol/vol) FBS. Before stimulation, cells were serum starved overnight in RPMI medium 1640 containing 20 mM Hepes and 0.5% insulin-free BSA. Cells were stimulated at 37°C with 100 nM insulin or 60 nM leptin or a combination of the two for the times indicated.

Western Blot Analysis. Cells were lysed in a buffer containing 50 mM Hepes (pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1 mg aprotinin, 1 μg/ml leupeptin, 35 mg/ml PMSF, 10 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, and 4 mM EDTA for 30 min and clarified by centrifugation. Equal amounts of protein were used for immunoprecipitation followed by Western blot analysis with the indicated antibodies and ^{125}I-Sepharose. Quantitative analysis of the blots was performed by using IMAGEQUANT software. The following antibodies were used in these experiments and were obtained from the following sources: OB-R (Santa Cruz Biotechnology, SC-1835), phosphotyrosine (Transduction Laboratories, Lexington, KY, pY20), p85α (Upstate Biotechnology, 06-195), Akt phosphoserine 473-specific (New England Biolabs, 9271) and glycogen synthase kinase 3α (GSK3α)/β-phosphoserine 21- and 9-specific (New England Biolabs, 9331). Anti-

Abbreviations: OB-R, leptin receptor; OB-R_L, long form of OB-R; OB-R_S, short form of OB-R; PEPCK, phospho*enol*pyruvate carboxykinase; PI 3-kinase, phosphatidylinositol 3-kinase; GSK3, glycogen synthase kinase 3; JAK, Janus kinase.

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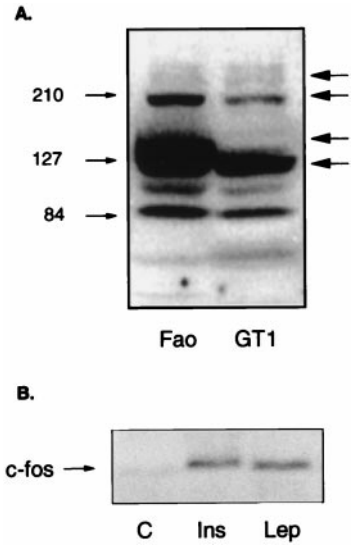

Fig. 1. (*A*) Different isoforms of OB-R are present in Fao cells. Lysates from Fao hepatoma cells and the choroid plexus cell line GT1 were separated on a 7.5% polyacrylamide gel and blotted with antibody raised against amino acids 32–51 in the extracellular domain of the OB-R. Several bands representing both the long and the short isoforms were detected. The long form migrates as a major band just below the 210-kDa marker, whereas a strong band in the 100- to 120-kDa area represents the short forms. The bands migrating at the 60- to 85-kDa range were not analyzed further and represent degradation products. The specificity for antibody recognition of all of these bands was confirmed by a peptide competition assay. (*B*) *c-fos* gene induction by insulin and leptin in Fao hepatoma cells. Fao cells were serum starved overnight and then stimulated with 100 nM insulin (Ins) or 60 nM leptin (Lep) for 30 min (C, control). Total RNA was isolated from control and treated cells, and Northern blotting was performed with *c-fos*-specific probe as described in *Experimental Procedures*. Both leptin and insulin were able to induce *c-fos* expression, proving the signaling capacity of the OB-R in Fao cells.

IRS-1 polyclonal antibody was raised against the C terminus of mouse IRS-1 in rabbits; anti-IRS-2 antibody was raised against peptide mixture of amino acids 618–747 and 976–1,094 of mouse IRS-2.

Northern Blot Analysis. Total RNA was prepared by using the Tri-Reagent kit (Molecular Research Center, Cincinnati). RNA (20 μg) was separated on 1.2% agarose formamide-formaldehyde gels, transferred to nitrocellulose membrane, and immobilized by UV crosslinking. After prehybridization, ^{32}P-labeled *c-fos*-specific probe was added, and the filters were hybridized at 42°C overnight. Stringent washing was performed by using standard protocols, and an autoradiograph was obtained by exposing the filters to x-ray film with an intensifying screen for 48 h.

