Regulation of Insulin-Stimulated Glucose Uptake in Rat White Adipose Tissue upon Chronic Central Leptin Infusion: Effects on Adiposity

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Leptin enhances the glucose utilization in most insulin target tissues and paradoxically decreases it in white adipose tissue (WAT), but knowledge of the mechanisms underlying the inhibitory effect of central leptin on the insulin-dependent glucose uptake in WAT is limited. After 7 d intracerebroventricular leptin treatment (0.2 μg/d) of rats, the overall insulin sensitivity and the responsiveness of WAT after acute in vivo insulin administration were analyzed. We also performed unilateral WAT denervation to clarify the role of the autonomic nervous system in leptin effects on the insulin-stimulated [^{3}H]-2-deoxyglucose transport in WAT. Central leptin improved the overall insulin sensitivity but decreased the *in vivo* insulin action in WAT, including insulin receptor autophosphorylation, insulin receptor substrate-1 tyrosine-phosphorylation, and Akt activation. In this tissue, insulin receptor substrate-1 and glucose transporter 4 mRNA and protein levels were down-regulated after central leptin treatment. Additionally, a remarkable up-regulation of resistin, together with an augmented expression of suppressor of cytokine signaling 3 in WAT, was also observed in leptin-treated rats. As a result, the insulin-stimulated glucose transporter 4 insertion at the plasma membrane and the glucose uptake in WAT were impaired in leptin-treated rats. Finally, denervation of WAT abolished the inhibitory effect of central leptin on glucose transport and decreased suppressor of cytokine signaling 3 and resistin levels in this tissue, suggesting that resistin, in an autocrine/paracrine manner, might be a mediator of central leptin antagonism of insulin action in WAT. We conclude that central leptin, inhibiting the insulin-stimulated glucose uptake in WAT, may regulate glucose availability for triacylglyceride formation and accumulation in this tissue, thereby contributing to the control of adiposity. **(*Endocrinology* 152: 1366–1377, 2011)**

Leptin modulates glucose homeostasis, acting through both central and peripheral pathways, as an insulin-sensitizing factor at the whole-body level (1, 2). Nevertheless, studies on glucose uptake by different tissues after *in vivo* leptin treatment suggest that the hormone exerts tissue-specific effects, stimulating glucose utilization in brown adipose tissue, heart, and skeletal muscle but inhibiting glucose uptake in white adipose tissue (WAT) (3,

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Abbreviations: AUC, Area under the curve; CYT, cytosolic fraction; eWAT, epididymal white adipose tissue; FFA, free fatty acid; GLUT, glucose transporter; IR, insulin receptor; IRS, insulin receptor substrate; KRH, Krebs-Ringer-HEPES; LM, light membrane; MCR, melanocortin receptor; mTORC, mammalian target of rapamycin complex; NE, norepinephrine; PM, plasma membrane; SOCS3, suppressor of cytokine signaling 3; rWAT, retroperitoneal white adipose tissue; TAG, triacylglyceride; WAT, white adipose tissue.

4). However, studies concerning the effects of leptin on insulin signaling in WAT have been not only scarce, but also controversial (5).

Whereas leptin microinjection into ventromedial hypothalamus decreases the insulin-stimulated glucose uptake in WAT (6), leptin incubation of isolated adipocytes has been reported to inhibit insulin stimulation of glucose transport (7), to impair only the lipogenic action of insulin (8, 9), and not to influence insulin-mediated effects (10, 11). Additionally, we have reported that *in vivo* chronic leptin administration impairs insulin-stimulated MAPK activity in isolated adipocytes (12), whereas the insulin-stimulated MAPK phosphorylation in WAT was increased after acute *in vivo* leptin treatment (13). Taken together, these data strongly suggest a complex regulatory cross talk between leptin and insulin signaling pathways.

Several studies have suggested that the WAT plays a pivotal role in regulating overall insulin sensitivity, although the mechanisms involved are still poorly understood. Thus, decreasing visceral fat by caloric restriction (14) or surgical removal (15) prevents the development of overall insulin resistance. Adipose-selective glucose transporter 4 (GLUT4) gene disruption causes glucose intolerance and hyperinsulinemia (16), whereas GLUT4 overexpression in fat tissue enhances whole-body insulin sensitivity and glucose tolerance in diabetic mice (17). Finally, the overexpression in WAT of suppressor of cytokine signaling 3 (SOCS3), known for its insulin inhibitory effects (18), causes local but not systemic insulin resistance (19). In fact, an increased SOCS3 expression in adipocytes had been proposed as a potential mediator of the insulin resistance induced by several hormones and cytokines, including leptin, insulin, TNF-α, and resistin (12, 20–22).

Compelling evidence suggests that leptin exerts its metabolic effects predominantly through different hypothalamic nuclei, where the leptin receptor expression is the highest compared with other tissues (23). By acting on the brain, leptin affects appetite, neuroendocrine pathways, and autonomic nerves, which transmit the hormone signal to the periphery (24). On the other hand, direct leptin effects have been reported in peripheral tissues that express the leptin receptor (5). However, the relative importance of peripheral *vs.* central actions of leptin on metabolic effects of the hormone is not well characterized.

Hence, the overall objective of the present work is to study the molecular mechanisms by which central leptin treatment impaired insulin-stimulated glucose uptake in WAT. To this end, we analyzed the effects of chronic central leptin infusion on early steps of the insulin signaling pathway implicated in glucose uptake in WAT. The role of SOCS3 and resistin as mediators of central leptin action regulating glucose availability for fatty acid esterification in this tissue are discussed. This work extends our previous observations examining the effects of *in vitro* and/or *in vivo* leptin administration on insulin signaling in isolated adipocytes (12).

Materials and Methods

Male 3-month-old Wistar rats were randomly housed in individual cages under conditions of climate-controlled quarters with a 12-h light cycle and fed *ad libitum* standard laboratory diet and water. The animals were handled according to the laws of the European Union and the guidelines of the National Institutes of Health, and the experimental protocols were approved by the institutional committee of bioethics.

WAT denervation

Unilateral autonomic nervous system denervation of the retroperitoneal fat pad (rWAT) was performed according to Kreier *et al.* (25). As we previously reported (26), local denervation in anesthetized rats, as below, was achieved by surgical removing nerves running along blood vessels from the diaphragm to the superior tip of the left rWAT, with the contralateral intact right pad used as control. Five weeks after the surgery, rats were subjected to an intracerebroventricular leptin administration as described below. In agreement with previous reports (27) and supporting the efficiency of the surgical denervation, the weight of the denervated left rWAT increased by ~27%, and the total norepinephrine (NE) content in the denervated fat pad was significantly reduced compared with the contralateral intact right pad (5.5 ± 0.4 *vs.* 9.7 ± 0.3 ng NE/g tissue, respectively; $n = 3$; $P < 0.001$).

Leptin administration

Intracerebroventricular leptin administration was performed as previously described (28). Briefly, rats were anesthetized with ip ketamine/diazepam/atropine used at 50 mg/kg, 4 mg/kg, and 0.2 mg/kg, respectively (Parke-Davis, Roche and Braun, Spain), and placed in a stereotaxic frame (David Koppf, Tujunga, CA). An opening in the skull was made with a dental drill at −1.6 mm lateral to the midline and 0.8 mm posterior to bregma. A cannula connected to an osmotic pump (Alzet, Palo Alto, CA) was implanted in the right lateral cerebral ventricle, with a releasing rate of 1 μl/h, and filled with 0.0082 μg/μl (0.2 μg/d) rat leptin (Sigma-Aldrich, St. Louis, MO), or its vehicle (PBS). Five groups of rats randomly distributed were investigated: 1) rats infused with leptin (Lep); 2) rats infused with vehicle and allowed to eat *ad libitum* (SS); 3) rats infused with vehicle and pair-fed to the amount of food consumed by the leptin-infused animals (PF); 4) unilateral denervated rats infused with leptin, and 5) unilateral denervated pair-fed rats infused with vehicle. After 7 d of treatment rats were killed by decapitation or subjected to an iv insulin (10 I.U/kg body weight) (Lily) or saline injection for 30 min and then killed by decapitation. rWAT and epididymal fat pads (eWAT) were dissected and weighed. An aliquot (per triplicate) from each fat pad was immediately used, independently, for glucose transport experiments as tissue explants. The rest of the tissue was flash frozen in liquid nitrogen and stored at −70 C until use. NE tissue content was measured using a NE research ELISA kit (Demeditec Diagnostics GmbH, Kiel, Germany), fol-

lowing the manufacturer's instructions. Plasma triacylglycerides (TAG), glucose, and TAG content in WAT were determined as previously described (29). Plasma leptin and insulin levels were assayed using specific rat kits from Spi-Bio (Montigny le Bretonneaux, France). Plasma resistin was assessed using a rat resistin ELISA kit (BioVendor, Brno, Czech Republic), following the manufacturer's instructions.

