

Central Resistin Overexposure Induces Insulin Resistance Through Toll-Like Receptor 4

Yacir Benomar,^{1,2} Arieh Gertler,³ Pamela De Lacy,⁴ Delphine Crépin,^{1,2} Hassina Ould Hamouda,^{1,2} Laure Riffault,^{1,2} and Mohammed Taouis^{1,2}

Resistin promotes both inflammation and insulin resistance associated with energy homeostasis impairment. However, the resistin receptor and the molecular mechanisms mediating its effects in the hypothalamus, crucial for energy homeostasis control, and key insulin-sensitive tissues are still unknown. In the current study, we report that chronic resistin infusion in the lateral cerebral ventricle of normal rats markedly affects both hypothalamic and peripheral insulin responsiveness. Central resistin treatment inhibited insulin-dependent phosphorylation of insulin receptor (IR), AKT, and extracellular signal-related kinase 1/2 associated with reduced IR expression and with upregulation of suppressor of cytokine signaling-3 and phosphotyrosine phosphatase 1B, two negative regulators of insulin signaling. Additionally, central resistin promotes the activation of the serine kinases Jun NH₂-terminal kinase and p38 mitogen-activated protein kinase, enhances the serine phosphorylation of insulin receptor substrate-1, and increases the expression of the proinflammatory cytokine interleukin-6 in the hypothalamus and key peripheral insulin-sensitive tissues. Interestingly, we also report for the first time, to our knowledge, the direct binding of resistin to Toll-like receptor (TLR) 4 receptors in the hypothalamus, leading to the activation of the associated proinflammatory pathways. Taken together, our findings clearly identify TLR4 as the binding site for resistin in the hypothalamus and bring new insight into the molecular mechanisms involved in resistin-induced inflammation and insulin resistance in the whole animal. *Diabetes* 62:102–114, 2013

The hypothalamus integrates hormonal and metabolic signals to respond to energy body requirements through the regulation of energy homeostasis (1,2). The disruption of this regulatory loop promotes the onset of obesity, currently considered a worldwide epidemic. Obesity is linked to common metabolic diseases including insulin resistance, which constitutes a principal risk factor for type 2 diabetes (3–5). Accumulating evidence indicates that changes in adipose-secreted factors in obesity, including release of inflammatory cytokines, dramatically affect insulin sensitivity (3–7). Among these adipokines, resistin is described as a potential factor in obesity-mediated insulin resistance and type 2

diabetes. Resistin is a cysteine-rich 12.5-kDa polypeptide secreted by adipose tissue in rodents and by macrophages in humans (7,8), promoting inflammation and insulin resistance (9–12). Circulating resistin is increased in obese insulin-resistant rodents (6) and humans (7), and fasting decreases resistin mRNA expression (6,13). Peripheral administration or transgenic overexpression of resistin impairs insulin action in insulin-sensitive tissues (14–16). Conversely, deletion of the resistin gene or infusing of resistin antibodies or antisense oligonucleotides restores insulin responsiveness (6,17–19). In humans, recent studies have linked resistin to insulin resistance, atherosclerosis, and inflammation (12, 20,21). More recently, it has been shown that resistin is expressed in the hypothalamus (22) and activates specific hypothalamic neurons (23). Central resistin also modulates glucose homeostasis, lipid metabolism, and food intake and impairs liver insulin sensitivity (24–27).

Resistin also regulates the synthesis and secretion of key proinflammatory cytokines such as tumor necrosis factor- α , interleukin (IL)-6, and IL-12 in macrophages via a nuclear factor- κ B-dependent pathway promoting insulin resistance (4,6,28,29). Moreover, recent studies have provided evidence for the contribution of Toll-like receptor-4 (TLR4) in the pathogenesis of obesity and insulin resistance. Saturated fatty acids activate both hypothalamic and peripheral TLR4 signaling, leading to proinflammatory cytokine production and endoplasmic reticulum stress (30–32). Conversely, TLR4 loss-of-function prevents saturated fatty acid-induced inflammation and insulin resistance (30,31,33). Resistin and TLR4 have been linked to a proinflammatory process in a human epithelial cell line in which resistin competes with lipopolysaccharide (LPS) for binding to TLR4 (34). Recently, an isoform of decorin was identified as a resistin receptor involved in white adipose tissue expansion (35). Another report has described that mouse resistin modulates glucose uptake and promotes adipogenesis in 3T3-L1 cells through the receptor tyrosine kinase-like orphan receptor-1 (36). In addition, in rheumatoid arthritis disease, resistin has been shown to use the IGF-1R signaling pathway (37). These data reveal a puzzling situation in which resistin could potentially interact with different receptors depending upon cellular model. However, in vivo at the neuronal level, the resistin receptor and its signaling have not yet been identified.