Results

Expression of OB-R Isoforms in Fao Cells. The presence of the various isoforms of the OB-R in Fao cells was analyzed by Western blotting by using a polyclonal antibody generated against amino acids 32–51 in the extracellular domain of the mouse OB-R. The predicted molecular masses of the long and short forms of the OB-R in the rat are 130 kDa and 101 kDa, respectively. However, stably transfecting Chinese hamster ovary cells with the cDNAs for these forms has indicated receptor heterogeneity and discrepancies between the predicted molecular mass and estimated mass of the receptor isoforms on SDS/PAGE, probably because of glycosylation of the receptors and differences in migration from the theoretical prediction (6, 8). Fao cells appear to express both the long and short isoforms of the OB-R (Fig. 1*A*). In agreement with data obtained by using transfected cells with OB-R_S and OB-R_L separately (6, 8), the long form in these cells migrates as a minor band above the 210-kDa marker and a major band below this marker; the short forms migrate as a broad band in the 110- to 130-kDa molecular mass range. The band at about 140 kDa could represent either the short or long forms of the receptor or a mixture of the two. The specificity of the antibody for all of these bands was established by competition with the OB-R peptide that was used to raise the antibody (data not shown). The bands at ≈60 and 85 kDa were not characterized further but were also specific and may represent the nonglycosylated short form of the OB-R or degradation fragments.

Leptin Induces *c-fos* Gene Expression in Fao Cells. *c-fos* is a member of the family of early growth response genes and can be induced by a number of different ligands including insulin and leptin (16, 17). There are conflicting data concerning the signaling capability of the different OB-R isoforms, but in general, stimulation of *c-fos* expression has been attributed to the signaling capacity of the long form of the receptor and can be demonstrated in PC-12 cells transfected with OB-R_L as well as in the hypothalamus and the jejunum *in vivo* (6, 17, 18). As shown on Fig. 1*B*, in Fao cells, both leptin and insulin can induce *c-fos* gene expression. Thus, Fao cells not only possess peptin receptors but also respond directly to these hormones with changes in gene expression.

Leptin Is Capable of Modifying the Insulin-Induced Tyrosine Phosphorylation of IRS-1 and IRS-2 in Opposite Fashion. Several studies have reported that leptin can modify insulin action in a variety of ways, but the results have been inconsistent in different cell lines (9, 14, 15, 19). Leptin has no effect on glucose metabolism in primary rat adipocytes (20) but stimulates glucose uptake in C_2C_{12} cells (15). Leptin also has varying effects on IRS protein phosphorylation and PI 3-kinase activation (reviewed above). These data point toward differences in the intracellular milieu and substrate availability of the different tissues and cell lines.

The two major immediate substrates of the insulin receptor in Fao cells are IRS-1 and IRS-2 (21). In Fao cells, leptin (60 nM) alone had no effect on IRS-1 or IRS-2 phosphorylation (see below), but pretreatment of these cells with leptin resulted in up to an 80% increase in insulin-induced IRS-1 tyrosine phosphorylation (Fig. 2 *A* and *B*). This effect was transient and was greatest after a 5-min pretreatment with leptin. By contrast, IRS-2 showed an opposite response to leptin pretreatment, with a 40–50% decrease in insulin-stimulated phosphorylation (Fig. 2 *A* and *C*). The onset of the latter effect was delayed compared with its effect on IRS-1 phosphorylation and remained constant up to 20 min after leptin treatment. These effects of leptin on IRS-1 and IRS-2 phosphorylation were also observed at physiological concentrations of the hormone, i.e., 1 or 3 nM (data not shown).

The transient nature of these changes in IRS-1 and IRS-2 phosphorylation could possibly reflect changes in IRS protein concentration or be secondary to changes in serine phosphorylation of these proteins, because both IRS-1 and IRS-2 contain many sites for serine/threonine phosphorylation (21, 22) and because phosphorylation of IRS-1 on serine residues has been shown to impair insulin-induced tyrosine phosphorylation (23). Previous studies have shown that serine phosphorylation of IRS-1 or IRS-2 results in a mobility shift of the IRS proteins on SDS gels (22, 23). After leptin pretreatment, no change in mobility of these proteins was observed (Fig. 2*A*), suggesting that the changes in tyrosine phosphorylation were not likely to be secondary to changes in serine phosphorylation of these proteins. Furthermore, the transient nature of the changes in IRS-1 and IRS-2 was not caused by an effect of leptin on the degradation or immunoprecipitability of the IRS proteins, be-