Glucose tolerance tests

Intraperitoneal glucose tolerance tests were performed in overnight-fasted animals as described (26). Rats from groups 1–3 were weighed and injected ip with 2 g of glucose/kg body weight. Blood samples were obtained from the tail tip at 0, 15, 30, 60, and 120 min after glucose administration, and glucose levels were determined using an Accutrend Glucose Analyzer (Roche Diagnostics Corp., Indianapolis, IN). Plasma insulin levels were determined as above. Overall changes in glucose and insulin during glucose tolerance test were calculated as the area under the curve (AUC) using the GraphPad Prism version 3.03 for Windows (GraphPad Software). The ratio of glucose AUC to insulin AUC was used as an index of whole body insulin sensitivity (30).

Glucose uptake in WAT explants

Glucose uptake was determined, independently, in rWAT and/or eWAT. Fat pad explants (approximately 20 mg), excluding visible connective tissue and blood vessels, were placed immediately after dissection in 0.5 ml Krebs-Ringer-HEPES (KRH) (pH 7.4) supplemented with 1.2 mM $CaCl_2$ and 2% BSA (KRH/Ca^{2+}-BSA). To analyze the effect of resistin (26–49, mouse resistin, Phoenix Pharmaceuticals Inc, Belmont, CA) on glucose uptake, WAT explants from control *ad libitum* rats were preincubated at 37 C in the absence or presence of resistin (200 ng/ml) for 4 h in 0.5 ml KRH/Ca^{2+}-BSA supplemented with 5.0 mM pyruvate before insulin stimulation. All explants were incubated in the absence or presence of 80 nM insulin for 10 min at 37 C. 2-Deoxyglucose (0.2 mM) and [^{3}H]-2-deoxyglucose (2 μCi/ml) (Amersham Pharmacia Biotech) were then added to the explants and incubated a further 10 min at 37 C. Explants were incubated with the transport stop solution (50 μM Cytochalasin B, 5 mM D-glucose) for 10 min at 37 C and washed twice in ice-cold KRH buffer to remove unbound label. Explants were dissolved in NaOH 1N, SDS 0.1%, at 50 C for 2 h, and radioactivity was determined using a Beckman Scintillation counter. Basal glucose uptake was taken as 2-deoxyglucose uptake in the absence of insulin. Data were expressed as pmol 2-deoxyglucose/mg wet weight. The small amount of extracellular 2-deoxyglucose remaining was corrected from the [^{3}H]-2-deoxyglucose content in samples incubated with the transport stop solution for 10 min at 37 C before the transport reaction.

Real-time RT-PCR

Total RNA was isolated from eWAT and/or rWAT using the Trizol reagent (Invitrogen, Carlsbad, CA). The cDNA was synthesized from 1.5 μg of DNase-treated RNA by using the reverse-transcriptase activity from Moloney murine leukemia virus (Life Technologies, Inc.-BRL, Carlsbad, CA), and p[dN]$_6$ (Boehringer Mannheim, Germany) as random primer. Relative quantitation of insulin receptor (IR)-A, IR-B, insulin receptor substrate (IRS)-1, GLUT4, SOCS3, and resistin gene expression was performed by TaqMan real-time PCR according to the manufacturer's protocol on an ABI PRISM 7500 FAST Sequence Detection System instrument and software (PE Applied Biosystem, Foster City, CA). IRS-1, GLUT4, SOCS3, and resistin were measured using Pre-Developed TaqMan Assay Reagents (PE Applied Biosystem). For IR-B, the primers and probe sets were designed using the manufacturer's software and sequences available in GenBank (M29014). The probe was 5′-TCTTCAGGCAATGGTGCT-3′ and spanned 18 nucleotides from the exon 11. For IR-A the primers used were 5′-CCCACCTTTTGAGTCTGACGAT-3′ and 5′-GGGATGGCCTGGGAACA-3′, flanking a fragment of 182 bp. To determine the accumulation of IR-A PCR product, SYBR Green dye was used as a real-time reporter of the presence of double-stranded cDNA.

To normalize the amount of sample cDNA added to the reaction, amplification of endogenous control 18S rRNA was included in separate wells using VIC (TaqMan Assay) or SYBR Green as real-time reporter. The $\Delta\Delta C_T$ method was used to calculate the relative differences between experimental conditions and control groups as fold change in gene expression (31).

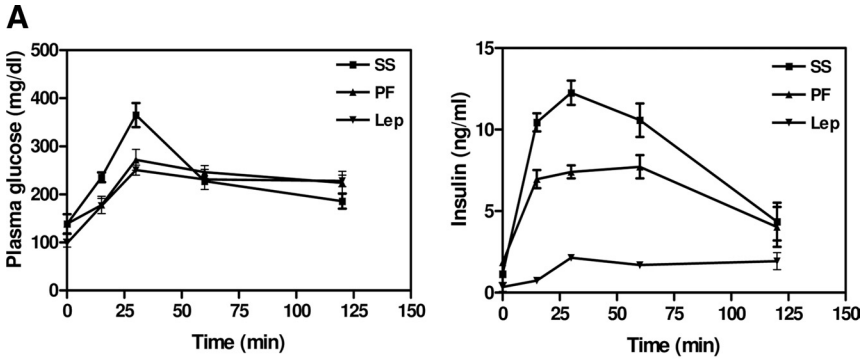
Subcellular fractionation and immunoblot analysis

The tissue was homogenized in 2 volumes of isolation medium [250 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM NaN_3, 5 mM NaF, 1 mM phenylmethylsulfonylfluoride, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin], centrifuged at 450 $\times$ *g* for 5 min, and the infranadant was considered as total extract. The purified

TABLE 1. Effects of central leptin administration on the biological characteristics of the animals

Treatment	SS	PF	Lep
Initial body weight (g)	353 ± 10^a	372 ± 20^a	359 ± 13^a
Final body weight (g)	380 ± 13^a	359 ± 11^b	344 ± 8^b
Daily food intake (g)	19 ± 1^a	13 ± 1^b	13 ± 1^b
Epididymal WAT weight (g)	5.5 ± 0.3^a	5.0 ± 0.4^a	3.7 ± 0.6^b
Retroperitoneal WAT weight (g)	4.9 ± 0.4^a	4.6 ± 1.1^a	2.9 ± 0.4^b
TAG in WAT (mg/g tissue)	18.3 ± 0.6^a	19.4 ± 1.1^a	15.7 ± 0.2^b
Plasma TAG (mg/dL)	68.2 ± 8.2^a	64.7 ± 5.6^a	44.3 ± 5.9^b
Plasma glucose (mM)	6.5 ± 0.8^a	5.9 ± 0.4^a	5.9 ± 0.6^a
Plasma insulin (ng/ml)	1.6 ± 0.5^a	1.4 ± 0.1^a	0.7 ± 0.1^b
Plasma leptin (ng/ml)	5.9 ± 0.5^a	5.4 ± 1^a	5.2 ± 0.8^a
Plasma resistin (ng/ml)	21 ± 1^a	24 ± 2^a	18 ± 2^a

After 7 d of central leptin (Lep) 0.2 μg/d or vehicle (SS or PF) infusion, glucose, insulin, leptin, and resistin were assessed in plasma. Results are the mean ± SEM of four to ten rats per group. Values that do not share the same superscript letter are significantly different ($P < 0.05$).



