

In vivo leptin infusion impairs insulin and leptin signalling in liver and hypothalamus

Yacir Benomar^{a,1}, Sandrine Wetzler^{b,1}, Christiane Larue-Achagiotis^b,
Jean Djiane^a, Daniel Tomé^b, Mohammed Taouis^{a,*}

^a Neuroendocrinologie Moléculaire de la Prise Alimentaire INRA, Université Paris XI, IBAIC, Bat447, 91405 Orsay, France

^b Physiologie de la Nutrition et du Comportement Alimentaire UMR INRA 914, INA-PG 16, Rue Claude Bernard 75231 Paris, France

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Abstract

Leptin resistance contributes to the pathogenesis of common obesity related metabolic diseases, including insulin resistance. However, the relationship between leptin and insulin resistance is not clearly established. Here, we show that induced hyperleptinemia by leptin infusion alters insulin signalling in rat liver. Leptin infusion clearly reduced the insulin or leptin dependent IRS-1/IRS-2 association to p85 regulatory subunit of PI 3-kinase. Leptin infusion also abolished STAT-3 phosphorylation in response to insulin or leptin and similar results were obtained for MAP-kinase phosphorylation. Hypothalamic leptin resistance was also induced by leptin infusion since leptin was unable to induce STAT-3 phosphorylation. These results provide evidence that induced hyperleptinemia can contribute to the onset of insulin resistance at least at the hepatic level.

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1. Introduction

Leptin, a hormone secreted by white adipose tissue, is involved in the regulation of energy balance and glucose homeostasis; and leptin plasma levels are well correlated with adipose tissue mass (Perez et al., 2004). In obese humans, plasma leptin concentrations are high leading to leptin-resistance which is believed to contribute to metabolic diseases, including insulin resistance and type 2 diabetes (Ceddia et al., 2002). Since the discovery of leptin gene, the relationship between hyperleptinemia and insulin resistance has been suggested (Sivitz et al., 1997). However, the link between hyperleptinemia and insulin resistance remains matter of controversy where diabetogenic or antidiabetogenic effects were attributed to leptin (Sivitz et al., 1997; Chinooswong et al., 1999). Furthermore, weight loss is

associated with reduction in leptinemia and increased insulin sensitivity (Sivitz et al., 1997; Chinooswong et al., 1999). Leptin treatment increases insulin sensitivity in normal, hyperinsulinemic or diabetic rats and corrects the diabetic phenotype of ob/ob mice (Muzzin et al., 1996). Other studies have showed insulin sensitivity was not affected by leptin (Sivitz et al., 1997; Widdowson et al., 1998; Cases et al., 2001; Zierath et al., 1998).

At the cellular level, several evidences support an interaction between leptin and insulin signaling networks. Both leptin and insulin receptors signal through common key intracellular signalling pathways such as JAK2/STAT-3, MAP-kinase and IRS/PI 3-kinase. It has been reported that leptin affects insulin signalling in insulin sensitive tissues (Kim et al., 2000) and also that insulin is able to modulate leptin signalling through JAK2/STAT-3 signalling cascade in rat hypothalamus (Carvalho et al., 2001). Other studies have showed an alteration of insulin action following leptin treatment in isolated rat adipocytes (Muller et al., 1997), human hepatic cell line (Cohen et al., 1996) and in skeletal

* Corresponding author. Tel.: +33 169157008; fax: +33 169157074.

E-mail address: mohammed.taouis@ibaic.u-psud.fr (M. Taouis).

¹ Authors have equally contributed to the present paper.

muscle (Sweeney et al., 2001). In HepG2 human hepatoma cell line, leptin inhibits the insulin-induced IRS-1 phosphorylation. In the same cells transfected with leptin receptor cDNA, leptin increased IRS-2 association with p85 subunit, regulatory subunit of PI 3-kinase (Wang et al., 1997). In FAO hepatoma cells, leptin pre-treatment transiently activated IRS/PI 3-kinase pathway (Szanto and Kahn, 2000). In rat adipocytes, leptin impairs insulin signalling at the level of MAP-kinase activity, insulin receptor phosphorylation and glycogen synthase-kinase phosphorylation (Perez et al., 2004).

In vivo, leptin alters skeletal muscle insulin stimulated PI 3-kinase activity and glucose transport (Singh et al., 2003). However, in liver, leptin seems to enhance insulin's action, such as reversing insulin resistance and hepatic steatosis in patients with severe lipodystrophy (Petersen et al., 2002). This is in good agreement with reports showing that leptin reduces hepatic glucose production by decreasing the synthesis of the key enzyme of gluconeogenesis PEPCK (Rossetti et al., 1997; Liu et al., 1998; Anderwald et al., 2002).

