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# Interaction between Leptin and Insulin Signaling Pathways Differentially Affects JAK-STAT and PI 3-Kinase-Mediated Signaling in Rat Liver

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**Chronic leptin treatment markedly enhances the effect of insulin on hepatic glucose production unproportionally with respect to body weight loss and increased insulin sensitivity. In the present study the cross-talk between insulin and leptin was evaluated in rat liver. Upon stimulation of JAK2 tyrosine phosphorylation, leptin induced JAK2 co-immunoprecipitation with STAT3, STAT5b, IRS-1 and IRS-2. This phenomenon parallels the leptin-induced tyrosine phosphorylation of STAT3, STAT5b, IRS-1 and IRS-2. Acutely injected insulin stimulated a mild increase in tyrosine phosphorylation of JAK2, STAT3 and STAT5b. Leptin was less effective than insulin in stimulating IRS phosphorylation and their association with PI 3-kinase. Simultaneous treatment with both hormones yielded no change in maximal phosphorylation of STAT3, IRS-1, IRS-2 and Akt, but led to a marked increase in tyrosine phosphorylation of JAK2 and STAT5b when compared with isolated administration of insulin or leptin. This indicates that there is a positive cross-talk between insulin and leptin signaling pathways at the level of JAK2 and STAT5b in rat liver.**

*Key words:* DNA-binding proteins / Drug effects / Insulin physiology / Leptin pharmacology / Liver / Metabolism / Protein-tyrosine kinase / Signal transduction.

## Introduction

Leptin, the protein encoded by the *ob* gene, is a hormone that is produced by adipocytes and acts through distinct receptors in target organs to control food intake and energy metabolism (Tartaglia *et al.,* 1995; Zhang *et al.,* 1994). Insulin resistance characterizes states of severe leptin deficiency or resistance, like in *ob*/*ob* or *db*/*db* mice, or genetic models of lipoatrophic diabetes. In some of these, administration of exogenous leptin improves glucose tolerance and insulin sensitivity independently of effects on food intake, probably by affecting neuroendocrine pathways that modulate insulin action in liver and muscle (Halaas *et al.,* 1995; Shimomura *et al.,* 1999), as this cytokine might also have direct effects on hepatic cells (Lee *et al.,* 2001). Furthermore, recent evidence indicates that leptin treatment in insulin-deficient diabetic rats restores normoglycemia (Chinookoswong *et al.,* 1999), and transgenic mice overexpressing leptin show increased insulin sensitivity (Ogawa *et al.,* 1999). Taken together, these observations suggest a potent antidiabetogenic effect of leptin *in vivo*.

Direct cross-talk between the leptin and insulin signaling systems remains unclear. Some data suggest that leptin can impair the early steps of insulin signaling including tyrosine phosphorylation of IRS-1 in hepatocytes (Cohen *et al.,* 1996). Other studies demonstrate that leptin can mimic effects of insulin, such as stimulation of glucose transport and glycogen synthesis in C2C12 myotubes, and that these effects may be mediated by stimulation of PI 3-kinase (Berti *et al.,* 1997; Kellerer *et al.,* 1997). In isolated muscle or adipocytes, short-term incubation with leptin does not stimulate glucose transport or lipogenesis (Ranganathan *et al.,* 1998; Zierath *et al.,* 1998).

Leptin exerts its effects through specific receptors of which five isoforms have been described, generated by alternative splicing of the primary transcript (Lee *et al.,* 1996). The leptin receptor is a member of the cytokine I receptor family, of which gp 130 is a prototype. This class of receptors stimulates gene transcription *via* activation of cytosolic STAT proteins (Darnell, 1996, 1997). The long form of the leptin receptor (OBRb) has the capacity to activate the JAK/STAT (Baumann *et al.,* 1996; Ghilardi *et al.,* 1996; Vaisse *et al.,* 1996; Ghilardi and Skoda, 1997; Tartaglia, 1997; White *et al.,* 1997) and MAPK (Bjorbaek *et al.,* 1997) pathways, stimulate tyrosine phosphorylation of IRS-1 (Bjorbaek *et al.,* 1997), and increase transcription of *fos*, *jun* (Bjorbaek *et al.,* 1997; Murakami *et al.,* 1997). OBRb is highly expressed in the hypothalamus, the primary site where leptin is thought to act (Tartaglia *et al.,* 1995). Leptin has been shown to activate STAT3 in the hypothalamus *via* JAK2 (Carvalheira *et al.,* 2001). Tyrosine-phosphorylated STAT is translocated to the nucleus where it is thought to bind to specific DNA sequences and activate genes important for energy homeostasis. Leptin may also exert direct effects at the level of gene expression or cellular function on nonhypo-

thalamic target tissues including hematopoietic cells, T cells, the endocrine pancreas, the pituitary, the ovary, adipocytes, skeletal muscle, and hepatocytes either through the short or long forms of the receptor (Bennett *et al.,* 1996; Emilsson *et al.,* 1997; Shimabukuro *et al.,* 1997; Siegrist-Kaiser *et al.,* 1997; Yu *et al.,* 1997; Cusin *et al.,* 1998; Liu *et al.,* 1998; Lord *et al.,* 1998;).