Insulin sensitivity index	SS	PF	Lep
Glucose AUC /Insulin AUC	35±8	48±7	136±13 **

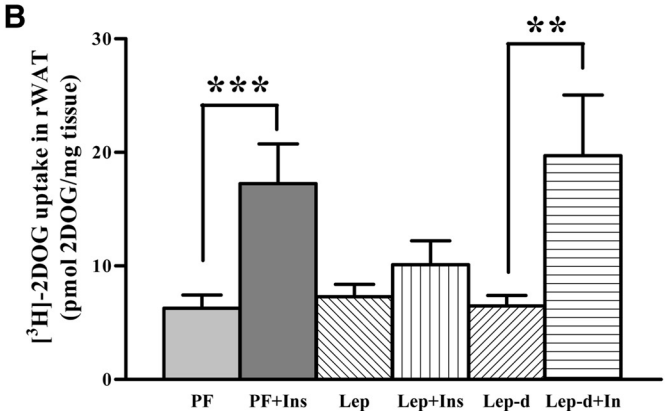


FIG. 1. Effects of central leptin administration on ip glucose tolerance test and glucose uptake in WAT explants. A, Intraperitoneal glucose tolerance test was carried out after 7 d of central leptin (Lep) 0.2 μg/d or vehicle (SS and PF) infusion (rat groups 1–3, see *Materials and Methods*) in overnight fasted rats before the test. Blood was taken from the tail at 0, 15, 30, 60, and 120 min after the glucose administration. Overall changes in glucose and insulin during glucose tolerance test were calculated as the AUC. The ratio of glucose AUC to insulin AUC was used as an index of whole body insulin sensitivity. Data are the mean ± SEM of three to four rats per group (**, $P < 0.05$). B, Glucose uptake in WAT explants after unilateral autonomic nervous system denervation (rat groups 4–5, see *Materials and Methods*). rWAT explants were used for [^{3}H]-2-deoxyglucose uptake measurements in the absence or presence of 80 nM insulin stimulation for 10 min. Results are the mean ± SEM of three to four rats per group, made in triplicate (**, $P < 0.005$; ***, $P < 0.001$). SS, vehicle-infused *ad libitum*-fed rats; PF, vehicle-infused per-fed rats; Lep, leptin-infused rats; Lep-d, leptin-infused in unilateral denervated rats.

plasma membrane (PM), light membranes (LM), and the cytosolic fraction (CYT) were obtained as described previously (32). Membrane protein concentrations were determined using the bicinchoninic (BCA) protein assay kit from Pierce Biotechnology (Cultek, Spain). As we have previously reported (26), β-actin, Na^+/K^+-ATPase, and TGN38 were used as controls for protein loading of the total tissue extract, PM, and LM fractions, respectively (data not shown). Samples from all experimental conditions were run on the same gel, immunoblotted, and detected as previously described (33). Primary polyclonal antibodies were anti-phospho-IR (Tyr^{1146}), anti-IRS-1, anti-phospho-IRS-1(Ser^{307}) (Cell Signaling, Beverly, MA), anti-IRβ, anti-SOCS3, anti-phospho-Akt (Thr^{308}) (Santa Cruz Biotechnology), anti-Akt2/PKBβ (Upstate Biotechnology), anti-resistin (Chemicon), monoclonal antibodies anti-phospho-tyrosine, anti-phospho-Akt (Ser^{473}) (Cell Signaling), anti-GLUT4 (IF8) (Biogenesis), anti-Na^+/K^+-ATPase, anti-TGN38, and anti-β-actin (Abcam, Cambridge, UK). The densitometric values of IR-PY, IRS-1-PY, IRS-1-PS, Akt-PS, or Akt-PT were normalized to the densitometric values of the corresponding amount of protein mass in the same sample. Data were expressed as a ratio of IR-PY/IR, IRS-1-PY/IRS-1, IRS-1-PS/IRS-1, Akt-PS/Akt2, and Akt-PT/Akt2.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 3.03 for Windows (GraphPad Software). Significant differences among groups were determined by one-way ANOVA followed by Tukey test.

Results

Central leptin reduces food intake, body weight, and adiposity

As expected, central leptin administration reduced the daily food intake, and this was accompanied by body weight loss in these rats. Moreover, leptin treatment decreased by 26% and 37% the weights of eWAT and rWAT, respectively, and according to its well-known lipostatic function, central leptin infusion significantly reduced TAG levels in plasma and in WAT. Finally, in agreement with our previous observations (29), fasting plasma insulin levels were also decreased compared with saline-treated pair-fed control rats (Table 1).

Effects of central leptin on insulin sensitivity and on glucose uptake in WAT

To investigate the consequences of central leptin treatment on the overall insulin sensitivity we performed an ip glucose tolerance test. As shown in Fig. 1A, the insulin secretion in leptin-treated rats was significantly lower than in saline-infused *ad libitum* and pair-fed control rats. Consistent with this, the insulin sensitivity index, calculated as the ratio of glucose AUC to insulin AUC, was improved significantly in leptin-treated rats. This result implies that leptin increases the whole-body insulin sensitivity.

Because leptin modulates the glucose utilization in a tissue-specific manner, we analyzed the effects of the hormone on 2-deoxyglucose uptake in WAT explants from saline-treated pair-fed control and leptin-treated rats.

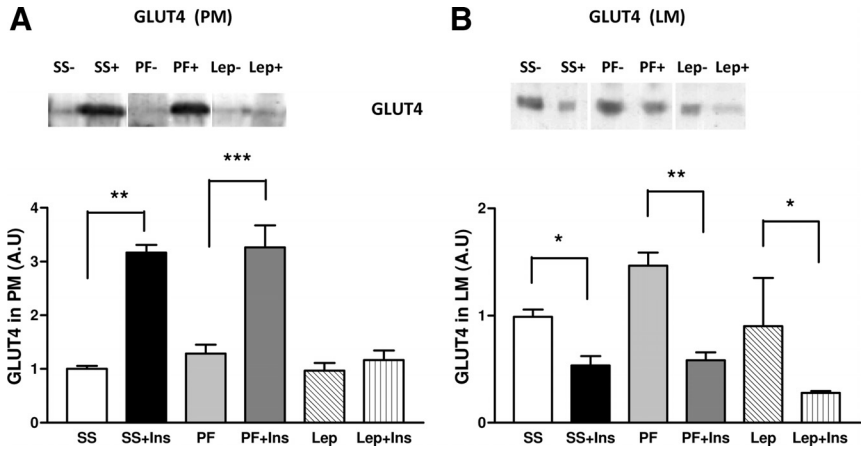


FIG. 2. Central leptin administration impairs the insulin-stimulated GLUT4 translocation to the PM in WAT. After 7 d of central leptin (Lep) 0.2 μg/d or vehicle (SS and PF) infusion, rats (groups 1–3) were injected iv with insulin (Ins) (10 IU/kg body weight) or vehicle injection. Thirty minutes after injection, the eWAT was removed and processed to analyze the amount of GLUT4 in PM and LM fractions. The GLUT4 content in 100 μg of PM (A) and 50 μg of LM (B) was determined by immunoblot. Representative immunoblots are shown. Results are the mean ± SEM of three to four rats per group (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$). SS, vehicle-infused *ad libitum*-fed rats; PF, vehicle-infused per-fed rats; Lep, leptin-infused rats.

Central leptin treatment had no effect on the basal but significantly decreased the insulin-stimulated glucose uptake in rWAT explants compared with saline-treated pair-fed control rats (Fig. 1B). Similar results were observed in eWAT (not shown). To investigate whether central leptin effects on WAT were mediated by neural circuits, we performed surgical denervation of the left rWAT, leaving the contralateral intact right fat pad as an internal control. As shown in Fig. 1B, local autonomic denervation abolished the inhibitory effect of central leptin on glucose transport and restored the insulin stimulation of glucose uptake in rWAT. Because the effect of central leptin took place in the face of unaltered circulating plasma leptin levels (Table 1), this result suggests that leptin action in WAT is indeed indirect and mediated by the autonomic nervous system.

We also examined the ability of insulin to induce the translocation of GLUT4 from intracellular compartments (LM) to the PM in eWAT upon *in vivo* insulin stimulation. In adipose tissue from saline-infused *ad libitum* and pair-fed control rats, *in vivo* insulin administration stimulated the GLUT4 translocation from LM to PM (Fig. 2). However, the ability of insulin to increase the amount of GLUT4 insertion at the PM was prevented by leptin even though the GLUT4 redistribution from LM was unaffected (Fig. 2, A and B).

Central leptin inhibits early steps of *in vivo* insulin signaling in WAT

To analyze the mechanism underlying the inhibitory effect of central leptin on glucose uptake and GLUT4 translocation upon acute *in vivo* insulin stimulation, we studied the effects of a 7-d central leptin infusion on early steps of insulin signaling pathway known to regulate these processes in WAT, such as IR and IRS-1. At the basal state we did not observe changes in IR and IRS-1 tyrosine-phosphorylation after chronic leptin treatment (Fig. 3, A and B). However, leptin treatment significantly augmented the basal content of IRS-1 phosphorylation on Ser307 (Fig. 3C).

The intravenous acute insulin administration stimulated both the IR autophosphorylation and the IRS-1 tyrosine phosphorylation in *ad libitum* and pair-fed control rats (Fig. 3, A and B). In contrast, in chronic leptin-treated rats insulin failed to promote the IR autophosphorylation and the tyrosine phosphorylation of IRS-1 (Fig. 3, A and B) but further increased the content of IRS-1 phosphorylation on the inhibitory Ser307 (Fig. 3C).