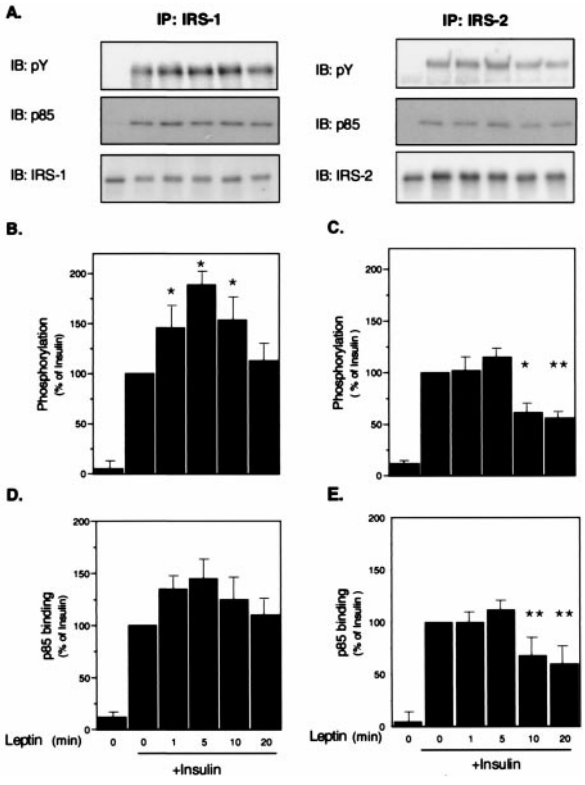

Fig. 2. Effect of leptin on insulin-induced IRS-1 and IRS-2 phosphorylation and p85 association. Fao cells were incubated with 60 nM leptin for the times indicated and then stimulated with 100 nM insulin for 1 min. (*A*) Western blots of anti-IRS-1 and anti-IRS-2 immunoprecipitates (IP) with anti-phosphotyrosine antibody (pY), anti-p85 antibody, or anti-IRS-1 or IRS-2 antibodies (IB, immunoblotting). (*B* and *C*) Quantitation of the blots measuring the phosphorylation of the IRS proteins. (*D* and *E*) Quantitation of the blots for p85 binding associated with the IRS proteins. The data in *B–E* represent the means of three to six independent experiments. Equal loading of proteins was verified by reblotting with the respective IRS antibodies. Statistical significance was analyzed by Student's *t* test: *, $P < 0.05$; **, $P < 0.01$.

cause reblotting with the respective IRS antibody indicated a stable level of proteins (Fig. 2*A*).

Leptin Modifies the Dynamics of Insulin-Induced Tyrosine Phosphorylation of the IRS Proteins. To characterize further the mechanisms underlying the changes in insulin-induced phosphorylation caused by leptin, we compared the kinetics of insulin-induced tyrosine phosphorylation of IRS proteins in the presence or absence of leptin prestimulation for a fixed time. For these studies, we chose 10 min of leptin pretreatment, because this time point allowed us to study the effects on IRS-1 and IRS-2 simultaneously. As noted above, prestimulation of the cells with leptin enhanced insulin-induced IRS-1 phosphorylation. This effect was observed at the earliest time point of insulin stimulation (1 min) but was lost by 10 min (Fig. 3*A*). By contrast, leptin pretreatment decreased the insulin-induced phosphorylation of IRS-2 at both 1 and 10 min (Fig. 3*B*). This difference in time course suggests a difference in the ratio of insulin-induced tyrosine phosphorylation of the two IRS proteins as well as their subsequent dephosphorylation. Also note that in these cells, which express only endogenous levels of leptin receptors, leptin treatment alone did not induce reproducible tyrosine phosphorylation of either of the IRS proteins (Fig. 3 *A* and *B*), nor did it affect tyrosine phosphorylation of the insulin receptor in either the presence or the absence of insulin stimulation (data not shown).