It is not yet completely clear whether, and by what mechanisms, parts of the important metabolic effects of leptin could be exerted directly at the level of peripheral tissues, as opposed to indirect action through the central nervous system. In the present study we have characterized leptin signal transduction in liver and determined whether insulin and leptin share common intracellular signal transduction pathways.

## Results

### *In vivo* Effect of Leptin on Tyrosine Phosphorylation in the JAK/STAT Pathway in Liver

To determine leptin-induced tyrosine phosphorylation of JAK2 we performed immunoprecipitation and Western blotting of liver extracts with anti-JAK2 and anti-phosphotyrosine antibodies, respectively. The time course experiments were performed injecting $10^{-6}$ M of leptin through the portal vein of rats and collecting liver specimens at different time points. As shown in Figure 1, JAK2 is strongly phosphorylated after 30 seconds of leptin treatment, with maximal phosphorylation occurring at 90 seconds and decaying after 3 minutes (Figure 1A).

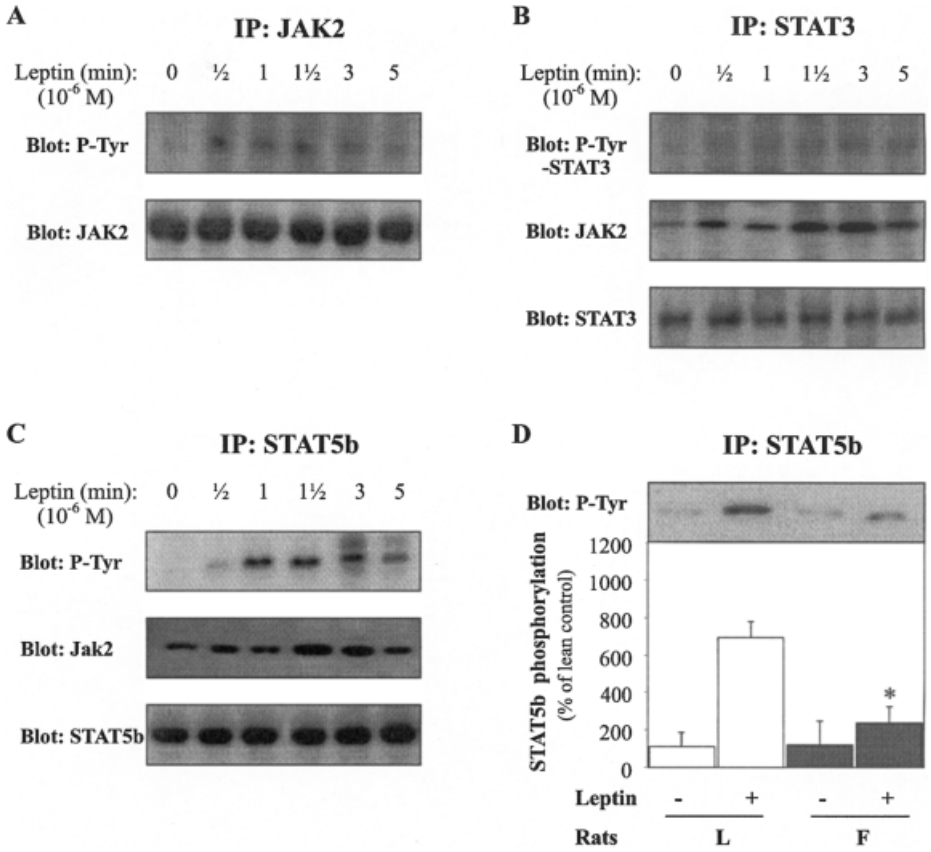


**Fig. 1** Effect of Leptin on JAK2, STAT3 and STAT5b Tyrosine Phosphorylation and STAT3/5b Association with JAK2 in the Liver *in vivo*. (A) Liver extracts from animals treated with leptin for the time points indicated were prepared as described in Materials and Methods. Proteins were immunoprecipitated from the extracts with anti-JAK2 antibody (IP, immunoprecipitation) and immunoblotted with anti-phosphotyrosine antibody (P-Tyr). Stripped membranes were reblotted with anti-JAK2 antibody ($n = 6$). (B) Leptin-stimulated tyrosine phosphorylation of STAT3. Tissue extracts from rats treated with leptin for the time points indicated were immunoprecipitated with anti-STAT3 and blotted with anti-phospho-specific STAT3 (P-Tyr-STAT3). The same membranes used for STAT3 tyrosine phosphorylation were stripped and reblotted with anti-JAK2 and anti-STAT3 antibodies ($n = 6$). (C) Leptin-stimulated tyrosine phosphorylation of STAT5b. Tissue extracts from rats treated with leptin for the time points indicated were immunoprecipitated with anti-STAT5b and blotted with anti-phosphotyrosine antibody. The same membranes used for STAT5 b tyrosine phosphorylation were stripped and reblotted with anti-JAK2 and anti-STAT5b antibodies ($n = 6$). (D) Liver extracts from lean (L) and fat (F) rats treated with leptin for 90 seconds were prepared as described in Materials and Methods. Tissue extracts were immunoprecipitated with anti-STAT5b and immunoblotted with anti-phosphotyrosine antibody. The bar diagram represents the quantitative tyrosine phosphorylation of STAT5b. Data (mean ± SEM, $n = 4$) are expressed relative to control, assigning a value of 100% to the lean control before leptin stimulation. $^*p < 0.01$, leptin lean *vs*. leptin obese.