Central leptin alters Akt phosphorylation in WAT in response to *in vivo* insulin stimulation

Next we examined the effect of leptin treatment on Akt phosphorylation in WAT upon *in vivo* insulin stimulation. In pair-fed rats, as expected, insulin administration stimulated Akt Ser473 and Thr308 phosphorylation at the PM, and this activated state of the enzyme was maintained at the cytoplasm fraction (Fig. 4, A–D). In contrast, leptin treatment increased the basal Akt Ser473 phosphorylation in both compartments (Fig. 4, A and C) without affecting basal Akt Thr308 phosphorylation (Fig. 4B). Surprisingly, upon insulin stimulation of leptin-treated rats, phosphorylation of Ser473 decreased near to the basal levels of control rats at the PM (Fig. 4A). Our data also indicate that the Akt distributed throughout the cytoplasm is unresponsive to insulin in leptin-treated rats (Fig. 4, C and D).

Effects of central leptin on IR, IRS-1, and GLUT4 mRNA and total protein levels in rat WAT

To further investigate the molecular mechanisms involved in central leptin action on insulin signaling in WAT, we examined the effects of a 7-d central leptin infusion on the mRNA and total protein levels of IR isoforms, IRS-1, and GLUT4 in rat eWAT.

The IR occurs in WAT as two alternatively spliced isoforms, IR-A (exon 11−) and IR-B (exon 11+) (34). To quantify the differential expression of IR-A and IR-B, specific primers were used in each case. Central leptin admin-

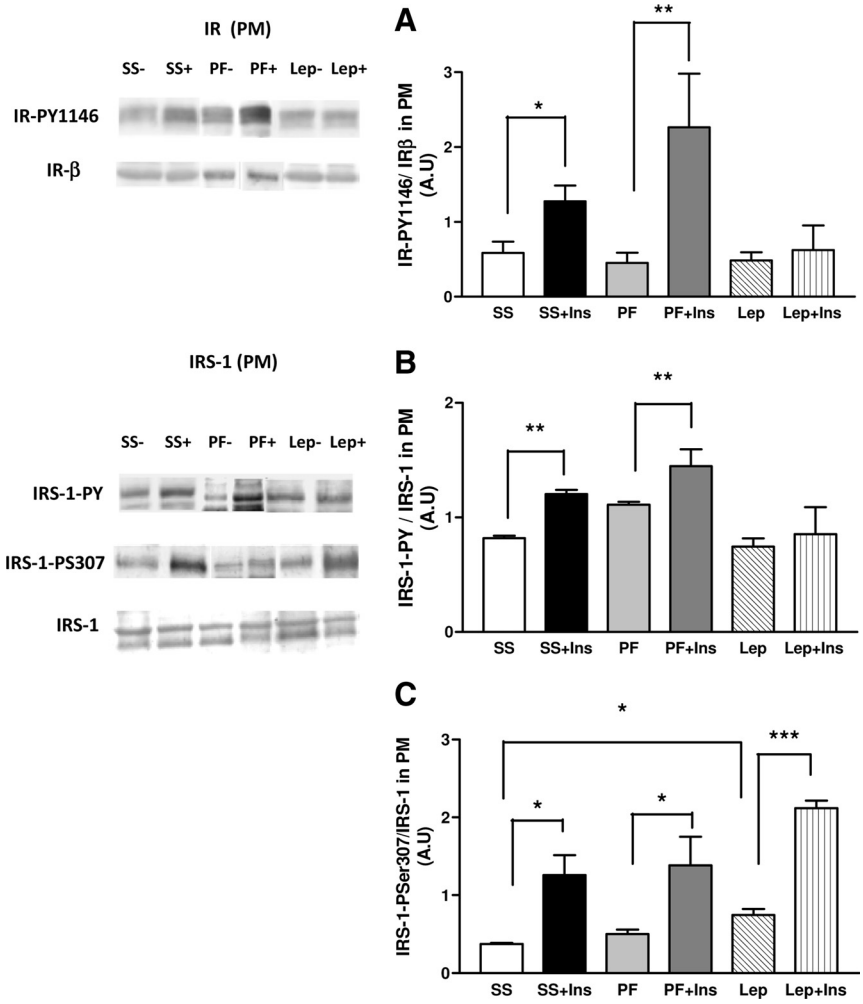


FIG. 3. Central leptin administration inhibits the *in vivo* insulin responsiveness of IR and IRS-1 at the PM of WAT. After 7 d of central leptin (Lep) 0.2 μg/d or vehicle (SS and PF) infusion, rats (groups 1–3) were injected iv with insulin (Ins) or vehicle. Thirty minutes after injection the eWAT was removed and used for immunoblotting analysis in 75 μg of PM using specific antibodies. Representative immunoblots are shown. The data are expressed as ratio of phosphorylated protein to the amount of each protein, respectively: IR-PY1146/IR (A), IRS-1-PY/IRS-1 (B), and IRS-1-PSer307/IRS-1 (C). Densitometric results are the mean ± SEM of three to four rats per group (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$). SS, vehicle-infused *ad libitum*-fed rats; PF, vehicle-infused per-fed rats; Lep, leptin-infused rats.

istration did not affect either the gene expression of the IR isoforms (Fig. 5D) or total IR protein levels (Fig. 5A) in WAT. However, both IRS-1 (Fig. 5, B and D) and GLUT4 (Fig. 5, C and D) mRNA and total protein levels significantly decreased in leptin-infused compared with pair-fed rats.

Chronic central leptin treatment increases SOCS3 and resistin mRNA and protein levels in rat WAT: effect of autonomic denervation

Having determined the effects of central leptin on early steps of insulin signaling pathway involved in glucose uptake in WAT, and because SOCS3 and resistin are two potentially negative regulators of insulin signaling, we decided to examine the expression levels of both proteins in rWAT upon central leptin administration. Our results show that SOCS3 mRNA (Fig. 6C) and protein levels (Fig. 6A) were increased in leptin-infused compared with pair-fed control rats. Moreover, a marked increase of 4.7-fold in mRNA (Fig. 6C) and 3.6-fold in resistin protein levels (Fig. 6B) was also observed in leptin-infused rats compared with the pair-fed ones. Central leptin treatment also induced similar changes in eWAT (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org/).

We have demonstrated that surgical autonomic denervation restored the insulin-stimulated glucose uptake in WAT in central leptin treated rats (Fig. 1B). Thus, we next assessed whether denervation of the rWAT would also attenuate the up-regulation of SOCS3 and resistin expression after central leptin administration. Surgical autonomic denervation resulted in a modest but statistically significant decrease in the mRNA and, to a higher extent, in the protein levels of both SOCS3 and resistin (Fig. 6). In fact, upon denervation SOCS3 protein levels were similar to pair-fed control rats, whereas there was a ~1.7-fold decrease in resistin protein levels. Collectively, these data support that these proteins could contribute to the inhibitory effect of central leptin on glucose uptake in WAT.

Effect of resistin on glucose uptake in WAT explants

Although central leptin administration for 7 d increases resistin mRNA and protein levels in WAT (Fig. 6 and Supplemental Fig. 1), interestingly, central leptin infusion did not alter plasma resistin levels (Table 1). These data suggest that resistin, acting nearby the WAT, is likely to cause local but not systemic insulin resistance. To directly address the effects of resistin on glucose uptake in this tissue, we preincubated rWAT explants with resistin (200 ng/ml) for 4 h and then analyzed the glucose uptake in the absence or presence of insulin. As shown in Fig. 7, no significant differences were found in basal glucose uptake values (14.39 ± 1.5 *vs.* 16.35 ± 1.8 pmol 2-deoxyglucose/mg tissue, n = 4; *P* not significant) in WAT explants preincubated with or without resistin, respectively. However,