Thus, we aimed to characterize hypothalamic resistin receptor and its signaling pathways involved in the impairment of insulin responsiveness. We show that resistin signals through TLR4 in the hypothalamus lead to the activation of Jun NH₂-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signaling pathways by the recruitment of the adaptor proteins myeloid differentiating factor 88 (MyD88) and Toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP), promoting overall inflammation. These findings reveal strong evidence for the

From the ¹Unité Mixte de Recherche 8195, University Paris-Sud, Orsay, France; the ²Centre National de la Recherche Scientifique, Center of Neurosciences Paris-Sud, Unité Mixte de Recherche 8195, Orsay, France; the ³Faculty of Agricultural, Food and Environmental Quality Sciences, The Institute of Biochemistry, Food Science, and Nutrition, The Hebrew University of Jerusalem, Rehovot, Israel; and ⁴Shenandoah Biotechnology, Inc. Warwick, Pennsylvania.

Corresponding author: Mohammed Taouis, mohammed.taouis@u-psud.fr. Received 28 February 2012 and accepted 6 July 2012.

DOI: 10.2337/db12-0237

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db12-0237/-/DC1>.

© 2013 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

direct role of hypothalamic TLR4 signal transduction in resistin-induced whole-body inflammation and insulin resistance.

RESEARCH DESIGN AND METHODS

Animals. Adult male Wistar rats weighing 250–300 g (Charles River Laboratories, Paris, France) were used and had unrestricted access to water and standard chow diet. Experimental procedures were performed according to the institutional guidelines approved by the governmental commission of animal research.

Chronic intracerebroventricular infusion of resistin and intraperitoneal insulin injection. Mini-osmotic pumps (model 2002; Alzet) were implanted under ketamine (150 mg/kg; Vibrac, Lyon, France)/xylazine (5 mg/kg; Rompun; Bayer, Lyon, France) anesthesia. Brain infusion cannulae were stereotaxically placed into the lateral brain ventricle. The osmotic pumps were housed in a subcutaneous pocket on the dorsal surface of the animal. The rats were then infused with either vehicle or resistin (1.2 $\mu\text{g}/12 \mu\text{L}/\text{day}$; pumping rate 0.5 $\mu\text{L}/\text{h}$) for 14 days. To test the impact of the central chronic resistin infusion on insulin sensitivity, overnight fasted rats were treated with insulin (1 U/kg of body weight) by intraperitoneal (IP) injection 30 min prior to euthanasia.

Measurement of blood glucose and plasma hormone levels. Blood glucose levels were measured with a blood monitoring system (Accu-Chek; Roche). Levels of plasma insulin, leptin, resistin, and adiponectin were determined by enzyme-linked immunosorbent assay using kits purchased from Millipore, according to the manufacturer's guidelines.

Hormones and chemicals. Human resistin was a generous gift of Protein Laboratories Rehovot Ltd. (Rehovot, Israel) and Shenandoah Biotechnology, Inc. (Warwick, PA). Resistin was produced in an *Escherichia coli* system, forming inclusion bodies. In purified resistin, the level of endotoxin was $<0.21 \text{ EU}/\mu\text{g}$. Human insulin Actrapid was provided by Novo Nordisk (Bagsvaed, Denmark). All cell-culture reagents were purchased from Invitrogen. Insulin, retinoic acid, secondary antibodies coupled to horseradish peroxidase, and other chemicals were purchased from Sigma-Aldrich. Polyvinylidene fluoride membranes and enhanced chemiluminescence (ECL) detection reagents were from Amersham/GE Healthcare.