To assess the ability of leptin to activate tyrosine phosphorylation of STAT3 and STAT5b we determined the time course for this activation. Initially, we measured leptin-induced tyrosine phosphorylation of STAT3 by immunoblotting with specific phospho-STAT3 antibodies. Figure 1B shows a moderate increase in leptin-stimulated STAT3 phosphorylation in rat liver which is maximal at 3 min. Co-immunoprecipitation between JAK2 and STAT3 in liver was observed in a similar fashion of JAK2 tyrosine phosphorylation. Subsequently, we measured leptin-induced tyrosine phosphorylation of STAT5b, by immunoprecipitation experiments using specific STAT5b antisera, and then the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. There was a time-dependent increase in STAT5b phosphorylation after leptin injection, which paralleled the increase of JAK2 phosphorylation after leptin stimulation (Figure 1C). Similar to STAT3, there was also co-immunoprecipitation of STAT5b and JAK2 after leptin stimulation. As expected, there was no alteration in STAT5b protein expression after leptin infusion.

Our results show a very rapid, and presumably direct induction of STAT5b phosphorylation by leptin in liver. To confirm these data we measured leptin-induced STAT5b tyrosine phosphorylation in the liver of *fa/fa* rats, which have a mutation that decreases the cell-surface expression and leptin affinity of the leptin receptor, as a negative control (White *et al.,* 1997; da Silva *et al.,* 1998). In *in vivo* experiments, there was an increase of 6.9-fold in leptin-induced tyrosine phosphorylation of STAT5b in the liver of lean rats (Figure 1D), compared with 2.4-fold increases in the liver of obese rats ($p<0.05$), representing a decrease of about 70% in STAT5b phosphorylation in obese rats.

### Effects of Leptin on IRS-1 and IRS-2 Tyrosine Phosphorylation, PI 3 Kinase Docking and Akt Serine Phosphorylation in Rat Liver

IRS proteins represent an important multifunctional interface between many receptors and intracellular pathways. A possible pathway toward IRS proteins phosphorylation is *via* JAK kinases (Berti *et al.,* 1997; Bjorbaek *et al.,* 1997;

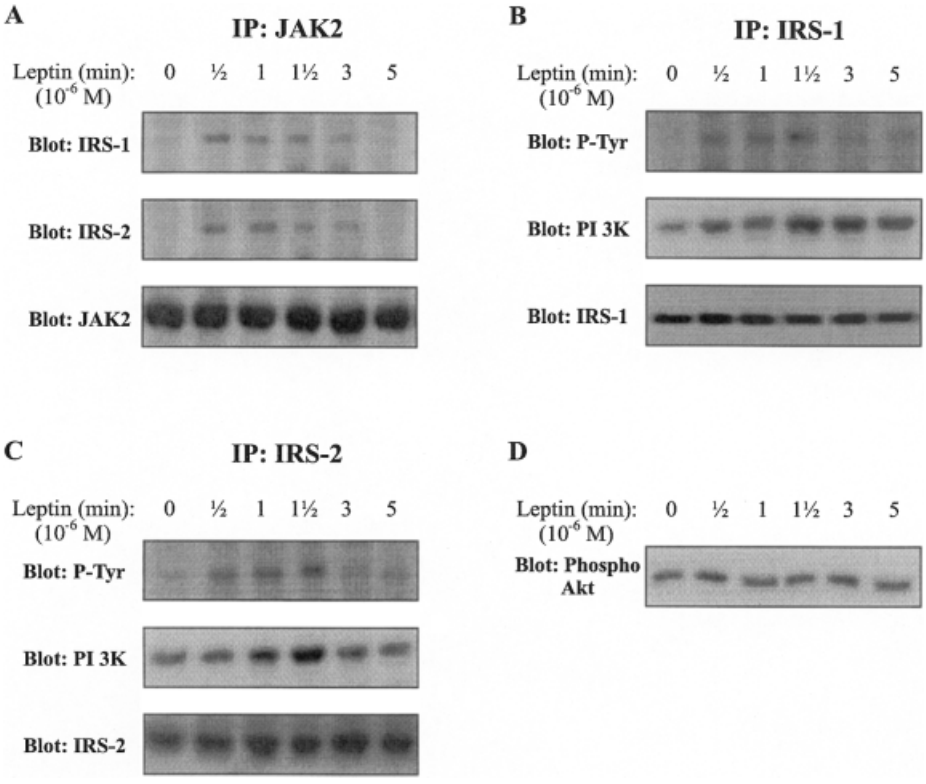


**Fig. 2** Leptin-Stimulated Tyrosine Phosphorylation of IRS-1 and IRS-2 and Their Association with the p85 Subunit of PI 3-Kinase in Liver *in vivo*.