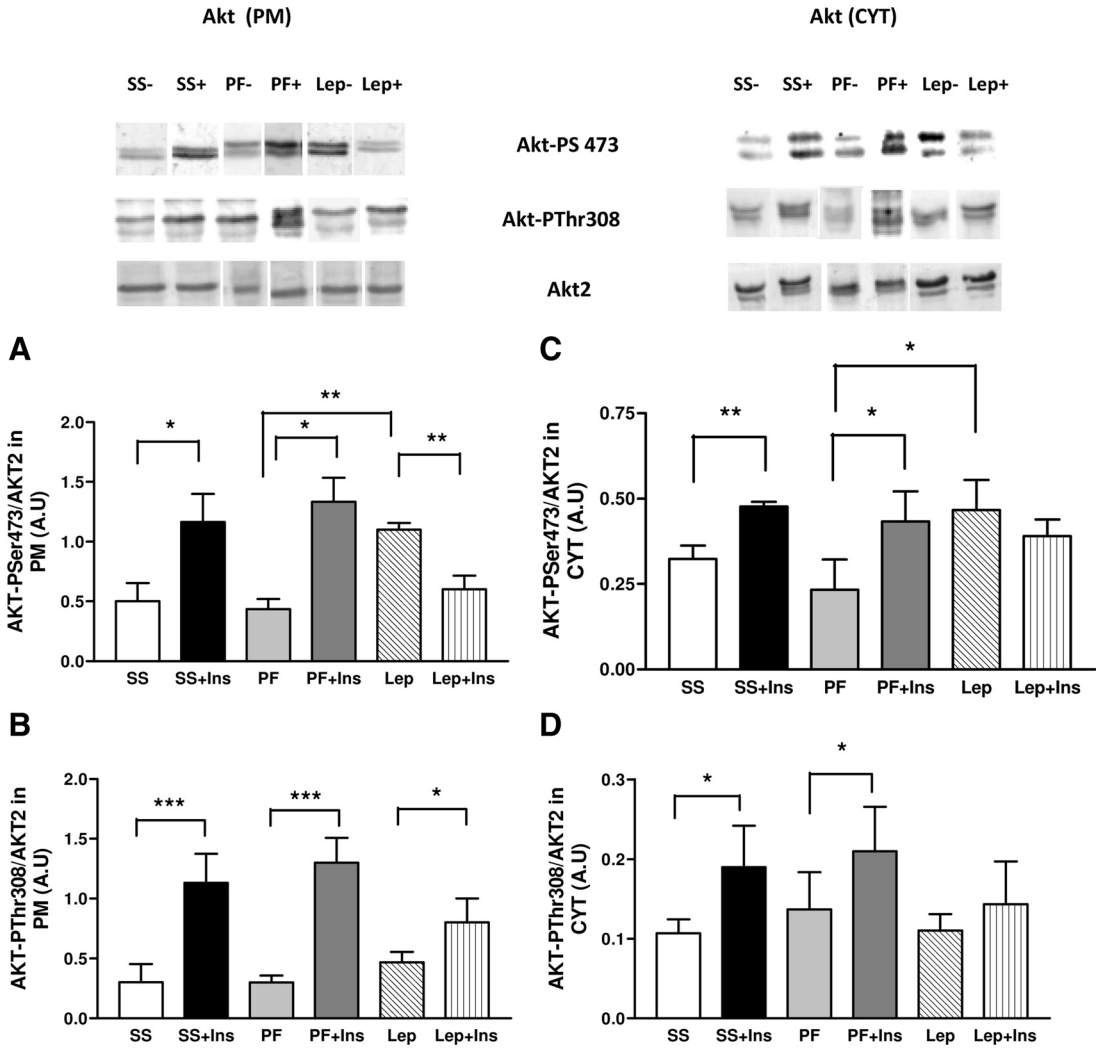


FIG. 4. Central leptin administration alters the *in vivo* insulin-induced Akt phosphorylation in WAT. After 7 d of central leptin (Lep) 0.2 μg/d or vehicle (SS and PF) infusion, rats (groups 1–3) were injected iv with insulin (Ins) or vehicle. Thirty minutes after injection, the eWAT was removed and used to analyze in PM and CYT fractions the Akt phosphorylation on Ser473 and Thr308 residues, respectively. Representative immunoblots are shown. The data are expressed as ratio of PSer473/Akt (A and C) and PT308Akt /Akt (B and D) in 75 μg of PM or 50 μg of CYT, respectively. Densitometric results are the mean ± SEM of three to four rats per group (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$). SS, vehicle-infused *ad libitum*-fed rats; PF, vehicle-infused per-fed rats; Lep, leptin-infused rats.

there was a 37% significant decrease in the insulin-stimulated glucose transport in WAT explants preincubated with resistin compared with untreated controls (17.05 ± 2.5 *vs.* 26.87 ± 3.1 pmol 2-deoxyglucose/mg tissue, respectively; n = 4; $P < 0.01$). Although insulin did increase glucose uptake above control in the presence of resistin the magnitude of the response was significantly attenuated (1.18-fold *vs.* 1.64-fold; $P < 0.01$) in the presence or in the absence of resistin, respectively, after insulin stimulation.

Discussion

Several studies have reported that central or peripheral leptin treatment enhances whole-body insulin sensitivity in terms of glucose metabolism but promotes insulin-resistance in WAT (2–4). While the peripheral actions of leptin are known to take place through leptin receptors, the neural pathways and the neurochemical phenotypes of the neurons constituting the autonomic nervous circuits which transmit leptin signal to WAT are largely unknown (35). Nevertheless, it is well established that central leptin, independently of its effects on food intake, regulates WAT lipid metabolism by increasing lipolysis and suppressing *de novo* lipogenesis (27, 29, 36). However, the mechanisms underlying the inhibitory effect of central leptin on the insulin-dependent glucose uptake in WAT, which may contribute to decrease glucose availability for free fatty acid (FFA) esterification and lipid deposition in this tissue, are not completely understood.

In the present study we provide new insights on the mechanisms by which central leptin regulates glucose uptake in WAT: inhibition of insulin-stimulated tyrosine

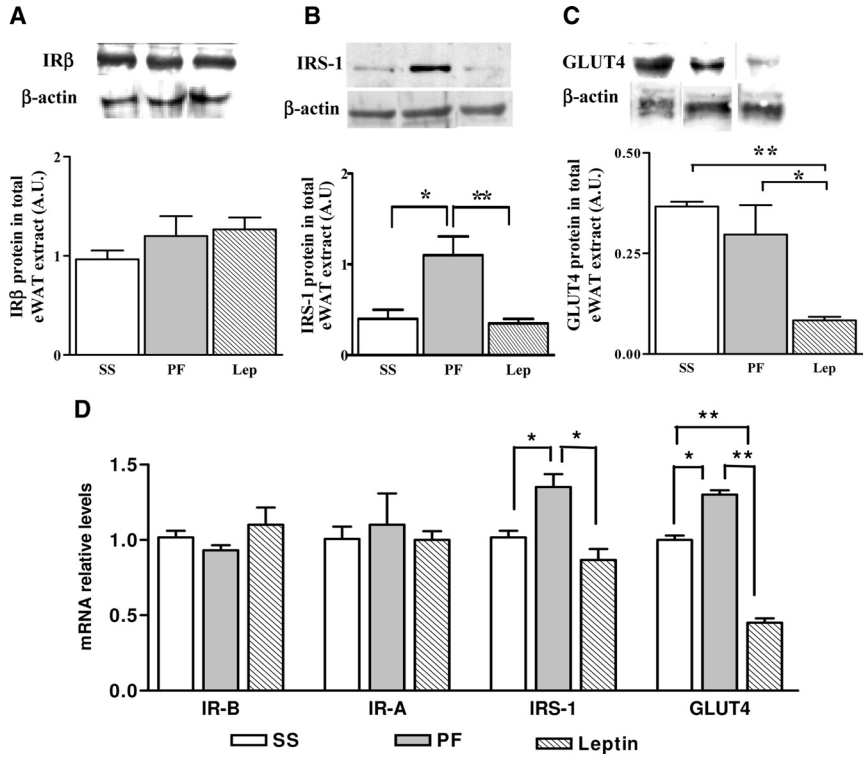


FIG. 5. Effect of centrally infused leptin on IR, IRS-1, and GLUT4 mRNA and total protein levels in WAT. The eWAT of rat (groups 1–3) infused for 7 d with central leptin (Lep) 0.2 μg/d or vehicle (SS and PF) was removed and processed to analyze total protein content and mRNA levels of IR-β, IRS-1, and GLUT4. IR-β (A), IRS-1 (B), and GLUT4 (C) protein content in 70 μg of total extract was determined by immunoblotting using specific antibodies for each protein. Representative immunoblots are shown. The data are expressed as ratio of each protein after correction for β-actin. Densitometric results are the mean ± SEM of three to four rats per group (*, $P < 0.05$; **, $P < 0.005$). D, Changes in mRNA levels in eWAT after central leptin treatment using 18S rRNA as the invariant control. The results are the mean ± SEM of three to four rats per group (*, $P < 0.05$; **, $P < 0.005$). SS, vehicle-infused *ad libitum*-fed rats; PF, vehicle-infused per-fed rats; Lep, leptin-infused rats.

phosphorylation of IR and IRS-1, suppression of Akt activation, the down-regulation of the mRNA and protein levels of both IRS-1 and GLUT4, and suppression of insulin-stimulated GLUT4 insertion at the PM. As a result, the glucose uptake in WAT was impaired in central leptin-treated rats, an effect that was dependent of an intact autonomic nervous system. In fact, surgical denervation of WAT abolished the inhibitory effect of leptin on glucose uptake and restored the insulin stimulation of glucose transport in this tissue. Our results agree with previous data showing that the inhibition of WAT lipogenesis in rats by central leptin is lost after surgical WAT denervation (27). The fact that plasma leptin does not increase after chronic central leptin administration excludes that the observed effects of this treatment were attributable to peripheral actions of the hormone.