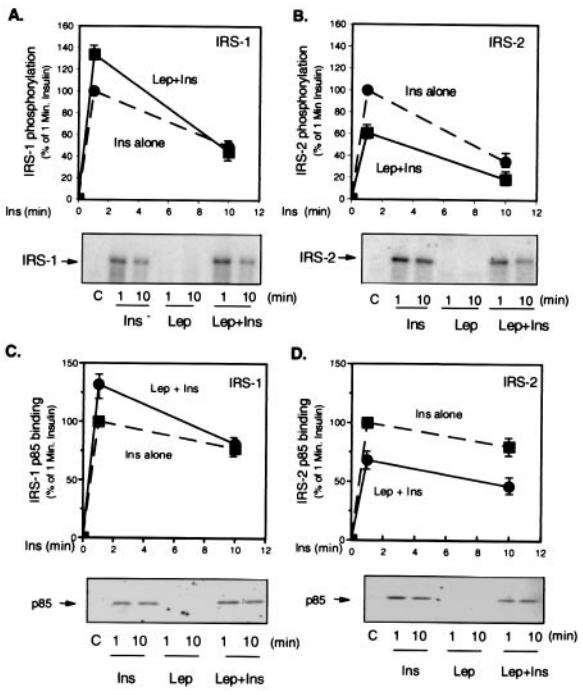

Fig. 3. Leptin has opposite effect on the dynamics of insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2. Cells were serum-starved overnight then treated with or without leptin (Lep; 60 nM) for 10 min, followed by a stimulation with 100 nM insulin (Ins) for 1 or 10 min. IRS protein phosphorylation and p85 binding were assessed as described for Fig. 2. The graphs represent the means of three to eight experiments. A representative blot is shown below each graph.

Leptin Modifies p85 Binding to IRS Proteins. PI 3-kinase plays a central role in insulin's action on glucose transport, glycogen and protein synthesis, as well as PEPCK gene expression (24, 25). PI 3-kinase is activated by binding through its regulator subunit to the phosphorylated IRS proteins. The regulatory subunit occurs in several forms. The major forms are derived from the p85α gene by alternative splicing and include p85α, p50α, and AS53/p55α (26, 27). To assess the effect of the altered IRS protein phosphorylation on the binding of p85α and of its various isoforms to IRS-1 and IRS-2, we performed immunoprecipitation and subsequent immunoblotting with an antibody raised against the N-terminal SH2 domain that is common for all splice variants. In Fao cells, only the full-length p85α isoform was detected. On insulin stimulation, p85α bound to both IRS proteins allowing for activation of the enzyme. Leptin caused a $\approx$40% increase in the insulin-induced association of p85α with IRS-1 (Fig. 2*C*). This increase paralleled the change is IRS-1 tyrosine phosphorylation but was not statistically significant. By comparison, there was a significant decrease in p85α association with IRS-2 (Fig. 2*D*). The time course and extent of decrease in p85 binding corresponded to the diminished insulin-induced tyrosine phosphorylation of IRS-2. Similar changes in p85 binding to IRS-1 and IRS-2 were observed when an insulin time course was performed with a fixed time of leptin pretreatment (Fig. 3 *C* and *D*). These findings support the significance of the divergent effects of leptin on the two different IRS proteins at the level of both tyrosine phosphorylation and signal transduction.

Leptin Induces Serine Phosphorylation of Akt and GSK3 but Does Not Modify Their Respective Insulin-Induced Phosphorylation. To investigate the effects of leptin on insulin signaling downstream of PI 3-kinase, we measured the serine phosphorylation of two major