(A) Liver extracts from animals treated with leptin for the time points indicated were prepared as described in Materials and Methods. Tissue extracts were immunoprecipitated with anti-JAK2 antibody (IP, immunoprecipitation) and immunoblotted with anti-IRS-1 and anti IRS-2. Stripped membranes were reblotted with anti-JAK2 antibody ($n = 4$). (B) Leptin-stimulated tyrosine phosphorylation of IRS-1. Tissue extracts from rats treated with leptin for the time points indicated were immunoprecipitated with anti-IRS-1 and blotted with anti-phosphotyrosine antibody (P-Tyr). The same membranes used for IRS-1 tyrosine phosphorylation were stripped and reblotted with anti-PI 3-kinase and anti IRS-1 antibodies ($n = 6$). (C) Leptin-stimulated tyrosine phosphorylation of IRS-2. Tissue extracts from rats treated with leptin for the time points indicated were immunoprecipitated with anti-IRS-2 and blotted with anti-phosphotyrosine antibody. The same membranes used for IRS-2 tyrosine phosphorylation were stripped and reblotted with anti-PI 3-kinase and anti IRS-2 antibodies ($n = 6$). (D) Leptin-stimulated serine phosphorylation of Akt. Liver extracts from rats that were stimulated with leptin for the time point indicated were prepared and proteins were separated by SDS-PAGE on 12% gels and blotted with phosphoserine-specific Akt antibodies.

Kellerer *et al.,* 1997; Carvalheira *et al.,* 2001). Since JAK2 is activated by leptin, we evaluated the ability of leptin to stimulate JAK2/IRS-1 and JAK2/IRS-2 association. Co-immunoprecipitation of JAK2 and IRS-1 or IRS-2 in liver was detected. In immunoprecipitates of JAK2 that were blotted with anti-IRS-1 or anti IRS-2 antibodies, there was an evident association between these proteins after leptin stimulation. These results demonstrate that JAK2 interacts with IRS-1 and IRS-2 by forming stable complexes in rat liver (Figure 2A).

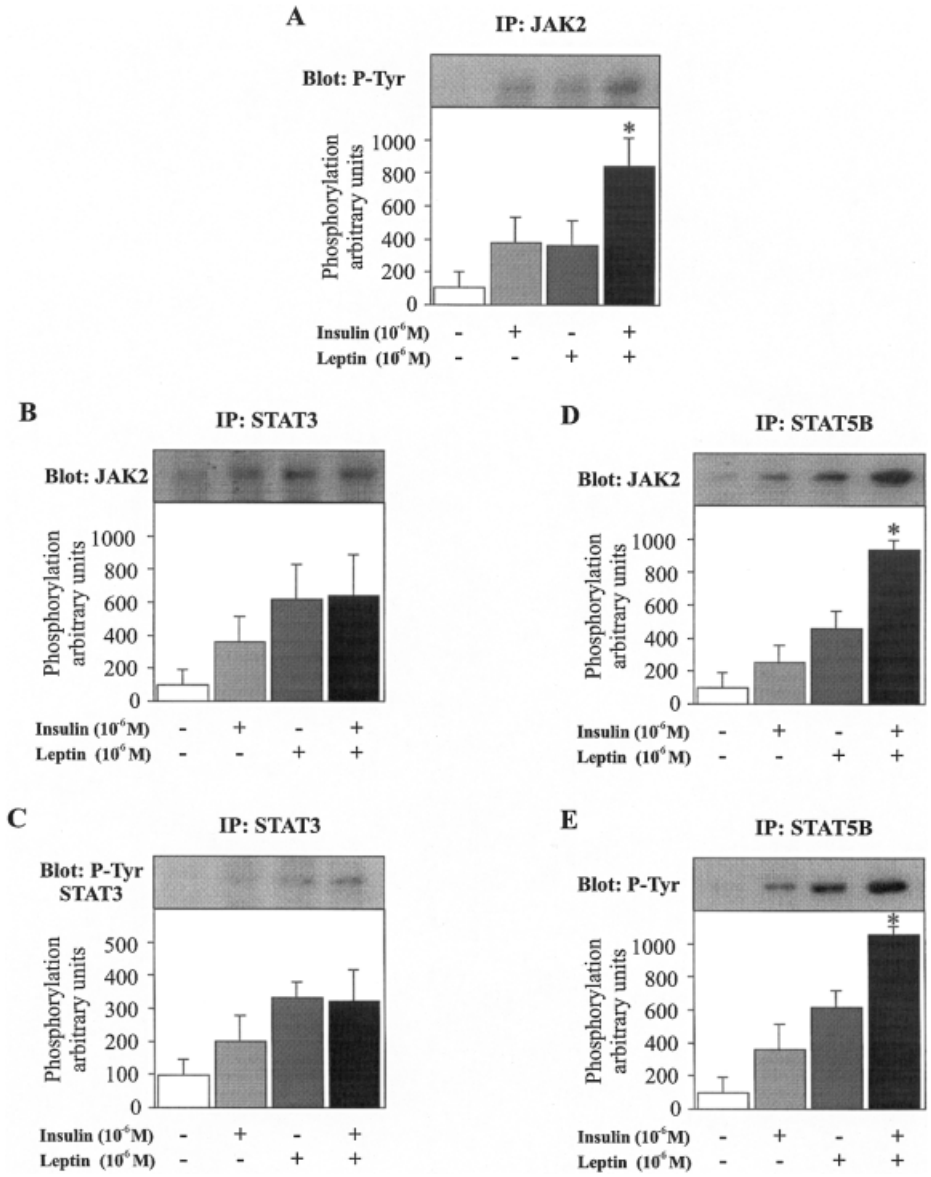


**Fig. 3** Effect of Insulin and Leptin on the JAK/STAT Pathway in Extracts of Livers from Rats Treated with Leptin, Insulin or a Combination of Both.