Cell culture and stimulation. SH-SY5Y human neuroblastoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mmol L-glutamine, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. Differentiated cells were used after 15 days of retinoic acid treatment. Serum-starved cells were incubated for 16 h in serum-free Dulbecco's modified Eagle's medium in the presence or absence of resistin (100 ng/mL). Cells were then stimulated for 10 min with insulin (100 nmol) to evaluate the impact of chronic resistin treatment on SH-SY5Y cell sensitivity to insulin.

Western blot analyses. After hormonal treatment, protein lysates from rats' tissues and SH-SY5Y cells were prepared and analyzed by Western blot. Briefly, protein extracts (50–100 μg) were subjected to SDS-PAGE and immunoblotted with primary antibodies raised against phospho (*p*)-AKT (ser⁴⁷³), AKT, *p*-extracellular signal-related kinase 1/2 (ERK1/2), ERK1/2, *p*-insulin receptor (IR), *p*-insulin receptor substrate (IRS)-1 (ser³⁰⁷), IRS-1, *p*-JNK, JNK, *p*-p38MAPK, p38MAPK, suppressor of cytokine signaling-3 (SOCS-3), β -tubulin (Cell Signaling Technology); IR, IL-6, phosphotyrosine phosphatase 1B (PTP-1B), TLR4, MyD88, TIRAP (Santa Cruz Biotechnology); and resistin (Abcam) overnight at 4°C. For protein detection, we used horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (Amersham Biosciences). The blots were finally scanned and quantified using the Carestream Molecular Imaging System 4000MM PRO (Carestream Health, Inc).

Immunoprecipitation. Protein lysates from rat tissues and SH-SY5Y cells were incubated with antibodies against TLR4 or MyD88 overnight at 4°C. The immune complexes were precipitated after incubation with a protein A-protein G-sepharose mix (Amersham Biosciences) for 2 h at 4°C and then heated in loading buffer at 100°C for 5 min. Immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti-TLR4, anti-resistin, anti-MyD88, or anti-TIRAP antibodies.

RNA extraction and quantitative real-time RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. One microgram of total RNA was reverse transcribed, and the cDNAs were submitted to quantitative PCR analysis using specific primer pairs using the Step One apparatus and Fast SYBR Green Master Mix (Applied Biosystems). A ratio of specific mRNA/18S rRNA amplification was calculated to correct for any difference in efficiency at reverse transcription.

Small interfering RNA silencing. A pool of specific small interfering RNA (siRNA) duplexes targeting human TLR4 gene and nontargeting siRNA (si-control) were purchased from Ambion, Inc. Transfection of TLR4 and control siRNA into SH-SY5Y cells was performed using Lipofectamine 2000 (Invitrogen)

transfection reagent according to the manufacturer's guidelines. Down-regulation of the TLR4 protein synthesis following siRNA transfection was proved by Western blot analysis.

Cross-linking experiments. SH-SY5Y cells were plated at a density of 6×10^6 /10-cm dish and serum-starved for 4 h before resistin treatment. Cells were then washed with ice-cold PBS (pH 8.0) to remove amine-containing culture media and proteins from the cells and treated with resistin (100 ng/mL) for 10 min or 1 h at 4°C. The cross-linker BS3 (Thermo Fisher Scientific) was added directly to the incubation mixture to a final concentration of 2.5 mmol/L. After an additional incubation for 1 h at 4°C, the cross-linking reaction was terminated by adding Tris-HCl solution (pH 7.4) to a final concentration of 20 mmol/L. Cells were solubilized in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 1% Nonidet P-40, 10% glycerol, proteases inhibitors, and phosphatases inhibitors. Protein lysates were then subjected to immunoprecipitation using anti-TLR4 antibody, and immunoprecipitates were analyzed by Western blot using appropriate antibodies.

Data analysis and statistics. Statistical analysis was performed using ANOVA (Statview Software program, version 5) to detect significant intergroup differences. Data are presented as means \pm SEM, and a *P* value <0.05 was considered statistically significant.