The present study aimed to investigate in normal rats the effect of chronic leptin treatment on the hypothalamic and liver sensitivity of JAK2/STAT-3, MAP-kinase and IRS/PI 3-kinase signalling pathways towards leptin and insulin. We show that after 7 days of leptin infusion (IP) in normal rats, there is a decrease in body fat weight and food intake concomitant with an augmentation of plasma leptin concentrations without significant changes in glycaemia or insulinemia. In the hypothalamus of leptin infused rats, leptin bolus completely abolished or attenuated STAT-3 or MAP-kinase phosphorylation, respectively. In liver of leptin infused rats, neither leptin nor insulin did increase IRS1 or IRS-2 association to p85. Furthermore, we show that insulin is able to activate STAT-3 and MAP-kinase phosphorylation in liver but not in the hypothalamus of untreated rats. Our data suggest that in normal rats the progressive augmentation of plasma leptin concentrations during leptin infusion induced hypothalamic resistance to leptin and importantly it induced both leptin and insulin resistance in liver.

2. Material and methods

2.1. Chemicals

Bovine serum albumin (fraction V radio immunoassay grade), leupeptin, aprotinin and protein A-agarose were purchased from Sigma Chemical Company (St. Louis, MO, USA). Pre-made polyacrylamide solution Protogel was from National Diagnostics (Prolabo, France). Antibodies directed towards IRS-1, IRS-2, total STAT-3, phospho-STAT-3, insulin receptor, were purchased from UBI (Euromedex, France); antibodies directed towards p44-42 MAP-kinase and phospho-p44-42 MAP-kinase were from cell signaling (Ozyme, France) and antibodies against p85 were from Santa

Cruz Biotechnology (Tebu, France). Nitrocellulose membranes were from Euromedex (France). Ovine leptin was produced in our laboratory as previously described (Gertler et al., 1998).

2.2. Animals

Eighteen male Wistar rats (Harlan, France, 150–160 g, 7–8 weeks old at the beginning of the experiment) were used. They were housed individually and placed in a temperature-, humidity- and light-controlled room ($22 \pm 1^\circ\text{C}$) with a 12–12 h light–dark cycle (light on: 10:00 am). Food (standard chow diet) and water were available ad libitum in their cages. The standard chow diet (P14 = 14.6 kJ/g) was composed of protein energy (14.8%), fat (10.5%) and CHO supplemented with minerals and fibres. Food intake and body weight were measured daily at 10:00 am and fresh diets were provided. Although spillage was minimal, when it did occur, the food lost from the stainless holders was collected and added to the total not yet consumed.

2.3. Chronic leptin infusion (minipumps) and treatments

After habituation for 1 week to the standard diet, the 18 rats were divided in two groups, nine rats (controls) received saline and nine rats received ovine leptin, dissolved in sterile bidistilled water (9/10, v/v) and sodium dihydrogen phosphate buffer (1/10, v/v, 1 M) and diluted to appropriate concentrations, via osmotic minipumps (1 mg/kg/day) for 7 days as previously described (Wetzler et al., 2004). The minipumps (Alzet no. 2001 model, constant rate 1 $\mu\text{l/h}$, were implanted under light ether anaesthesia, in the intraperitoneal cavity. At the end of the experiment, 20 min before lethal anaesthesia, rats of each group were injected either with IP leptin (1 mg/kg) ($n=3$), or insulin (1 IU/kg) ($n=3$) or saline ($n=3$) in order to test hypothalamic and liver response to those injections.

2.4. Sample collection and analysis

On the 7th day after minipump implantation, and after 6 h of food deprivation, a drop of blood was taken from the femoral vein of each rat for glycaemia evaluation. Each blood drop was applied to a blood glucose test strip and inserted in a glucose meter (Glucostrend 2, Accu-Chek System, Roche, limit of sensitivity 0.6 mmol/l). Then rats were anesthetized with a lethal dose of pentobarbital (50 mg/kg BW by i.p injection) and heparinized (100 units heparin/100 g BW). Blood samples were collected from the inferior vena cava and centrifuged, and the plasma was frozen at -20°C until insulin and leptin assays were carried out. Then, the liver and hypothalamus were quickly removed and immediately frozen into liquid nitrogen for further analysis. Three depots of white adipose tissue: epididymal, retroperitoneal and subcutaneous, were carefully removed and weighed.

Serum leptin and insulin concentrations were measured using a double-antibody radioimmunoassay kit (leptin: Linco research, insulin: CIS Bioindustries, Gif/Yvette, France).

2.5. Iodination of ovine leptin

Ovine leptin (10 μ g) was radio-iodinated (IMS 50, Amersham Pharmacia Biotech, France) by the chloramine T method.