(A) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-JAK2 and blotted with anti-phosphotyrosine antibody. The bar diagram shows the quantitative phosphorylation of JAK2. Data (mean ± SEM, $n = 6$) are expressed as relative to control, assigning a value of 100% to the control mean. (B) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-STAT3 and blotted with anti-JAK2 antibody. The bar diagram shows the quantitative association of STAT3 with JAK2. Data (mean ± SEM, $n = 6$) are expressed as relative to control, assigning a value of 100% to the control mean. (C) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated (IP, immunoprecipitation) with anti-STAT3 antobodies and blotted with anti-phospho-specific STAT3 (P-Tyr-STAT3). The bar graph shows the quantitative phosphorylation of STAT3. Data (mean ± SEM, $n = 6$) are expressed as relative to control, assigning a value of 100% to the control mean. (D) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-STAT5b and blotted with anti-JAK2 antibody. The diagram shows the quantitative association of STAT5b with JAK2. Data (mean ± SEM, $n = 6$) are expressed relative to control, assigning a value of 100% to the control mean. (E) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-STAT5b and blotted with anti-phosphotyrosine antibody (P-Tyr). The diagram shows the quantitative phosphorylation of STAT5b. Data (mean ± SEM, $n = 6$) are expressed as relative to control, assigning a value of 100% to the control mean. *$p < 0.05$ *vs*. leptin-treated rats.

Figure 2B shows a clear increase in leptin-stimulated IRS-1 phosphorylation, which was maximal at 90 s and almost vanished completely after 3 minutes. To better define the levels of IRS-2 phosphorylation, we performed Western blot analysis of tyrosyl-phosphorylated proteins in anti-IRS-2 immunoprecipitates before and after stimulation with leptin. Figure 2C shows that there was a marked increase in leptin-stimulated IRS-2 phosphorylation in rat liver, which was maximal at 90 s followed by a striking decay.

Previous studies (Backer *et al.,* 1992; Folli *et al.,* 1992) have suggested that there is a relatively stable, high affinity interaction between IRS-1 or IRS-2 and the 85 kDa subunit of the PI 3-kinase such that both proteins can be co-precipitated by antibodies to either protein. Blots that had been previously immunoprecipitated with antibodies against IRS-1 or IRS-2 were subsequently incubated with antibodies against the 85 kDa subunit of PI 3-kinase (Figure 2B and C). There was little PI 3-kinase immunoreactivity in the basal state of control rats. After 30 s of stimulation with leptin, there were significant increases in IRS-1 or IRS-2/PI 3-kinase association, which slightly decreased thereafter and returned to basal levels after 3 minutes. These observations are consistent with the formation of a stable association between IRS-1 or IRS-2 and PI 3-kinase after leptin stimulation. As expected, there were no changes in IRS-1 and IRS-2 protein levels.

Using antibodies against serine-phosphorylated Akt, the levels of Akt activation were examined in liver after leptin stimulation. As shown in Figure 2D, Akt phosphorylation in liver was unaltered by leptin injection.

### The Effect of Simultaneous Administration of Leptin and Insulin on the JAK/STAT Pathway

First we measured tyrosine phosphorylation of JAK2 in the liver from rats that were stimulated with insulin, leptin or both hormones for 90 s. The phosphorylation of JAK2 in liver was increased approximately 3.8-fold by insulin stimulation and approx, 3.6-fold by leptin. Insulin and leptin together showed ~8.4-fold increase in JAK2 tyrosine phosphorylation, representing increases of ~230% above the effects of leptin alone (Figure 3A). In a second experiment, we evaluated insulin- and/or leptin-stimulated JAK2/STAT3 association and STAT3 tyrosine phosphorylation in the liver from rats that were stimulated with insulin, leptin or both hormones for 3 min. Insulin-stimulated STAT3 association with JAK2 showed an increase of ~3.5-fold and STAT3 phosphorylation 2.0-fold over the basal levels, while leptin led to a ~6.2- and 3.3-fold increase in JAK2/STAT3 association and STAT3 tyrosine phosphorylation, respectively. However, simultaneous stimulation did not induce additive effects on JAK2/STAT3 association and STAT3 tyrosine phosphorylation (Figure 3B and C). Finally, we assessed the association between JAK2 and STAT5b and STAT5b tyrosine phosphorylation in the liver from rats that were stimulated with insulin, leptin or both hormones for 3 minutes. Insulin led to a ~2.5 fold increase in JAK2/STAT5b association and to a 4.6-fold in tyrosine phosphorylation of STAT5b over the basal levels, whereas leptin induced a ~4.6-fold increase in JAK2/STAT5b association and a 6.1-fold increase in STAT5b tyrosine phosphorylation. Insulin and leptin together showed a ~9.3-fold increase in JAK2/STAT5b association and ~10-fold increase in STAT5b tyrosine phosphorylation, representing enhancements of ~200% and ~170%, respectively, above the effects of leptin alone (Figure 3D and E).