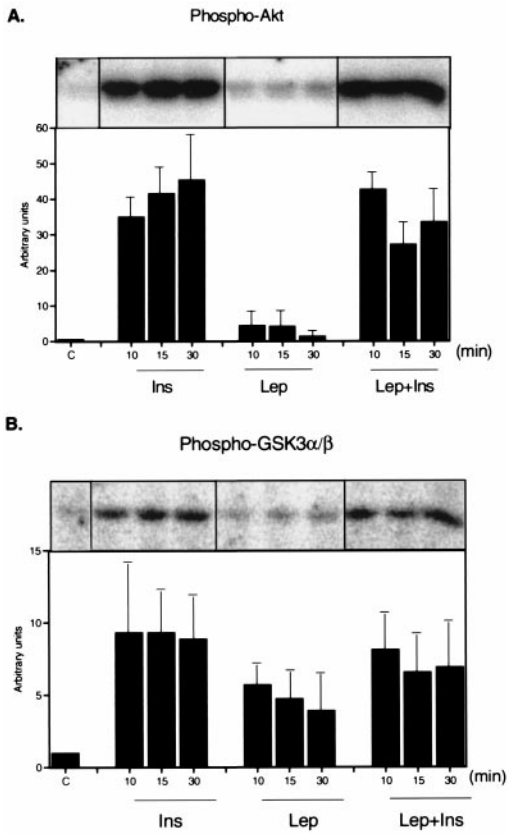

Fig. 4. Leptin (Lep) induces Akt and GSK3 phosphorylation but does not alter insulin-induced phosphorylation of the proteins. Cells were stimulated with 100 nM insulin (Ins), 60 nM leptin, or a combination of the two for the times indicated. Cells were lysed; the proteins were separated on SDS/12% PAGE and blotted with phosphoserine-specific Akt and GSK3 antibodies. Insulin and, to a lesser extent, leptin caused an increase in the phosphorylation of both proteins; the effect of the combination of the two hormones was identical of that of insulin alone. A representative Western blot of each is shown. The bar graphs represent the means of two or three independent experiments.

metabolic intermediate targets, Akt and GSK3. Both of these enzymes play multifunction roles in insulin action, and for both, serine phosphorylation has been shown to correlate with the activity of the enzyme (28–30). In Fao cells, insulin induced a 60- to 80-fold increase in the serine phosphorylation of Akt. Leptin treatment caused a 2- to 8-fold elevation in phospho-Akt but had no effect on insulin-induced phosphorylation of the enzyme (Fig. 4*A*).

GSK3 is a serine kinase that has been implicated to play a role in several divergent pathways of insulin action, because it can phosphorylate glycogen synthase, as well as ATP-citrate lyase, tau, C/EBPα, and heat shock transcription factor 1 (31–33). GSK3 is regulated by phosphorylation on both serine and tyrosine residues, with phosphorylation on tyrosine required for full activity of the enzyme and phosphorylation on serine decreasing its activity (34). Insulin stimulates serine phosphorylation of residues 21 and 9 in GSK3α and GSK3β, respectively, via the serine kinase activity of Akt and thus decreases its activity. In Fao cells, GSK3 is highly tyrosine phosphorylated in basal state, but neither insulin nor leptin induced tyrosine dephosphorylation (data not shown). By contrast, insulin induced a 10- to 15-fold elevation in the serine phosphorylation of GSK3. Leptin alone caused a 4- to 5-fold increase in serine phosphorylation of GSK3 but failed to enhance insulin's effect further (Fig. 4*B*).

Discussion

The discovery of leptin, a hormone produced by adipocytes that acts at the level of the brain to control appetite, has created a seminal change in thinking about the nature of regulation of body mass (1, 2, 4). Several animal models of obesity have shown that obesity can be caused by genetic defects in either leptin or the OB-R (1, 5), and the OB-R system has been shown to interact with other central regulators of energy expenditure and food intake in the control of body fat (2, 3). However, in humans, such mutations are extremely rare, and in general, human obesity is characterized by high circulating levels of leptin (35, 36). Obesity is also accompanied by insulin resistance with high circulating insulin levels and a predisposition to development of type 2 diabetes (37). Thus, in obesity and type 2 diabetes, tissues are often exposed to high concentrations of both of leptin and insulin. Insulin receptors and some forms of the OB-R are present in most tissues of the body, raising the possibility that these two hormonal signaling systems could interact to modify each other's actions in these disease states.