2.6. Western blot analysis

Starved Con or Lep rats were subjected to leptin, insulin or saline acute IP treatment and sacrificed 30 min later. Tissues (liver and hypothalamus) were immediately frozen in liquid nitrogen and stored at -80°C . Frozen tissues were homogenized in the solubilization buffer containing containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 1% nonidet-P40, 10% glycerol, Protease inhibitors (0.35 mg/ml PMSF, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin) and phosphatase inhibitors (10 mM sodium fluoride, 1mM sodium orthovanadate, 20 mM sodium β -glycerophosphate, 10 mM benzamidine). After lysis in ice for 90 min, insoluble materials was removed by centrifugation (15,000 rpm at 4°C for 30 min) and protein concentrations of the resulting lysates were determined using a protein assay kit (Pierce, Perbio Science, France). Solubilized materials were either directly subjected to Western blotting or to immunoprecipitation before Western blotting. For direct application, liver or hypothalamus extracts (150 μ g) were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were then blocked with 3% non-fat dry milk. After incubation with appropriate primary and secondary antibodies, nitrocellulose membranes were washed and targeted protein were detected using enhanced chemiluminescence's reagents (ECL; Amersham Biosciences). For immunoprecipitations, equal amounts of proteins (500 μ g) were incubated with the appropriate antibodies overnight at 4°C . Immune complexes were collected following incubation with protein-A agarose for 2 h at 4°C . The complexes were washed extensively, and then subjected to Western blotting as described above. For the quantification of STAT-3 and MAP-kinase phosphorylation, obtained bands were quantified using Scion Image Software and normalized to total amount of STAT-3 or MAP-kinase, respectively.

2.7. Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA, STATVIEW Software, version 5) to detect significant inter-group differences. Values are expressed as means \pm S.E.M., and $p < 0.05$ was considered statistically significant. Differences in body composition and food intake between the two groups were tested using Student's *t*-test.

3. Results

3.1. Glycaemia, insulinemia, leptinemia

At time of sacrifice glycaemia and insulinemia were not significantly different between Con and Lep rats. Plasma glucose concentrations were 6.1 ± 0.2 mmol/l and 5.7 ± 2.3 mmol/l in Con and Lep rats, respectively. Plasma insulin concentrations were 64.4 ± 10.3 μ U/ml and 74.9 ± 18.8 μ U/ml in Con and Lep rats, respectively. Whereas,

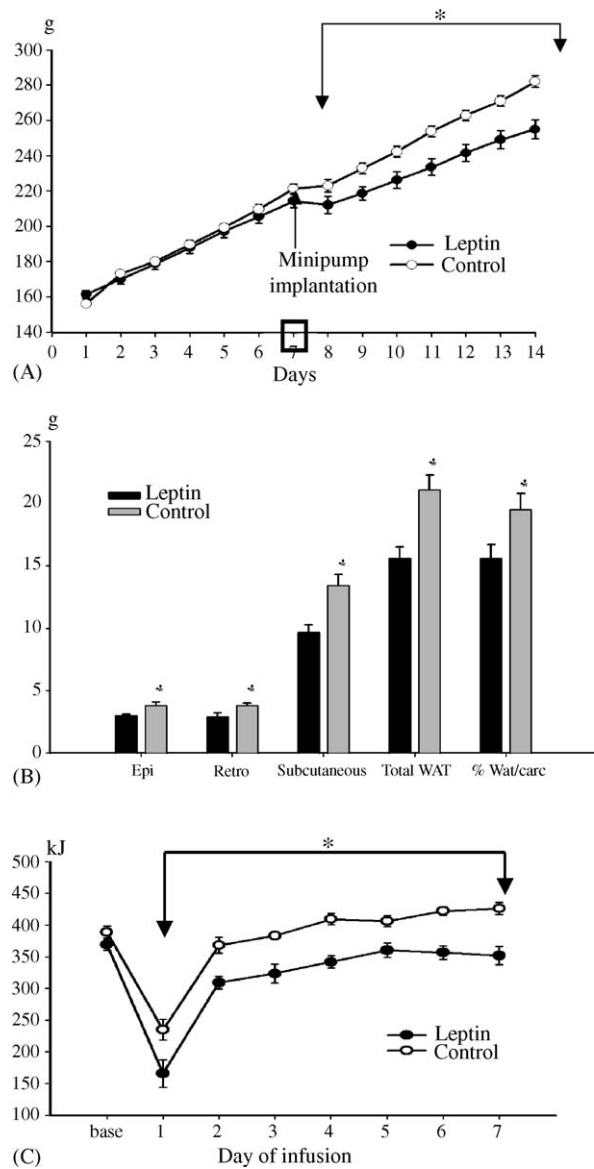


Fig. 1. Response of Wistar rats to leptin infusion. The animals were infused with saline (Con) or ovine leptin (Lep) (1 mg/kg/day) using osmotic minipumps implanted into the intraperitoneal cavity. Infusion was performed from day 7 to day 14 and body weight (A) was measured. Fat mass deposit in various anatomical locations (Epi: epididymal fat; Retro: retroperitoneal fat; subcutaneous fat) was also measured (B). Total energy intake was also measured from day 1 of infusion to the end and expressed as kJ/100 g of weight (C). Results are expressed as mean \pm S.E.M., and **p* < 0.05 significant differences between leptin saline infusion.