### The Effect of Simultaneous Administration of Leptin and Insulin on the PI 3-Kinase/Akt Pathway

We next examined whether simultaneous stimulation with leptin and insulin could affect the early steps of the

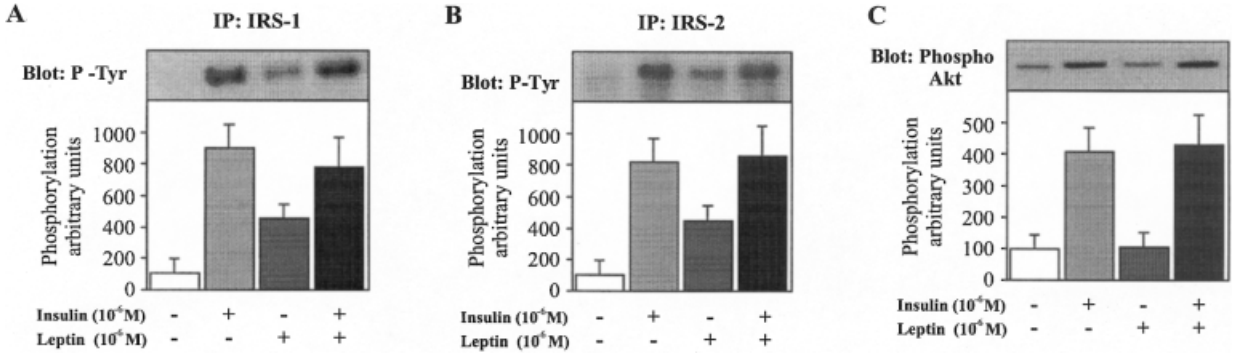


**Fig. 4** Effect of Insulin and Leptin on IRS-1, IRS-2 and Akt Phosphorylation in Extracts of Livers from Rats Treated with Leptin, Insulin or a Combination of Both.
(A) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-IRS-1 and blotted with anti-phosphotyrosine antibody. The bar diagram shows the quantitative phosphorylation of IRS-1. Data (mean ± SEM, $n = 6$) are expressed as relative to control, assigning a value of 100% to the control mean. (B) Tissue extracts from rats treated with saline, leptin, insulin or both hormones were immunoprecipitated with anti-IRS-2 and blotted with anti-phosphotyrosine antibody. Shown is the quantitative phosphorylation of IRS-2. Data (mean ± SEM, $n = 6$) are expressed as relative to control, assigning a value of 100% to the control mean. (C) Western blots of liver extracts from rats treated with leptin for 5 minutes with phosphoserine-specific Akt antibodies. The bar diagram shows the quantitative phosphorylation of Akt. Data (mean ± SEM, $n = 6$) are expressed as relative to control, assigning a value of 100% to the control mean.

insulin signal transduction pathways. We measured tyrosine phosphorylation of IRS proteins in the liver from rats that were stimulated with insulin, leptin or both hormones for 90 s. The phosphorylation of IRS-1 in liver showed an approx. 9.0-fold increase with insulin stimulation and approx. 3.3-fold with leptin. The effect of insulin and leptin was similar to insulin alone (Figure 4A). IRS-2 tyrosine phosphorylation upon insulin and/or leptin stimulation was comparable with that obtained in IRS-1 immunoprecipitates (Figure 4B). Akt serine phosphorylation was examined in the liver from rats that were stimulated with insulin, leptin or both hormones for 5 min. Leptin alone had no effect on Akt tyrosine phosphorylation in liver. Insulin induced a ~4-fold increase in Akt phosphorylation and no additive effect was observed by the addition of leptin (Figure 4C).

## Discussion

In the present study we have investigated the possibility of direct interactions between insulin and leptin action in liver, focusing on some key intermediate steps in these signaling pathways. Our results show that leptin, upon stimulation of JAK2 tyrosine phosphorylation, induced JAK2 co-immunoprecipitation with STAT3, STAT5b, IRS-1 and IRS-2. These phenomena paralleled the leptin-induced tyrosine phosphorylation of STAT3, STAT5b, IRS-1 and IRS-2. Insulin stimulated a mild increase in tyrosine phosphorylation of JAK2, STAT3 and STAT5b in liver. Leptin was much less effective than insulin at stimulating IRS/PI 3-kinase pathways. Akt phosphorylation was not changed by leptin but increased by insulin. Whereas there was no change in maximal phosphorylation of IRS-1, IRS-2, Akt and STAT3, simultaneous stimulation with both hormones resulted in a marked increase in tyrosine phosphorylation of JAK2 and STAT5b when compared with isolated administration of insulin or leptin. Thus, there is direct cross-talk between the insulin and leptin signaling pathways at the level of JAK2 and STAT5b, in a way that the simultaneous administration of both hormones modulates the signal that is transduced through the common elements of these pathways.

The molecular mechanisms by which OBR couples to tyrosine phosphorylation events in liver are not known. In the present study the rapid tyrosine phosphorylation and association of JAK2 with IRS-1, IRS-2, STAT3 and STAT-5b proteins suggests that a large signaling complex is formed with the OBR upon leptin treatment. Co-immunoprecipitation of JAK2 and IRSs, STAT3 and -5b could be due the direct association of JAK2, IRSs, STAT3 and STAT5b with the OBR, or indirect association of IRSs, STAT3 and -5b with the JAK2 kinase. There are several mechanisms by which JAK2, IRSs and STATs may associate with OBR. One possibility is that JAK2 initially associates with the OBR and leads to recruitment of IRSs, STAT3 and -5b proteins. A second is that OBR recruit IRSs and/or STAT proteins, which serve as adapter molecules for binding JAK2. A third possibility is that JAK2, IRSs and STATs associate with the receptor, and upon ligand binding to the receptor, JAK2 phosphorylates the associated IRSs and STAT proteins. Further studies (mutational analysis of the OBR and/or experiments in JAK2 deficient cell lines) will be required to assess this issue.