RESULTS

Central chronic resistin infusion affects metabolic and endocrine parameters. Recombinant human resistin was infused in the lateral ventricle of Wistar rats for 14 days. This treatment markedly decreased both food intake and body weight (Fig. 1A) associated with the upregulation of proopiomelanocortin (POMC) and the down-regulation of the neuropeptide Y (NPY) gene expression in the arcuate nucleus (Fig. 1B). In addition, we also assessed the effect of chronic central resistin administration on overnight-fasted serum leptin, insulin, adiponectin, resistin, and blood glucose levels. Fasting serum insulin, resistin, and leptin levels exhibited no differences among control and resistin-treated rats. However, the intracerebroventricular (ICV) administration of resistin markedly decreased the circulating levels of adiponectin (16.8 ± 2.1 vs. $12.9 \pm 2.6 \mu\text{g}/\text{mL}$; $P < 0.05$) and increased the blood glucose levels (114 ± 11.8 vs. $139 \pm 12.1 \text{ mg}/\text{dL}$; $P < 0.05$). Next, the expression levels of key genes involved in energy homeostasis and insulin sensitivity such as uncoupling protein 2 (UCP-2) and adiponectin receptors (AdipoR1/2) were examined. ICV resistin treatment reduced the expression levels of UCP-2 and AdipoR1/2 in the hypothalamus, muscle, and adipose tissue. In the liver, this treatment altered only the expression of AdipoR1/2 but not UCP-2 (Fig. 1C).

Chronic resistin treatment alters insulin responsiveness in vivo and in SH-SY5Y human neuronal cells. Following chronic ICV vehicle or resistin infusion and an IP insulin bolus, insulin-dependent phosphorylation of AKT and ERK1/2 in the hypothalamus, skeletal muscle, liver, and adipose tissue was examined. In control rats, insulin markedly increased both AKT and ERK1/2 phosphorylations in all studied tissues (Fig. 2A and B). In resistin-treated rats, insulin-dependent phosphorylation of ERK1/2 was completely abolished with increased basal phosphorylation in the hypothalamus and muscle (Fig. 2B). The same results were obtained for insulin-dependent Akt phosphorylation in the hypothalamus, whereas in muscle, liver, and adipose tissue, the effect of insulin was maintained, but to a lesser extent as compared with control rats (Fig. 2A). To interrogate the direct contribution of resistin overexposure to the impairment of insulin signaling, we examined in SH-SY5Y cells the impact of resistin treatment on insulin-dependent Akt and ERK phosphorylation. Resistin clearly abolished Akt and ERK1/2 phosphorylation in response to insulin. As noticed for the hypothalamus, the basal