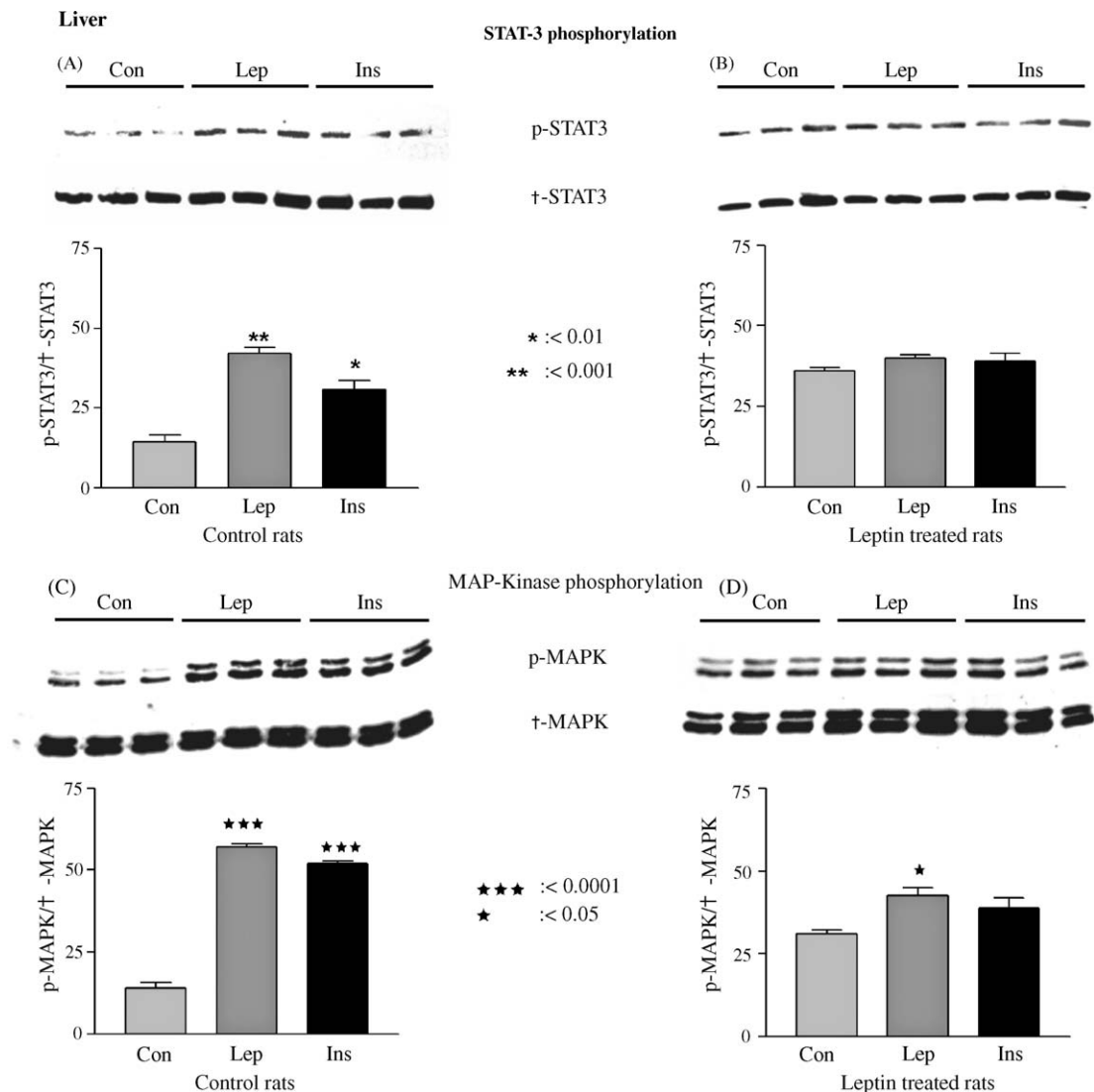


Fig. 2. Phosphorylation of STAT-3 (A and B) and MAP-kinase (C and D) in the hypothalamus of rats infused with saline or leptin (1 mg/kg/day) for 7 days and killed 30 min following an IP injection of saline, insulin (1 U/kg) or leptin (1 mg/kg). Proteins were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes. Phosphorylated STAT-3 or MAP-kinase was visualized by immunoblotting with antibodies specific for phospho STAT-3 or phospho MAP-kinase. The same blots were immunoblotted with antibodies specific to total STAT-3 or total MAP-kinase. Bands were quantified using Scion Image software and results were expressed as ratio of phosphorylated/total STAT-3 or MAP-kinase. Data are means \pm S.E.M. for three rats.

plasma leptin levels were increased by almost three-fold in Lep rats (22.9 ± 8.6) as compared to Con rats (7.5 ± 2.2 ng/ml), but however, due to individual variations this increase is nearly significant ($p < 0.07$).