A previous *in vivo* study implicated only STAT3 in hypothalamic leptin signaling, whereas experiments involving transfected cells provide evidence for the activation of other STAT isoforms, depending on the cellular model (Baumann *et al.,* 1996; Ghilardi *et al.,* 1996; Vaisse *et al.,* 1996; White *et al.,* 1997). The nature of the STAT isoforms required for transducing the leptin signal is still controversial and may depend on the cellular context and the concomitant presence of other stimuli *in vivo*. Here we show that not only tyrosine phosphorylation of STAT3, but also STAT5b, is markedly activated in hepatic tissue by leptin administration. These results are in accordance with a recent study showing that leptin administration i.v. induces a STAT5b gel shift in nuclear extracts of hepatocytes (Briscoe *et al.,* 2001). The current study is the first demonstration of leptin-induced STAT5b activation in liver *in vivo*.

Originally it was thought that the hypothalamus was the only tissue expressing OBRb. However, recent evidence at the level of messenger RNA expression and cellular function suggest that peripheral organs including adipose tissue also express OBRb (Cohen *et al.,* 1996; Emilsson *et al.,* 1997; Siegrist-Kaiser *et al.,* 1997; Yu *et al.,* 1997). Unlike OBRb, the short isoform of the leptin receptor (OBRa) appears incapable of activating STATs, although it can mediate tyrosine phosphorylation of JAK2 (Bjorbaek *et al.,* 1997). Several studies have analyzed alteration in leptin signaling induced by the *fa* mutation in OBR, demonstrating a defective activation of STAT5b by the mutant receptor (White *et al.,* 1997). Thus, our data showing leptin-induced STAT5b phosphorylation in liver of lean rats, but a mild STAT5b phosphorylation in liver of Zucker *fa/fa* rats, support the notion that hepatocytes express OBRb as suggested previously (Kim *et al.,* 2000).

Intracellular interactions between different signaling systems may function as mechanisms for enhancing or counter-regulating hormone action. In the case of insulin, the cross-talk with leptin-mediated pathways results in direct interactions between insulin and leptin signaling systems at the level of JAK2 and STAT5b. Simultaneous stimulation with both hormones led to increased tyrosine phosphorylation of JAK2 and STAT5b and the association of JAK2 with STAT5b. In contrast, no effect on STAT3 phosphorylation or JAK2/STAT3 association was observed when compared with acute insulin or leptin administration. These results suggest that the positive cross-talk between insulin and leptin signaling is due to additive effects on JAK2 activation and divergence of association between JAK2 and STAT3 and -5b. Another possible reason for this difference is differential insulin/leptin signal amplification. STAT5b can be activated either by insulin through the insulin receptor in a JAK-

independent fashion (Sawka-Verhelle *et al.,* 1997, 2000) or with OBRb by leptin (Briscoe *et al.,* 2001). In contrast, the role of insulin in the activation of the STAT3 is controversial (Ceresa and Pessin, 1996; Kim *et al.,* 2000;Carvalheira *et al.,* 2001) and may be tissue-specific. Here we demonstarted a mild activation of STAT3, but this effect was not additive with leptin.

Although leptin has an insulin-sensitizing effect, which is evident from the rapid reduction of glucose and insulin levels in leptin-deficient, insulin-resistant *ob*/*ob* mice after leptin administration (Pelleymounter *et al.,* 1995) and the enhanced insulin-stimulated glucose disposal in normal rats infused with leptin (Sivitz *et al.,* 1997), in the present study we found that leptin and insulin elicit overlapping but distinct signaling pathways toward the PI 3-kinase/AKT pathway. Notably, i.v. injection of leptin results in phosphorylation of JAK2 and a mild phosphorylation of IRS-1 and IRS-2 with a parallel increase in IRSs/PI 3-kinase association compared with the large effects of insulin on these signaling steps. In liver, Akt shows enhanced serine phosphorylation after insulin, but not after leptin stimulation.

The effect of leptin stimulation on IRS-1 and IRS-2 tyrosine phosphorylation and its effects on insulin-induced signaling is dependent of the cell line used. Cohen *et al.* (1996) reported that leptin caused attenuation of several insulin-induced activities, including tyrosine phosphorylation of the IRS-1 on human hepatic cells. On the other hand, Wang *et al.* (1997) showed that leptin treatment increase IRS-1 and IRS-2 tyrosine phosphorylation on hepatoma cells. Furthermore, Kellerer *et al.* (1997) demonstrated that leptin has insulin-like effects on glucose transport and glycogen synthesis in $C_2C_{12}$ myotubes and that these effects are dependent on IRS-2 tyrosine phosphorylation. In accordance with Kim *et al.* (2000), here we showed that in the liver the treatment with the combination of the two hormones is not additive, synergistic or inhibitory in the PI 3-Kinase-Akt pathway.