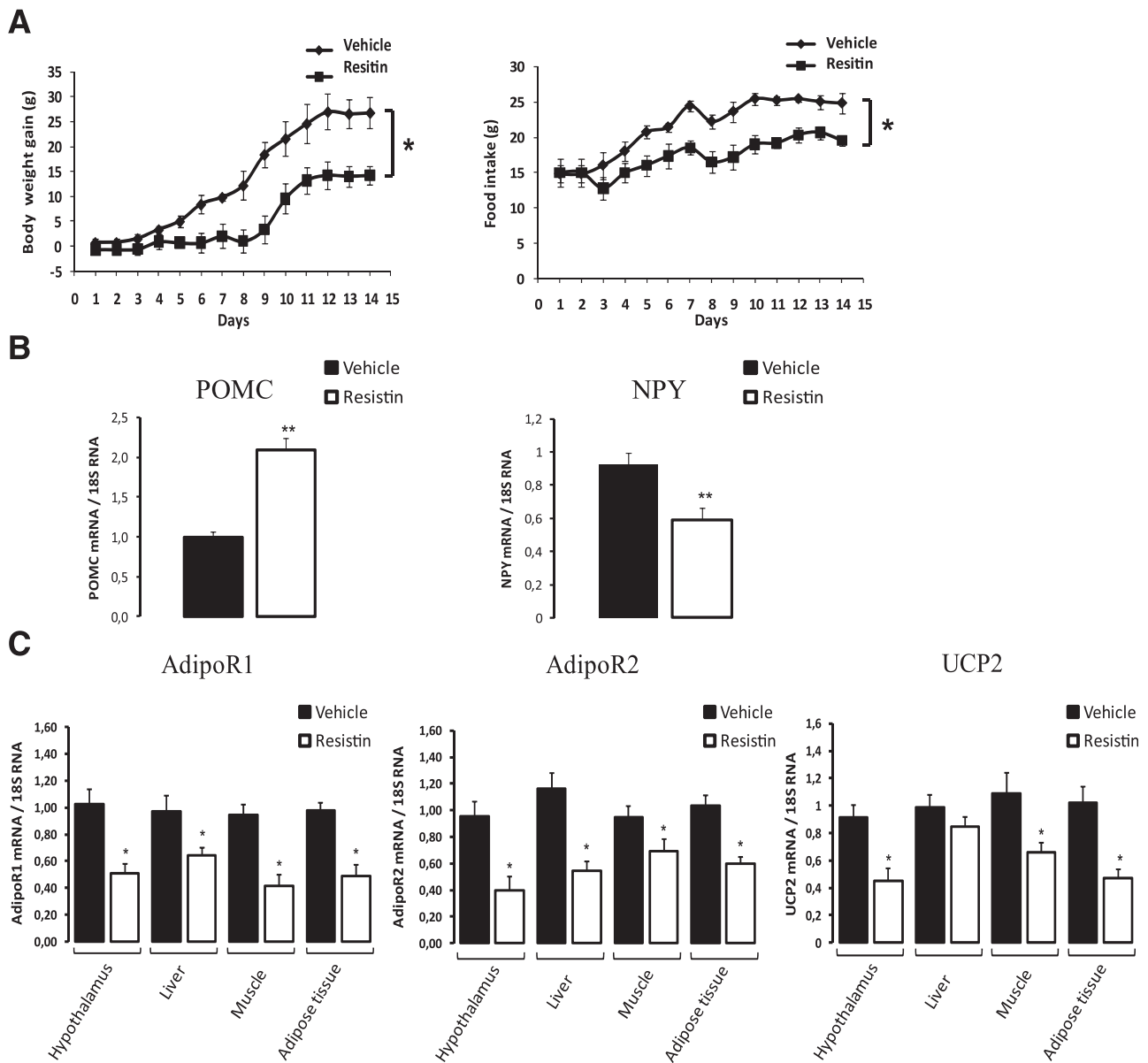


FIG. 1. Effect of central chronic resistin infusion on metabolic and endocrine parameters. *A–C*: Male Wistar rats received an ICV of vehicle or resistin (1.2 µg/day) during a period of 2 weeks, and body weight gain and food intake were measured daily (*A*). At the end of the infusion period, the mRNA levels of POMC and NPY were measured in the hypothalamus (*B*). UCP-2, adipoR1, and adipoR2 mRNA levels also measured in the hypothalamus and peripheral insulin-sensitive tissues using SYBR Green real-time RT-PCR (Applied Biosystems). Results were normalized to 18S RNA. All data are presented as means ± SEM (*n* = 3–6/group). **P* < 0.05, ***P* < 0.01 compared with vehicle.

phosphorylation of Akt and ERK1/2 was increased as compared with untreated cells (Fig. 2*A* and *B*).

Chronic resistin treatment inhibits insulin-stimulated IR phosphorylation and reduced IR expression. We next examined the effect of chronic resistin ICV treatment on both IR phosphorylation and expression. In vehicle-treated rats, insulin increased the tyrosine phosphorylation of the IR-β subunits in all studied tissues. Conversely, ICV resistin treatment almost totally abolished the insulin-dependent tyrosine phosphorylation of the IR-β in these tissues (Fig. 3*A*). A similar effect was observed in SH-SY5Y cells overexposed to resistin (Fig. 3*A*). We also found that resistin treatment markedly downregulates both mRNA and protein levels of IR in the hypothalamus, liver, and adipose tissue. In the skeletal muscle, this treatment significantly reduced the protein level of IR but not mRNA

level (Fig. 3*B* and *C*). Furthermore, in SH-SY5Y cells, the overexposure to resistin caused a significant decrease in IR expression at both the mRNA and protein levels (Fig. 3*B* and *C*).