3.2. Impact of leptin infusion on food intake, body weight and body composition

During the basal period (7 days) prior to minipump implantation, control group (Con) and leptin group (Lep) had similar daily body weight gain (BWG) with 10.3 ± 0.5 and 9 ± 0.3 g, respectively. Following mini-pump implantation and from day 1 of leptin infusion BWG of Lep rats progressively decreased to reach a reduction of 36% ($+39 \pm 2.4$ g) as

compared to Con rats ($+60.8 \pm 1.9$ g) after 7 days of leptin infusion ($p < 0.0001$) (Fig. 1A).

At the end of leptin or placebo infusion adipose tissue from different locations was weighed. Leptin infusion significantly reduced white adipose tissue (WAT) by 26% ($p < 0.005$); particularly retroperitoneal WAT (-25% , $p < 0.05$), epididymal WAT (-22% , $p < 0.05$) and subcutaneous WAT (-28% , $p < 0.005$) (Fig. 1B). WAT/carcass ratio was significantly lower in Lep rats as compared to Con rats (Fig. 1B).

In parallel to the measurement of body weight gain, feed intake was measured during leptin or placebo infusion. During basal period prior to leptin infusion, both Con and Lep rats ingested similar amount of feed as expressed as kJ/100 g BW. The energy intake was expressed as energy intake