In conclusion, we have presented evidence for rapid, direct effects of leptin administration *in vivo* on intracellular signaling pathways in the liver and we observed a convergence of leptin and insulin signaling at the level of the PI 3-Kinase-Akt pathway without synergism. Moreover, our results indicate a direct and positive cross-talk between insulin and leptin at the level of JAK2 and STAT-5b tyrosine phosphorylation and association. This mechanism may serve to potentiate the activity of both insulin and leptin pathways and to increase stimulation in physiological processes such as the regulation of carbohydrate and lipid metabolism, which are under the combined control of insulin and leptin.

## Materials and Methods

### Materials

The reagents for SDS-PAGE and immunoblotting were obtained from Bio-Rad (Richmond, USA). Tris base, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween-20, glycerol, affinity-purified rabbit anti-mouse IgG and bovine serum albumin (BSA, fraction V) were obtained from Sigma Chemical Co. (St. Louis, USA). Leptin was acquired from Calbiochem (La Jolla, USA). Sodium amobarbital and human recombinant insulin (Humulin R) were purchased from Eli Lilly & Co. (Indianapolis, USA). Protein A-Sepharose 6Mb, [$^{125}$I]protein A and nitrocellulose paper (Hybond ECL, 0.45 µm) were obtained from Amersham (Piscataway, USA). Antibodies to IRS-1 (SC-559), IRS-2 (SC-8299), JAK2 (SC-294G), STAT3 (SC-483), STAT5b (SC-835) and phosphotyrosine (SC-508) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Antibodies against the p85 subunit of PI 3-kinase (06 – 195) were from Upstate Biotechnology (Lake Placid, USA). Anti-Akt-Ser-473 (9271L) and anti-STAT3 phosphotyrosine 705-specific antibodies (9131) were from New England Biolabs (Beverly, USA).

### Animals

Eight week-old male Wistar and Zucker rats were allowed access standard rodent chow and water *ad libitum*. All experiments involving animals were in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA). Room temperature was maintained between 21 – 23 °C, and a 12-hour light, 12-hour-dark cycle was used.

### Surgical Procedures and Tissue Preparation

After 7 hours fast, rats were anesthetized with sodium amobarbital (15 mg/kg body weight, intraperitoneally), and submitted to the surgical procedure as soon as the anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened, the portal vein was exposed, and *in vivo* stimulation of the liver was obtained by injection of 500 µl of normal saline (0.9% NaCl), insulin ($10^{-6}$ M), leptin ($10^{-6}$ M) or an equimolar mixture of insulin ($10^{-6}$ M) and leptin ($10^{-6}$ M) into the portal vein. Fragments of the livers were excised in a time dependent manner. The tissue was minced coarsely, and homogenized immediately in extraction buffer [1% Triton X-100, and 100 mM Tris (pH 7.4) containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.1 mg aprotinin/ml] at 4 °C with a Polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Inc., Westbury, USA) operated at maximum speed for 30 s. The extracts were centrifuged at 30 000 *g* and 4 °C in a Beckman 70.1 Ti rotor (Palo Alto, USA) for 20 min to remove insoluble material, and the supernatant of these tissues was used for immunoprecipitation with the indicated antibodies.

### Protein Analysis by Immunoblotting

The precipitated proteins were treated with Laemmli sample buffer (Laemmli, 1970) containing 100 mM dithiothreitol and heated in a boiling water bath for 4 min, after which they were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus (Mini-Protean, Bio-Rad Laboratories, Inc., Richmond, USA). Electrotransfer of proteins from the gel to nitrocellulose was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described (Towbin *et al.,* 1979) except for the addition of 0.02% SDS to the transfer buffer to enhance the elution of high molecular mass proteins. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h at room temperature at 4 °C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween-20). The nitrocellulose blot was incubated with the indicated antibodies, diluted in blocking buffer (0.3% BSA instead of nonfat dry milk) overnight at 4 °C,

and then washed for 20 min with blocking buffer without milk. An amplification step with rabbit anti-mouse IgG (1:1000 final dilution) was added to the standard protocol when monoclonal anti-phosphotyrosine antibodies were employed in Western blotting experiments. The blots were subsequently incubated with 2 mCi [$^{125}$I]protein A (30 mCi/mg) in 10 ml blocking buffer for 2 h at room temperature and then washed again for 20 min as described above. [$^{125}$I]Protein A bound to the antipeptide antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak Co., Rochester, USA) with Cronex Lightning Plus intensifying screens at –80°C for 12–48 h. Band intensities were quantified by optical densitometry (Scion Image software) of the developed autoradiographs. To exclude unspecific interactions between the investigated proteins and protein-A Sepharose control precipitations without the specific antibodies were performed, followed by immunoblotting with anti-JAK2, anti IRS-1, anti-IRS-2, anti-STAT3 and anti-STAT5b antibodies, and no band was detected. The results indicated that the immunoprecipitation performed in the experiments described here do not allow the detection of unspecific interactions.

#### Statistical Analysis

Where appropriate, the results were expressed as the mean ± SEM accompanied by the indicated number of experiments. Kruskal-Wallis 1-way ANOVA test was used in the statistical comparisons with $p<0.05$ indicating significance.

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