A major finding of the present work is that the effects of central leptin on insulin signaling in WAT were paralleled with the marked up-regulation of SOCS3 and resistin mRNA and protein levels in WAT upon central leptin administration. Because plasma resistin levels were not modified upon central leptin treatment but were significantly increased in WAT, these data suggest that central leptin, increasing resistin and SOCS3 levels in WAT, could contribute to tissue-specific but not systemic insulin resistance. Supporting this contention, it has been reported that resistin, inducing SOCS3 expression, attenuates early steps of insulin signaling pathway (IR autophosphorylation, IRS-1 tyrosine phosphorylation, and Akt activation) in 3T3-L1 adipocytes, while the inhibition of SOCS3 function, expressing a dominant negative SOCS protein, prevented resistin from antagonizing insulin action in adipocytes (22).

The inhibitory effect of central leptin on insulin-stimulated glucose uptake in WAT could be mediated by the melanocortin receptor (MCR) system. In fact, the intracerebroventricular infusion of SHU9119, a MCR antagonist, significantly decreased the glucose uptake in brown adipose tissue, heart, and skeletal muscle while increasing glucose uptake as well as TAG synthesis and deposition in WAT in rats, under clamp conditions (37). On the other hand, the intracerebroventricular injection of SHU9119 abolished the stimulatory effect on glucose uptake in peripheral tissues induced by leptin injection into the ventromedial hypothalamus except in WAT (38). These results suggest that MCR system is necessary for the control of glucose uptake by peripheral tissues induced by central leptin.

We have previously shown that in hyperleptinemic aged insulin resistant rats a reduction in the expression of the IR-B isoform occurs, without changes in IR-A (34). Thus, we hypothesized that chronic central leptin could affect the mRNA levels of the IR isoforms. Central leptin administration for 7 d did not affect the IR isoform gene expression or total IR protein levels but inhibited the *in vivo* insulin stimulation of IR autophosphorylation. Our data also indicate that chronic central leptin inhibited the insulin-stimulated IRS-1 tyrosine phosphorylation, increased IRS-1 phosphorylation on Ser307, which is well known to promote IRS-1 degradation (39), and this was accompanied by reduced IRS-1 mRNA and protein levels. These changes occurred in parallel with the marked up-regulation of SOCS3 upon central leptin administration

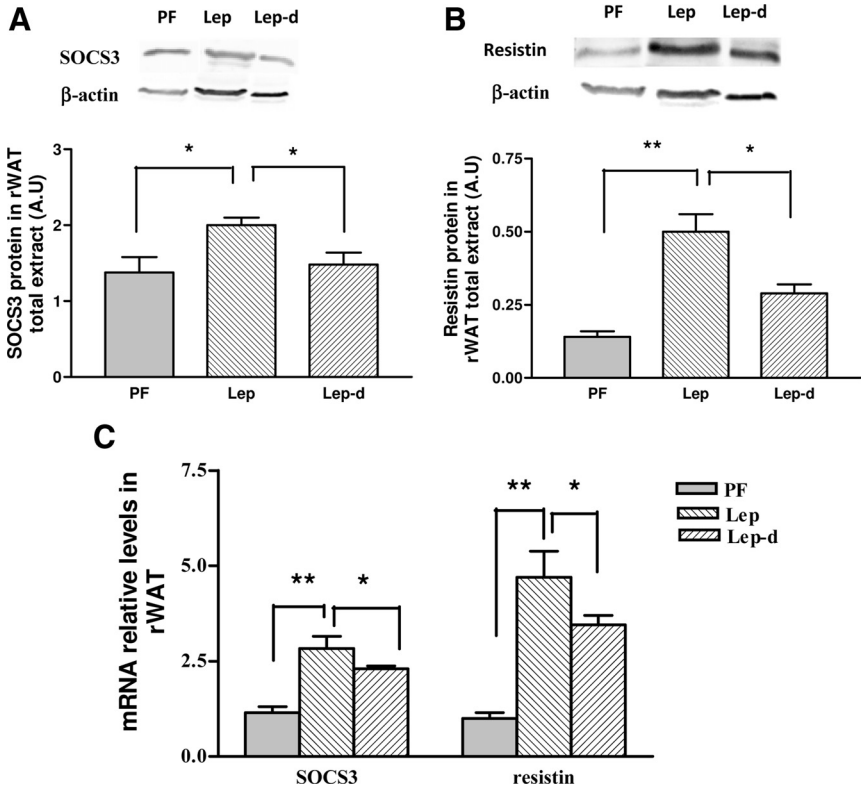


FIG. 6. Central leptin administration increases SOCS3 and resistin expression in rWAT. After unilateral autonomic nervous system denervation, the rWAT of rat (groups 4–5) infused for 7 d with central leptin (Lep) 0.2 μg/d or vehicle (PF) was removed and processed to analyze total protein content levels of SOCS3 (A) and resistin (B) in 70 μg of total extract using specific antibodies. Representative immunoblots are shown. The data are expressed as ratio of each protein after correction for β-actin. Densitometric results are the mean ± SEM of three to four rats per group (*, $P < 0.05$; **, $P < 0.005$). C, Changes in SOCS3 and resistin mRNA levels in rWAT after central leptin treatment using 18S rRNA as the invariant control. The results are the mean ± SEM of three to four rats per group (*, $P < 0.05$; **, $P < 0.005$). PF, vehicle-infused per-fed rats; Lep, leptin-infused rats; Lep-d, leptin-infused in unilateral denervated rats.

reported herein. SOCS3 is known to act as a negative regulator of insulin signaling, inhibiting the insulin-stimulated tyrosine phosphorylation of IR and IRS-1 (19, 20, 40), and promoting IRS-1 protein degradation (39, 41). Although we have not directly addressed IRS-1 protein degradation, our results suggest that central leptin could drive IRS-1 to proteasome degradation. In fact, central leptin promotes a ~2.2-fold higher decrease in IRS-1 total protein than in mRNA levels. Taken together, our results demonstrate that central leptin inhibits early steps of the signaling pathway normally used by insulin in the regulation of glucose uptake in WAT.

Full activation of Akt by insulin requires hierarchical phosphorylation of two residues, Thr308 and Ser473, by the PDK1 protein kinase and the Rictor-mammalian target of rapamycin complex (mTORC)2, respectively (42, 43). Thus, we next examined the effect of central leptin on *in vivo* insulin stimulation of Akt phosphorylation and GLUT4 translocation to the PM. It has previously been reported that Akt Ser473-phosphorylation in liver and muscle in rodents was unaltered (13) or increased upon peripheral leptin treatment *in vivo* (44, 45). The results presented herein demonstrate that central leptin elicits an increase in the basal content of Akt Ser473-phosphorylation and inhibits normal Akt activation after insulin stimulation. Consequently, central leptin impairs the *in vivo* insulin-stimulated GLUT4 insertion at the PM in WAT. Moreover, leptin decreases GLUT4 mRNA and protein levels, which agrees with previous data in sc leptin-treated rats (4). As a result, the insulin-stimulated glucose uptake in WAT was impaired in central leptin-treated rats. As the main role of insulin in WAT is to promote the incorporation of glucose into TAG, the inhibition of glucose uptake by central leptin may contribute to regulate glucose availability for FFA esterification and to control lipid deposition in this tissue. In addition, there is evidence reporting that central leptin administration decreased both lipoprotein lipase expression and FFA uptake in WAT (27). All these observations, together with the decrease in the visceral fat weight and in the content of TAG in WAT in leptin-treated rats reported herein, are consistent with the putative function of the hormone in regulating adiposity.