Resistin treatment increases IRS-1 serine phosphorylation. The resistin-induced insulin resistance could also be attributed to increased phosphorylation of IRS-1 on serine residue, which is considered as a peculiarity of insulin resistance. To test this hypothesis, we examined the impact of chronic resistin ICV infusion on IRS-1 phosphorylation on serine 307 residue. Resistin treatment significantly increased the serine³⁰⁷ phosphorylation of IRS-1 in the hypothalamus, liver, skeletal muscle, and adipose tissue (Fig. 4*A*). Similarly, the overexposure of SH-SY5Y cells to resistin markedly increased the IRS-1 phosphorylation on serine³⁰⁷ residue (Fig. 4*A*).

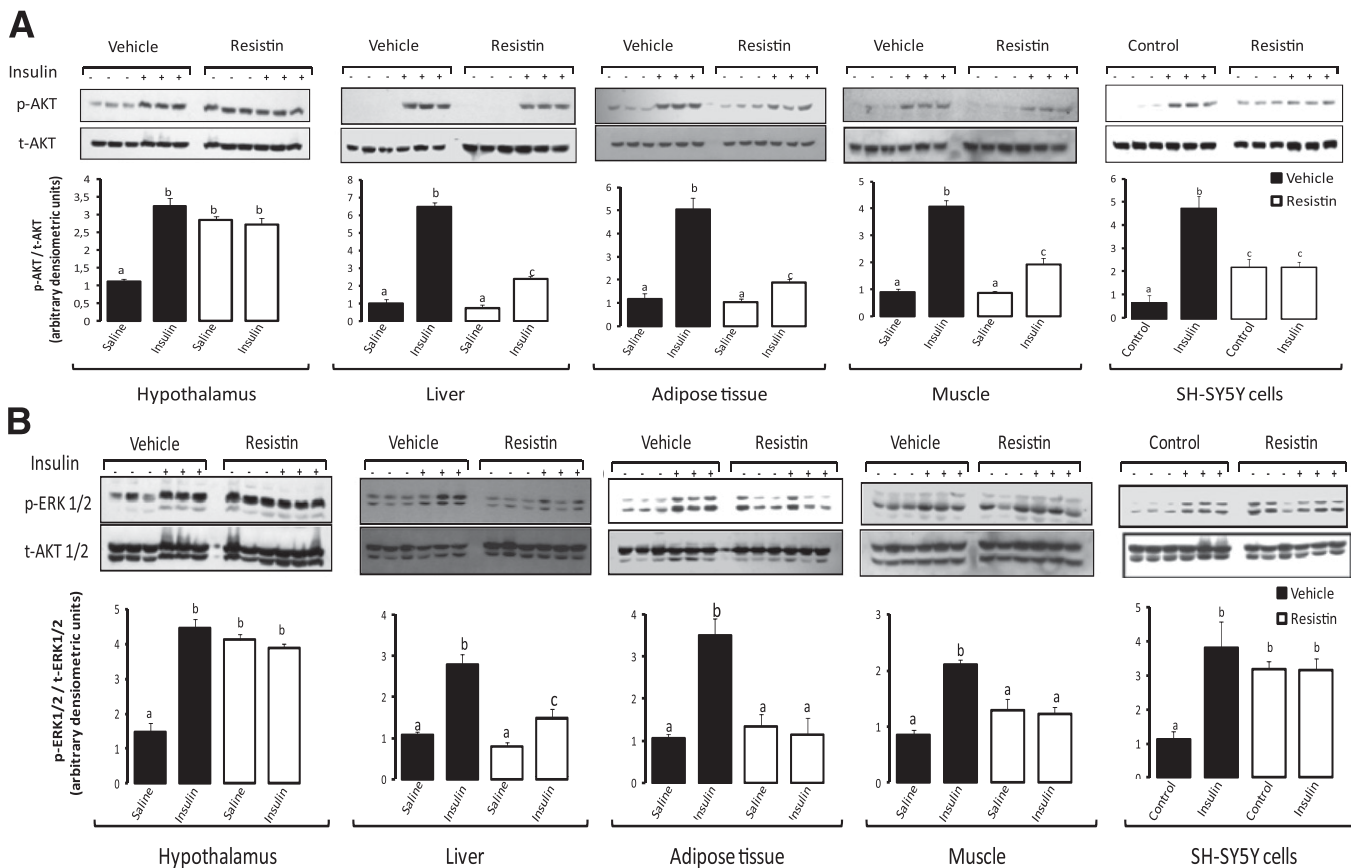


FIG. 2. Effect of chronic resistin treatment on insulin signaling. Male Wistar rats received an ICV of vehicle or resistin (1.2 $\mu\text{g}/\text{day}$) during a period of two weeks. At the end of the infusion period, vehicle and resistin-treated rats received IP human insulin (1 U/kg body weight) or saline bolus 30 min before euthanasia to test insulin responsiveness. Serum-deprived SH-SY5Y cells were incubated for 16 h with resistin (100 ng/mL) and then stimulated for 10 min with or without insulin (100 nmol/L). Protein lysates from hypothalamus, liver, muscle, adipose tissue, and SH-SY5Y cells were subjected to Western blot analysis. Membranes were probed sequentially with anti-p-AKT followed by anti-AKT antibodies (A) or anti-p-ERK1/2 followed by anti-ERK1/2 (B). The proteins on the blots were revealed by ECL and bands quantified by densitometry. The results are expressed as means \pm SEM ($n = 3\text{--}6/\text{group}$). ^{a,b,c} denote significant differences at $P < 0.05$ by ANOVA and Fisher post hoc test.

Resistin treatment activates JNK and p38 MAPK. To investigate the potential link between resistin and proinflammatory pathways, we studied the impact of chronic resistin ICV treatment on the phosphorylation of JNK and p38 MAPK. Resistin treatment increases the phosphorylation of both kinases in the hypothalamus, liver, skeletal muscle, and adipose tissue (Fig. 4B and C). Furthermore, resistin overexposure of SH-SY5Y cells significantly increased the phosphorylation of both JNK and p38 MAPKs as compared with control cells (Fig. 4B and C).