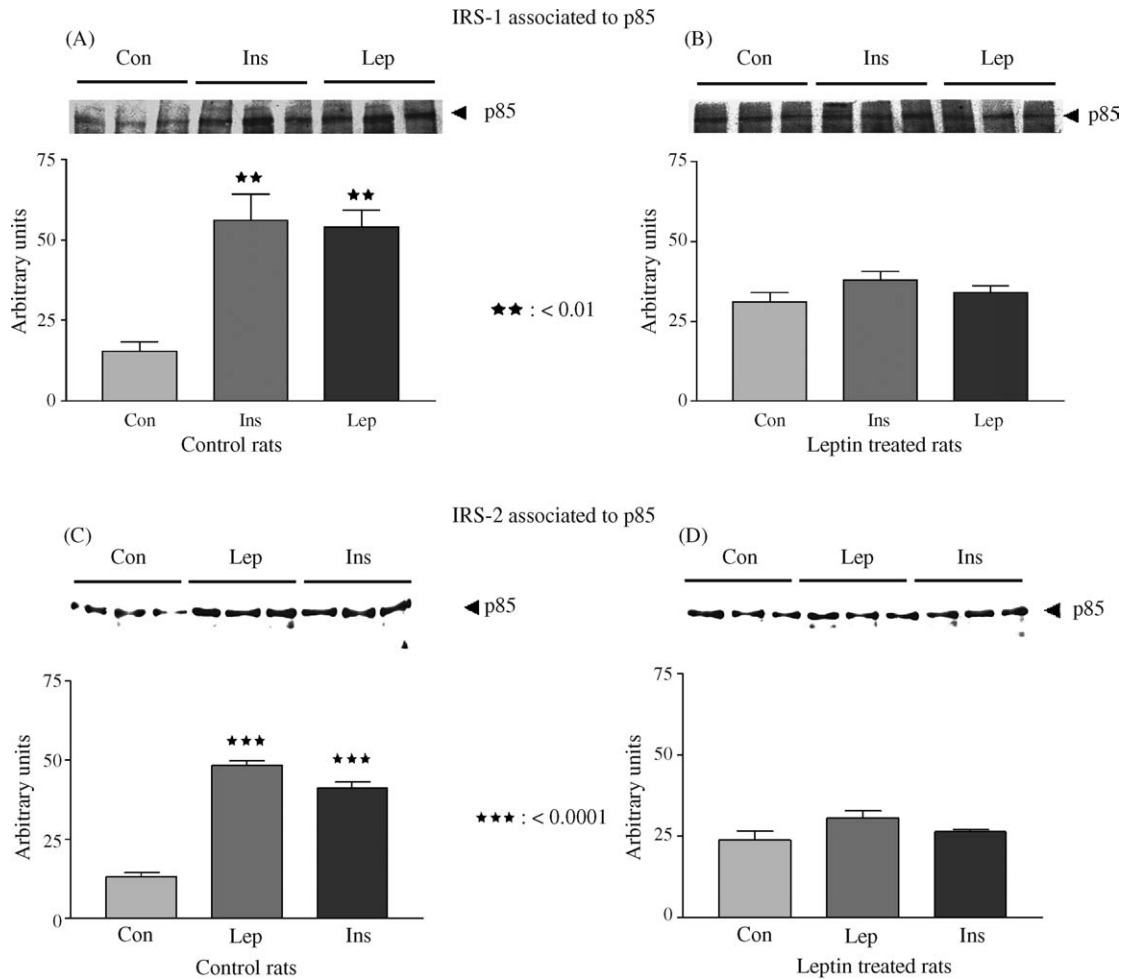


Fig. 3. Phosphorylation of STAT-3 (A and B) and MAP-kinase (C and D) in the liver of rats infused with saline or leptin (1 mg/kg/day) for 7 days and killed 30 min following an IP injection of saline, insulin (1 U/kg) or leptin (1 mg/kg). Proteins were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes. Phosphorylated STAT-3 or MAP-kinase was visualized by immunoblotting with antibodies specific for phospho STAT-3 or phospho MAP-kinase. The same blots were immunoblotted with antibodies specific to total STAT-3 or total MAP-kinase. Bands were quantified using Scion Image software and results were expressed as ratio of phosphorylated/total STAT-3 or MAP-kinase. Data are means \pm S.E.M. for three rats.

prior to minipump implantation. During the whole period of leptin infusion (7 days), Lep rats significantly ($p < 0.05$) decreased their total energy intake by 16% as compared to Con rats (528.8 ± 18.8 kJ/100 g BW for Lep rats versus 634 ± 11.2 kJ/100 g BW for Con rats) (Fig. 1C).

4. Leptin chronic treatment alters liver STAT-3 and MAP-kinase sensitivity to leptin and insulin

To investigate the possible action of leptin chronic treatment on leptin or insulin sensitivity in liver, we studied the phosphorylation of STAT-3 in response to insulin or leptin. Following, SDS-PAGE and Western blotting, STAT-3 phosphorylation was quantified as described in Section 2. In Con rats, leptin or insulin significantly ($p < 0.001$ and 0.01 , respectively) increased STAT-3 phosphorylation (Fig. 2A). In Lep rats, both insulin and leptin were unable to increase STAT-3 phosphorylation and their effect is similar to that produced in rats receiving saline (Fig. 2B).

The effect of leptin chronic treatment was also investigated in liver MAP-kinase. MAP-kinase phosphorylation was also estimated by Western blotting using specific antibodies directed towards phospho-MAP-kinase, and results were normalized to total MAP-kinase. In Con rats, leptin and insulin significantly ($p < 0.0001$) increased MAP-kinase phosphorylation by six- and five-fold, respectively, as compared to saline treatment (Fig. 3C). In Lep rats, the effect of insulin was completely abolished and that of leptin was clearly attenuated with a slight, but significant ($p < 0.05$), increase in MAP-kinase phosphorylation (Fig. 3D).

5. Leptin chronic treatment alters liver IRS1 and IRS-2 association to the regulatory subunit of PI 3-kinase (p85) in response to acute leptin or insulin

The liver plays a central role in regulating glucose and lipid homeostasis, and is one of the major sites of insulin action. One of the important signalling pathways controlled