In addition to the role of Akt on insulin-stimulated glucose uptake, it has been shown that the pharmacological inhibition of Akt resulted in a reduction in basal and insulin-stimulated lipogenesis in isolated rat adipocytes (46). Thus, the suppression of insulin-stimulated Akt activation in leptin-treated rats reported herein could be consistent with previous data showing the inhibition of WAT lipogenesis by central leptin (27). Moreover, insulin signaling via PI3K/Akt activates mTORC1 leading to increased hepatic lipogenesis (47). In addition, the activation of mTORC1 signaling in 3T3-L1 adipocytes by ectopic expression of Rheb increases *de novo* lipogenesis and promotes intracellular accumulation of TAG, while the inhibition of mTORC1 signaling by rapamycin or by knock down of raptor stimulates lipolysis primarily via activation of ATGL expression (48). Consequently, it can be speculated that the inhibition by central leptin of insulin-stimulated Akt activation reported herein could affect mTORC1 activation and ATGL expression in WAT. In support of this, we have previously shown that

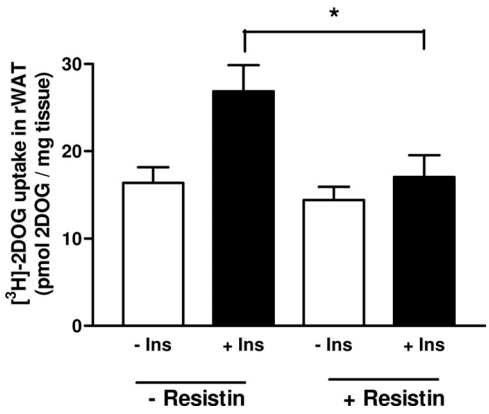


FIG. 7. Resistin inhibits insulin-stimulated glucose uptake in WAT explants. rWAT explants from ad libitum control rats preincubated without or with resistin (200 ng/ml) for 4 h were used for [^{3}H]-2-deoxyglucose uptake measurements in the absence (*white bars*) or in the presence (*black bars*) of 80 nM insulin stimulation for 10 min. Results are the mean ± SEM of three to four rats, made in triplicate (*, $P < 0.01$ *vs.* insulin in the absence of resistin).

central leptin administration significantly increased ATGL expression in WAT (29) and impaired the insulin-mediated phosphorylation of both mTOR and S6K in this tissue (unpublished data). These results are consistent with the inhibition by leptin of the insulin-stimulated Akt activation reported herein. Nevertheless, these suggestions need further work before a final conclusion may be drawn.

Several hormones and cytokines associated with insulin resistance, such as leptin, insulin, IL-6, TNF-α, and resistin, induce SOCS3 expression acting directly on adipocytes *in vitro* (12, 20–22). Because SOCS3 expression in WAT in mice is induced after an ip resistin injection (22), we hypothesized that resistin expression in WAT could be up-regulated by central leptin. Contrary to previous data showing either no change or decreased resistin mRNA levels in WAT upon central leptin infusion (49, 50), we found that central leptin, infused at a 50-fold lower concentration than in previous studies (49, 50), markedly increased resistin mRNA and total protein levels in WAT, without affecting plasma resistin levels. Although we cannot establish a direct association between central leptin effects on insulin signaling and resistin up-regulation in WAT, several lines of evidences suggest that resistin, acting in an autocrine/paracrine manner, might lead to WAT insulin resistance. First, local autonomic denervation of WAT restored the insulin stimulation of glucose uptake, and this was paralleled by the marked decrease in SOCS3 and resistin protein levels in this tissue. Although the resistin protein content was still higher in leptin-treated than in pair-fed control rats upon denervation, it can be argued that resistin, at the protein levels reported herein, might not be sufficient to inhibit the glucose transport in response to insulin. In fact, it has been reported that the complete inhibition of insulin-stimulated glucose uptake was observed between 100 to 500 nM resistin in L6 skeletal muscle cells (51). Because the immunoblot analyses provide relative protein data, additional studies have to be done before a final conclusion may be drawn. Second, in agreement with previous data analyzing the direct effect of resistin on glucose uptake in 3T3-L1 adipocytes (52) and L6 cells (53) or in 3T3-L1 adipocytes stably expressing resistin (54), we demonstrate that resistin decreased insulin stimulation of glucose uptake in WAT explants. This observation provides direct support for the evidence that, upon central leptin administration, the up-regulation of resistin levels in WAT can contribute to the inhibition of the insulin-stimulated glucose uptake in this tissue. Finally, our data are consistent with previous reports showing that resistin inhibits the early steps of insulin signaling pathway involved in glucose uptake in 3T3-L1 adipocytes (52, 54) and L6 cells (53). Nevertheless, the contribution of other factors promoting insulin resistance in WAT upon central leptin administration should be taken into account. In fact, central leptin increases both SOCS3 and IL-6 mRNA levels in WAT (27). Thus, we suggest that the adipose-derived factor resistin and the cytokine suppressor SOCS3 could be implicated in the local attenuation of the insulin action in WAT upon chronic central leptin treatment.

In conclusion, we have shown that leptin, acting at a central level, negatively modulates early steps of the signaling pathway normally used by insulin in the regulation of glucose uptake in WAT. Because local autonomic denervation of WAT abolished the inhibitory effect of central leptin on glucose transport and decreased SOCS3 and resistin levels in this tissue, our data also suggest that resistin might be a mediator of central leptin antagonism of insulin action in WAT. Because glucose uptake from extracellular sources is an important step to promote TAG formation and *de novo* lipogenesis in WAT, the inhibition of glucose uptake by central leptin may contribute to regulate excessive fat mass enlargement, a factor known for playing a key role in the development of overall insulin resistance.