The liver plays a central role in regulating glucose and lipid homeostasis and is one of the major sites of insulin action. In type 2 diabetes with fasting hyperglycemia, insulin resistance is manifest in decreased insulin stimulation of glucose uptake in muscle and fat and, most importantly, uncontrolled hepatic glucose output (24). *In vivo*, leptin has been shown to enhance insulin's ability to inhibit hepatic glucose output, increase gluconeogenesis, and suppress glycogenolysis, as well as to increase PEPCK and decrease glucokinase gene expression (12, 13). Whether these actions of leptin are mediated directly or indirectly, however, remains controversial. For example, a redistribution of hepatic glucose flux occurs after intracerebroventricular injection of leptin, suggesting a central and indirect action of leptin on liver (13). However, peripheral administration of leptin (13) and studies with isolated perfused liver (38) indicate the possibility of a direct action of leptin on this tissue. In addition, leptin effects and cross talk between the insulin and leptin signaling network have been demonstrated *in vitro* by using cultured human hepatoma cells, freshly isolated hepatocytes, and C_2C_{12} muscle cells (9, 14, 15, 19). In the present study, we have further investigated the possibility of direct interactions between insulin and leptin action in liver, focusing on some key intermediate steps in these signaling pathways.

Fao cells are a well differentiated hepatoma cell line that provides a useful model in which to ask this question, because these cells are highly insulin responsive and contain both forms of the OB-R. In addition, Fao cells can mediate both leptin and insulin signaling as demonstrated by the ability of both of these hormones to induce *c-fos* gene expression. Thus, this cell line produces a stable system that allows direct studies of leptin–insulin signaling interaction without the need to transfect in high, nonphysiological levels of receptor. This cell line also provides a model for addressing the signaling capacity of the OB-R in peripheral tissues where the short forms of receptor are much more abundant than the long form. Indeed, there remains considerable debate as to the signaling capacity of the short forms of the receptor, but these short forms clearly may compete for leptin binding and possibly also compete for some of the immediate signaling molecules, such as the JAK kinases, nevertheless permitting signaling by the long form of OB-R (10).

The first intracellular step in insulin signaling is phosphorylation of the two major substrate proteins of the receptor, IRS-1 and IRS-2 (21, 24, 25). These proteins share a high degree of sequence homology but do have differences in specific phosphorylation sites, in kinetics of phosphorylation, and in subcellular localization that suggest distinct roles in the postreceptor actions of insulin (39). Indeed, disruption of the IRS-1 gene in mice led to growth retardation and insulin resistance; IRS-2

knockout mice have normal growth but also have insulin resistance and develop overt diabetes because of retarded β-cell development (40, 41). In cells from knockout mice, IRS-1 and IRS-2 also have some differences in their ability to support DNA synthesis versus intermediate metabolic enzymes (42). The IRS proteins can also serve as substrates for some cytokine receptors (21), and in cells transfected with high levels of the OB-R and JAK kinase, leptin can also stimulate IRS protein phosphorylation (6).

In Fao cells, leptin alone has no effect on IRS-1 or IRS-2 phosphorylation but does produce significant and differential effects on the insulin-induced phosphorylation of these two major insulin receptor substrates. Thus, leptin induces a very transient increase in insulin-stimulated IRS-1 tyrosine phosphorylation, while producing a decrease in insulin-induced tyrosine phosphorylation of IRS-2 that persists for at least the first 20 min of hormone stimulation. The mechanisms underlying these acute alterations by leptin of insulin-induced phosphorylation are not yet completely clear. These effects are not the result of any modification of insulin receptor autophosphorylation or IRS protein concentration. Tyrosine phosphorylation of IRS proteins has been shown to modulate after changes in serine phosphorylation of the proteins (22, 23); however, this possibility also seems unlikely, because serine phosphorylation usually results in decreased mobility of IRS proteins on SDS/PAGE and such decreased mobility did not occur after leptin prestimulation. The SH2 domain containing phosphatase SHP-2 has been implicated in leptin's action as well as in the dephosphorylation of IRS proteins (43–45); however, no changes in IRS-1- or IRS-2-associated SHP-2 could be identified after leptin treatment (data not shown). It is possible that leptin modifies the intracellular trafficking of the IRS proteins, changing the rates of phosphorylation and dephosphorylation (39). Further studies will be needed to address this question.