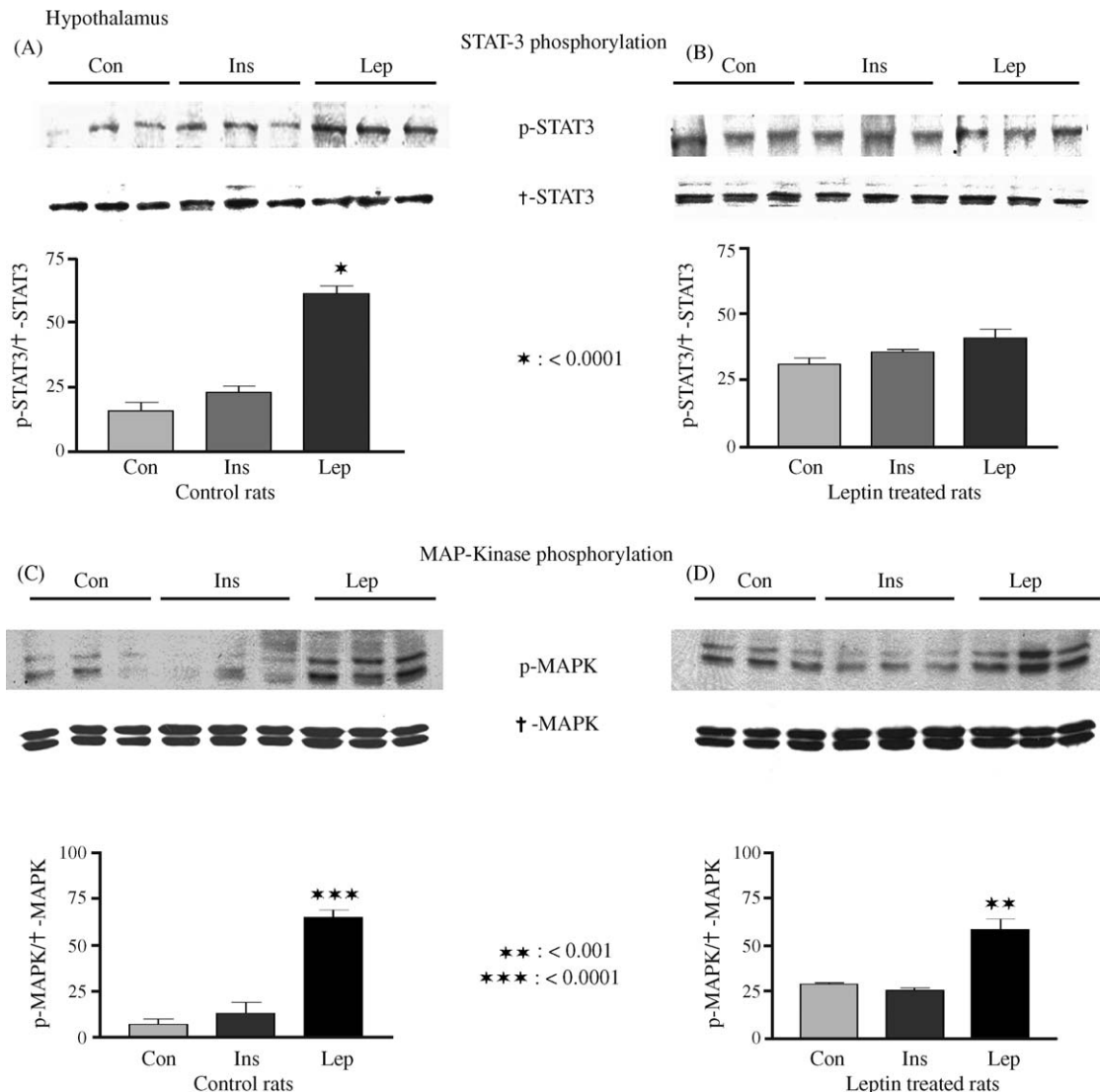


Fig. 4. IRS-1 (A and B) or IRS-2 (C and D) association to p85 subunit of PI 3-kinase in the liver of rats infused with saline or leptin (1 mg/kg/day) for 7 days and killed 30 min following an IP injection of saline, insulin (1 U/kg) or leptin (1 mg/kg). Liver proteins were immunoprecipitated with either anti-IRS-1 or anti-IRS-2 antibodies and immunoprecipitates were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes. The association of p85 with either IRS-1 or IRS-2 was visualized by immunoblotting with antibodies specific for p85 subunit. Bands were quantified using Scion Image software and results were expressed as means \pm S.E.M. for three rats.

by insulin is IRS/PI 3-kinase cascade and its alteration may lead to insulin-resistance. Thus we studied the impact of leptin chronic treatment on insulin or leptin action on IRS-1 or IRS-2 association to the regulatory subunit of the PI 3-kinase. Following leptin chronic treatment, starved rats were treated by IP with leptin, insulin or saline and sacrificed 30 min later. Solubilized liver samples were subjected to immunoprecipitation using either IRS-1 or IRS-2 antibodies, and blots were revealed with an anti-p85 antibody. In Con rats, insulin or leptin significantly increase IRS-1 ($p < 0.01$) (Fig. 3A) and IRS-2 ($p < 0.0001$) (Fig. 3C) association to the p85 subunit. In Lep rats, both leptin and insulin were unable to increase IRS-1 (Fig. 3B) or IRS-2 (Fig. 3D) association to p85.

5.1. Leptin chronic treatment alters hypothalamus STAT-3 but not MAP-kinase phosphorylation in response to acute leptin injection

The impact of leptin chronic treatment on hypothalamic insulin and leptin sensitivity was studied. Lep or Con rats were subjected to acute leptin, insulin or saline treatment (IP) and sacrificed 30 min later. STAT-3 phosphorylation was measured as described in Section 2. In Con rats, leptin significantly ($p < 0.0001$) increased STAT-3 phosphorylation by at least four-fold, whereas insulin and saline treatments were without effect (Fig. 4A). In Lep rats, STAT-3 phosphorylation in response to leptin was completely abolished and its

effect is similar to that obtain with saline or insulin treatment (Fig. 4B).