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References

1. **Sivitz WI, Walsh SA, Morgan DA, Thomas MJ, Haynes WG** 1997 Effects of leptin on insulin sensitivity in normal rats. Endocrinology 138:3395–3401
2. **Cusin I, Zakrzewska KE, Boss O, Muzzin P, Giacobino JP, Ricquier D, Jeanrenaud B, Rohner-Jeanrenaud F** 1998 Chronic central leptin infusion enhances insulin-stimulated glucose metabolism and favors the expression of uncoupling proteins. Diabetes 47:1014–1019
3. **Minokoshi Y, Haque MS, Shimazu T** 1999 Microinjection of leptin into the ventromedial hypothalamus increases glucose uptake in peripheral tissues in rats. Diabetes 48:287–291
4. **Wang JL, Chinookoswong N, Scully S, Qi M, Shi ZQ** 1999 Differential effects of leptin in regulation of tissue glucose utilization in vivo. Endocrinology 140:2117–2124
5. **Ceddia RB, Koistinen HA, Zierath JR, Sweeney G** 2002 Analysis of paradoxical observations on the association between leptin and insulin resistance. FASEB J 16:1163–1176
6. **Haque MS, Minokoshi Y, Hamai M, Iwai M, Horiuchi M, Shimazu T** 1999 Role of the sympathetic nervous system and insulin enhancing glucose uptake in peripheral tissues after intrahypothalamic injection of leptin in rats. Diabetes 48:1706–1712
7. **Müller G, Ertl J, Gerl M, Preibisch G** 1997 Leptin impairs metabolic actions of insulin in isolated rat adipocytes. J Biol Chem 272:10585–10593
8. **Ceddia RB, William Jr WN, Lima FB, Curi R** 1998 Leptin inhibits insulin-stimulated incorporation of glucose into lipids and stimulates glucose decarboxylation in isolated rat adipocytes. J Endocrinol 158:R7–R9
9. **Ceddia RB, William Jr WN, Lima FB, Flandin P, Curi R, Giacobino JP** 2000 Leptin stimulates uncoupling protein-2 mRNA expression and Krebs cycle activity and inhibits lipid synthesis in isolated rat white adipocytes. Eur J Biochem 267:5952–5958
10. **Zierath JR, Frevert EU, Ryder JW, Berggren PO, Kahn BB** 1998 Evidence against a direct effect of leptin on glucose transport in skeletal muscle and adipocytes. Diabetes 47:1–4
11. **Ranganathan S, Ciaraldi TP, Henry RR, Mudaliar S, Kern PA** 1998 Lack of effect of leptin on glucose transport, lipoprotein lipase, and insulin action in adipose and muscle cells. Endocrinology 139:2509–2513
12. **Pérez C, Fernández-Galaz C, Fernández-Agulló T, Arribas C, Andrés A, Ros M, Carrascosa JM** 2004 Leptin impairs insulin signaling in rat adipocytes. Diabetes 53:347–353
13. **Kim YB, Uotani S, Pierroz DD, Flier JS, Kahn BB** 2000 In vivo administration of leptin activates signal transduction directly in insulin-sensitive tissues: overlapping but distinct pathways from insulin. Endocrinology 141:2328–2339
14. **Barzilai N, Banerjee S, Hawkins M, Chen W, Rossetti L** 1998 Caloric restriction reverses hepatic insulin resistance in aging rats by decreasing visceral fat. J Clin Invest 101:1353–1361
15. **Gabriely I, MA XH, Yang XM, Atzmon G, Rajala MW, Berg AH, Scherer P, Rossetti L, Barzilai N** 2002 Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process? Diabetes 51:2951–2958
16. **Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, Minnemann T, Shulman GI, Kahn BB** 2001 Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 409:729–733
17. **Tozzo E, Gnudi L, Kahn BB** 1997 Amelioration of insulin resistance in streptozotocin diabetic mice by transgenic overexpression of GLUT4 driven by an adipose-specific promoter. Endocrinology 138:1604–1611
18. **Emanuelli B, Peraldi P, Filloux C, Sawka-Verhelle D, Hilton D, Van Obberghen E** 2000 SOCS3 is an insulin-induced negative regulator of insulin signaling. J Biol Chem 275:15985–15991
19. **Shi H, Cave B, Inouye K, Bjørbaek C, Flier JS** 2006 Overexpression of suppressor of cytokine signaling 3 in adipose tissue causes local but not systemic insulin resistance. Diabetes 55:699–707
20. **Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E** 2001 SOCS3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-α in the adipose tissue of obese mice. J Biol Chem 276:47944–47949
21. **Shi H, Tzameli I, Bjørbaek C, Flier JS** 2004 Suppressor of cytokine signaling 3 is a physiological regulator of adipocyte insulin signalling. J Biol Chem 279:34733–34740
22. **Steppan CM, Wang J, Whiteman EL, Birnbaum MJ, Lazar MA** 2005 Activation of SOCS-3 by resistin. Mol Cell Biol 25:1569–1575
23. **Fei H, Okano HJ, Li C, Lee GH, Zhao C, Darnell R, Friedman JM** 1997 Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proc Natl Acad Sci USA 94:7001–7005
24. **Prodi E, Obici S** 2006 Minireview: The brain as a molecular target for diabetic therapy. Endocrinology 147:2664–2669
25. **Kreier F, Kap YS, Mettenleiter TC, van Heijningen C, van der Vliet J, Kalsbeek A, Sauerwein HP, Fliers E, Romijn JA, Buijs RM** 2006 Tracing from fat tissue, liver, and pancreas: a neuroanatomical framework for the role of the brain in type 2 diabetes. Endocrinology 147:1140–1147
26. **Bonzón-Kulichenko E, Schwudke D, Gallardo N, Moltó E, Fernández-Agulló T, Shevchenko A, Andrés A** 2009 Central leptin regulates total ceramide content and SREBP- 1C proteolytic maturation in rat white adipose tissue. Endocrinology 150:169–178
27. **Buettner C, Muse ED, Cheng A, Chen L, Scherer T, Pocai A, Su K, Cheng B, Li X, Harvey-White J, Schwartz GJ, Kunos G, Rossetti L** 2008 Leptin controls adipose tissue lipogenesis via central, STAT3-independent mechanisms. Nat Med 14:667–675
28. **Fernández-Galaz C, Fernández-Agullo T, Pérez C, Peralta S, Arribas C, Andrés A, Carrascosa JM, Ros M** 2002 Long-term food restriction prevents aging-associated central leptin resistance in wistar rats. Diabetologia 45:997–1003
29. **Gallardo N, Bonzón-Kulichenko E, Fernández-Agulló T, Moltó E, Gómez-Alonso S, Blanco P, Carrascosa JM, Ros M, Andrés A** 2007 Tissue-specific effects of central leptin on the expression of genes involved in lipid metabolism in liver and white adipose tissue. Endocrinology 148:5604–5610
30. **Levy JR, Davenport B, Clore JN, Stevens W** 2002 Lipid metabolism and resistin gene expression in insulin-resistant Fischer 344 rats. Am J Physiol Endocrinol Metab 282:E626–E633
31. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408
32. **Simpson IA, Yver DR, Hissin PJ, Wardzala LJ, Karnieli E, Salans LB, Cushman SW** 1983 Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. Biochim Biophys Acta 763:393–407
33. **Villar M, Serrano R, Gallardo N, Carrascosa JM, Martinez C, Andrés A** 2006 Altered subcellular distribution of IRS-1 and IRS-3 is associated with defective Akt activation and GLUT4 translocation in insulin-resistant old rat adipocytes. Biochim Biophys Acta 1763:197–206
34. **Serrano R, Villar M, Martínez C, Carrascosa JM, Gallardo N, Andrés A** 2005 Differential gene expression of insulin receptor isoforms A and B and insulin receptor substrates 1, 2 and 3 in rat tissues:

modulation by aging and differentiation in rat adipose tissue. J Mol Endocrinol 34:153–161
35. **Bartness TJ, Song CK** 2007 Brain-Adipose tissue neural crosstalk. Physiol Behav 91:343–351
36. **Nogueiras R, Wilson H, Rohner-Jeanrenaud F, Tschöp MH** 2008 Central nervous system regulation of adipocyte metabolism. Regul Pept 149:26–31
37. **Nogueiras R, Wiedmer P, Perez-Tilve D, Veyrat-Durebex C, Keogh JM, Sutton GM, Pfluger PT, Castaneda TR, Neschen S, Hofmann SM, Howles PN, Morgan DA, Benoit SC, Szanto I, Schrott B, Schürmann A, Joost HG, Hammond C, Hui DY, Woods SC, Rahmouni K, Butler AA, Farooqi IS, OŘahilly S, Rohner-Jeanrenaud F, Tschöp MH** 2007 The central melanocortin system directly controls peripheral lipid metabolism. J Clin Invest 117:3475–3488
38. **Toda C, Shiuchi T, Lee S, Yamato-Esaki M, Fujino Y, Suzuki A, Okamoto S, Minokoshi Y** 2009 Distinct effects of leptin and a melanocortin receptor agonist injected into medial hypothalamic nuclei on glucose uptake in peripheral tissues. Diabetes 58:2757–2765
39. **Greene MW, Sakaue H, Wang L, Alessi DR, Roth RA** 2003 Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by serine 312 phosphorylation. J Biol Chem 278:8199–8211
40. **Ueki K, Kondo T, Kahn CR** 2004 Suppressor of cytokine signaling 1 (SOCS-1) and (SOCS3) cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol 24:5434–5446
41. **Rui L, Yuan M, Frantz D, Shoelson S, White MF** 2002 SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 277:42394–42398
42. **Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT** 1997 Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science 277:567–570
43. **Hresko RC, Mueckler M** 2005 mTOR.RICTOR is the ser473 kinase for AKT/Protein kinase B in 3T3–L1 adipocytes. J Biol Chem 280:40406–40416
44. **Maroni P, Bendinelli P, Piccoletti R** 2003 Early intracellular events induced by in vivo leptin treatment in mouse skeletal muscle. Mol Cell Endocrinol 201:109–121
45. **Huang W, Dedousis N, Bandi A, Lopaschuk GD, O'Doherty RM** 2006 Liver triglyceride secretion and lipid oxidative metabolism are rapidly altered by leptin in vivo. Endocrinology 147:1480–1487
46. **Berggreen C, Gormand A, Omar B, Degerman E, Göransson O** 2009 Protein kinase B activity is required for the effects of insulin on lipid metabolism in adipocytes. Am J Physiol Endocrinol Metab 296:E635–E646
47. **Li S, Brown MS, Goldstein JL** 2010 Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. Proc Natl Acad Sci USA 107:3441–3446
48. **Chakrabarti P, English T, Shi J, Smas CM, Kandror KV** 2010 The mTOR complex 1 suppresses lipolysis, stimulates lipogenesis and promotes fat storage. Diabetes 59:775–781
49. **Lin J, Choi YH, Harztell DL, Li C, Della-Fera MA, Baile CA** 2003 CNS melanocortin and leptin effects on stearoyl-CoA desaturase-1 and resistin expression. Biochem Biophys Res Commun 311:324–328
50. **Asensio C, Cettour-Rose P, Theander-Carrillo C, Rhoner-Jeanrenaud F, Muzzin P** 2004 Changes in glycemia by leptin administration or high-fat feeding in rodent models of obesity/type 2 diabetes suggest a link between resistin expression and control of glucose homeostasis. Endocrinology 145:2206–2213
51. **Moon B, Kwan JJ, Duddy N, Sweeney G, Begum N** 2003 Resistin inhibits glucose uptake in L6 cells independently of changes in insulin signaling and GLUT4 translocation. Am J Physiol Endocrinol Metab 285:E106–E115
52. **Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA** 2001 The hormone resistin links obesity to diabetes. Nature 409:307–312
53. **Palanivel R, Maida A, Liu Y, Sweeney G** 2006 Regulation of insulin signaling, glucose uptake and metabolism in rat skeletal muscle cells upon prolonged exposure to resistin. Diabetologia 49:183–190
54. **Fernández CM, del Arco A, Gallardo N, Aguado L, Rodriguez M, Ros M, Carrascosa JM, Andrés A, Arribas C** 2010 S-resistin inhibits adipocyte differentiation and increases TNFalpha expression and secretion in 3T3–L1 cells. Biochim Bipohys Acta 1803:1131–1141