Leptin has been shown to have varying effects on PI 3-kinase activity. Acute intravenous infusion of leptin (46) and high fat feeding (47), which increase leptin levels, have been shown to increase IRS-1-associated PI 3-kinase in liver *in vivo*, whereas leptin treatment of C_2C_{12} cells *in vitro* increases IRS-2-associated PI 3-kinase (12). In Fao cells, the leptin-induced alterations in the insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 resulted in corresponding and divergent changes in the association of the p85 subunit of the PI 3-kinase with these substrates. Thus, insulin-induced association of the p85 with IRS-1 was increased, whereas that with IRS-2 was significantly diminished after leptin preincubation, indicating that leptin pretreatment can differentially modify insulin signaling through these two substrates. Previous studies have suggested that IRS-1 and IRS-2 are differently involved in mediating insulin's mitotic and metabolic actions (39, 41, 42). An increase in p85 association with IRS-1 and decrease in association with IRS-2 could change the balance or nature of insulin signaling. The differential effects on IRS-1 and IRS-2, as well as differences in time course of these effects, could contribute to some of the divergence in observations in the literature. *Ob/ob* mice, with obesity secondary to a genetic deficiency of leptin, show a decrease in IRS-1-associated and, to an even greater extent, IRS-2-associated PI 3-kinase as well as alterations in the alternatively spliced forms of the regulatory subunit of PI 3-kinase (27). Thus, differential effects in these early steps in insulin signaling can also be observed in obese states without the effect of leptin.

PI 3-kinase activation is central to most of insulin's metabolic effects, including stimulation of glucose transport, lipogenesis, glycogen synthesis, inhibition of gluconeogenesis by attenuation of PEPCK gene expression, promotion of protein synthesis, and protection against apoptosis (24, 25). At a molecular level, PI 3-kinase mediates these events by an activation of two kinases—PDK1 that phosphorylates Akt on Thr-308 and a putative PDK2 that phosphorylates Akt on Ser-473—leading to an increase in Akt kinase activity (29). With regard to glycogen synthesis, Akt phosphorylates GSK3α and GSK3β on serine residues 21 and 9, respectively. This phosphorylation leads to a deactivation of GSK3, reducing phosphorylation and thus enhancing the activity of glycogen synthase (48). In Fao cells, both Akt and GSK3 have enhanced serine phosphorylation after insulin and, to a lesser extent, leptin stimulation. This enhanced serine phosphorylation would result in an increase in Akt activity and a decrease in GSK3 activity. Treatment with the combination of the two hormones is not additive, synergistic, or inhibitory. GSK3 is tyrosine phosphorylated in the basal state on Tyr-216 and requires this phosphorylation for full activation (34, 49). In Fao cells, GSK3 is highly tyrosine phosphorylated; however, neither insulin nor leptin changes the level tyrosine phosphorylation (data not shown). Thus, the regulation of GSK3 in Fao cells, similar to that in muscle, occurs mainly via serine phosphorylation (49).

The impact of the changes in Akt and GSK3 phosphorylation and activity could be multiple. Using [^{13}C]NMR, Cohen *et al.* (50) have shown that leptin can acutely enhance hepatic glycogen synthesis *in vivo*, and leptin has been shown to enhance glycogen synthesis in isolated hepatocytes (51). This latter effect, however, seems to occur via an inhibition of phosphorylase rather than an increase in synthase activity (19). Acute infusions of leptin in rats have also been shown to enhance insulin inhibition of hepatic glucose production, increase hepatic PEPCK mRNA, and decrease abundance of the glucokinase message (12). In perfused liver, leptin can mimic insulin action to suppress epinephrine-induced glucose release, while stimulating lactate-induced gluconeogenesis (38). In addition to its effects on glycogen synthase, GSK3 is involved in a great variety of cellular responses, including phosphorylation of ATP-citrate lyase and the transcription factors C/EBPα and heat shock transcription factor 1 (31–33, 52). Alterations in the activity of these molecules would raise the possibility that leptin can differentially modify hepatic function by altering lipid metabolism or changing patterns of gene expression mediated through C/EBPα, heat shock transcription factor 1, or other transcription factors.

In summary, our results show a unique differential interaction between the leptin and insulin signaling networks. This cross talk may contribute to some of the alterations in the metabolic and mitotic effects of insulin action that are involved in the development of insulin resistance characteristic of type 2 diabetes.

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