To investigate whether, leptin chronic treatment affects other signalling pathways in the hypothalamus; we studied the activation of MAP-kinase. Starved Con or Lep rats were subjected to leptin, insulin or saline acute IP treatment and sacrificed 30 min later. The MAP-kinase phosphorylation was measured as described in Section 2. The results indicate that in both Lep (Fig. 4D) and Con (Fig. 4C) rats, leptin but not insulin increased MAP-kinase phosphorylation. Leptin increased MAP-kinase phosphorylation by almost six-fold in Con rats and only by two-fold in Lep rats, as compared to their respective control (saline treated rats).

6. Discussion

Numerous studies reported paradoxical observations on the link between hyperleptinemia and insulin resistance (Ceddia et al., 2002). In the present paper we investigate the impact of a chronic increase in plasma leptin concentrations on leptin and insulin signalling in hypothalamus and liver. Following 7 days of leptin infusion, plasma leptin concentration was increased by almost three-fold and this was concomitant with a significant reduction in food intake and fat mass without changes in carcass weight. This results agree with the idea that leptin specifically affects adipose tissue whatever its localization (i.e. subcutaneous or intra-abdominal) (Barzilai et al., 1997). Moreover, leptin treatment reduced leptin-sensitivity of the hypothalamus and both leptin and insulin-sensitivity in the liver. This is the first evidence, in our knowledge, demonstrating that chronic leptin treatment induces insulin resistance of both STAT-3 and MAP-kinase signaling pathways in liver.

Our data also show that chronic leptin treatment significantly altered leptin-sensitivity of the hypothalamus as estimated by STAT-3 and MAP-kinase phosphorylation following an acute leptin challenge. In addition, we also show that acute insulin treatment did not affect STAT-3 or MAP-kinase phosphorylation in the hypothalamus of Lep or Con rats. These data indicated that chronic leptin treatment led to leptin-resistance in the hypothalamus, despite the significant reduction in food intake and fat body weight. This could originate from the increase in both STAT-3 and MAP-kinase basal phosphorylation during chronic leptin infusion in Lep rats as compared to Con rats. This state may mimic the early stage of the onset of leptin resistance characterized by an increase in plasma leptin concentrations (Considine et al., 1996). It is noteworthy that the hypothalamic leptin resistance appeared without significant changes in plasma glucose or insulin levels. The present study show most likely a state of leptin-resistance that may reflect a pre-obese state where subjects are not resistant to insulin and their glycemia is normal. The insulin plasma levels of Lep rats is higher than Con rats, although this difference is not statistically significant, but indicates a tendency which may probably be ampli-

fied whether the leptin chronic treatment was maintained longer.

In liver, our results show that in normal rats both leptin and insulin acutely injected stimulate STAT-3 and MAP-kinase phosphorylation. These effects were abolished following chronic leptin treatment indicating both leptin and insulin resistance of the liver. The alteration of insulin signalling in liver of Lep rats may be attributed to a direct action of leptin on liver or indirect through the central nervous system, mainly hypothalamus. Recently, it has been showed that leptin impairs insulin signalling in isolated adipocytes and in vivo following intracerebroventricular injection (Perez et al., 2004). In addition, leptin chronic treatment abolished leptin effects on STAT-3 and MAP-kinase phosphorylation. The alteration of insulin signalling in liver cannot be attributed to an increase in plasma insulin levels, since the chronic leptin treatment did not significantly affect insulinemia. Thus, the alteration of insulin signalling in liver is most likely attributed to hyperleptinemia.

To investigate whether other signalling pathways were affected by leptin treatment in liver, IRS/PI 3-kinase pathway was studied. In control rats, both leptin and insulin were able to increase IRS-1 or IRS-2 association to p85, regulatory subunit of PI 3-kinase. Following chronic leptin treatment, both leptin and insulin actions on IRS-1 or IRS-2 association with p85 were abolished. Our results clearly show that chronic leptin treatment impairs insulin signalling in vivo in liver. Interestingly, basal association of IRS1/IRS2 with PI 3-kinase is increased Lep rats, leading probably to “constitutive” activation of PI 3-kinase, which may explain insulin and leptin resistance of this signalling pathway. In addition, the constitutive activation of PI 3-kinase may, at least partially, explain the normal glycemia observed in Lep rat group. As described previously, this may reflect a pre-obese state that may lead to hyperinsulinemia and hyperglycemia whether leptin chronic treatment have been maintained longer.

Taken together, these results show that leptin chronic treatment affects insulin signalling in rat liver by altering three major signalling pathways: STAT-3, MAP-kinase and IRS/PI 3-kinase. This is new evidence that links leptin resistance to insulin resistance in vivo in rat.

In conclusion, this study shows that leptin chronic infusion impairs (1) leptin signalling in the hypothalamus (STAT-3 and MAP-kinase signalling pathways), (2) leptin and insulin signalling in the liver (STAT-3, MAP-kinase and IRS/PI 3-kinase signaling pathways), and finally this study demonstrates that leptin resistance may contribute to the onset of insulin resistance.

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