Resistin treatment upregulates SOCS-3, PTP-1B, and IL-6 expression. The impact of resistin on negative modulators of insulin signaling such as SOCS-3 and PTP-1B was also investigated. First, we found that chronic resistin ICV treatment markedly increased both mRNA and protein levels of SOCS-3 in the hypothalamus, liver, skeletal muscle, and adipose tissue as in SH-SY5Y cells (Fig. 5A). This treatment also increased PTP-1B expression, at both the mRNA and protein levels, in the hypothalamus, liver, and SH-SY5Y cells but not in skeletal muscle and adipose tissue (Fig. 5B). Furthermore, we examined the effect of chronic resistin ICV treatment on the expression of a key proinflammatory cytokine, IL-6. Our data showed that resistin significantly increases the mRNA and protein levels of IL-6 in the hypothalamus and peripheral insulin-sensitive tissues: liver, muscle, and adipose tissue (Fig. 5C).

Resistin activates TLR4 signaling both in the hypothalamus and SH-SY5Y neuronal cells. We examined the effect of resistin treatment on TLR4 signal transduction by measuring the association of TLR4 with MyD88 and TIRAP, two adaptors critically involved in TLRs signal transduction. Our data showed that resistin treatment markedly increased the association of MyD88 with TLR4 in the hypothalamus and SH-SY5Y cells. Additionally, we showed that this treatment increases the association of TLR4/MyD88 with TIRAP in the hypothalamus (Fig. 6A). **Resistin directly binds to TLR4 in SH-SY5Y human neuronal cells.** Next, we tested the hypothesis of a direct binding of resistin on TLR4 in SH-SY5Y cells. Following the cross link of resistin on cell-surface proteins, TLR4 was immunoprecipitated and immunoblotted using anti-TLR4 antibodies. As shown in Fig. 6B, analysis of lysates from SH-SY5Y cells treated with resistin for 10 or 60 min revealed a major band of a molecular mass of ~ 116 kDa corresponding most likely to the complex resistin/TLR4 receptor. The band intensity increases after incubation with resistin for 60 min as compared with 10 min. In lysates from untreated cells, the 116-kDa band was not detected, whereas another band representing free TLR4 (~ 80 kDa) was revealed. When binding was carried out without cross-linker BS3, only the band corresponding to TLR4 was detected (Fig. 6B). To demonstrate the specificity of the binding of

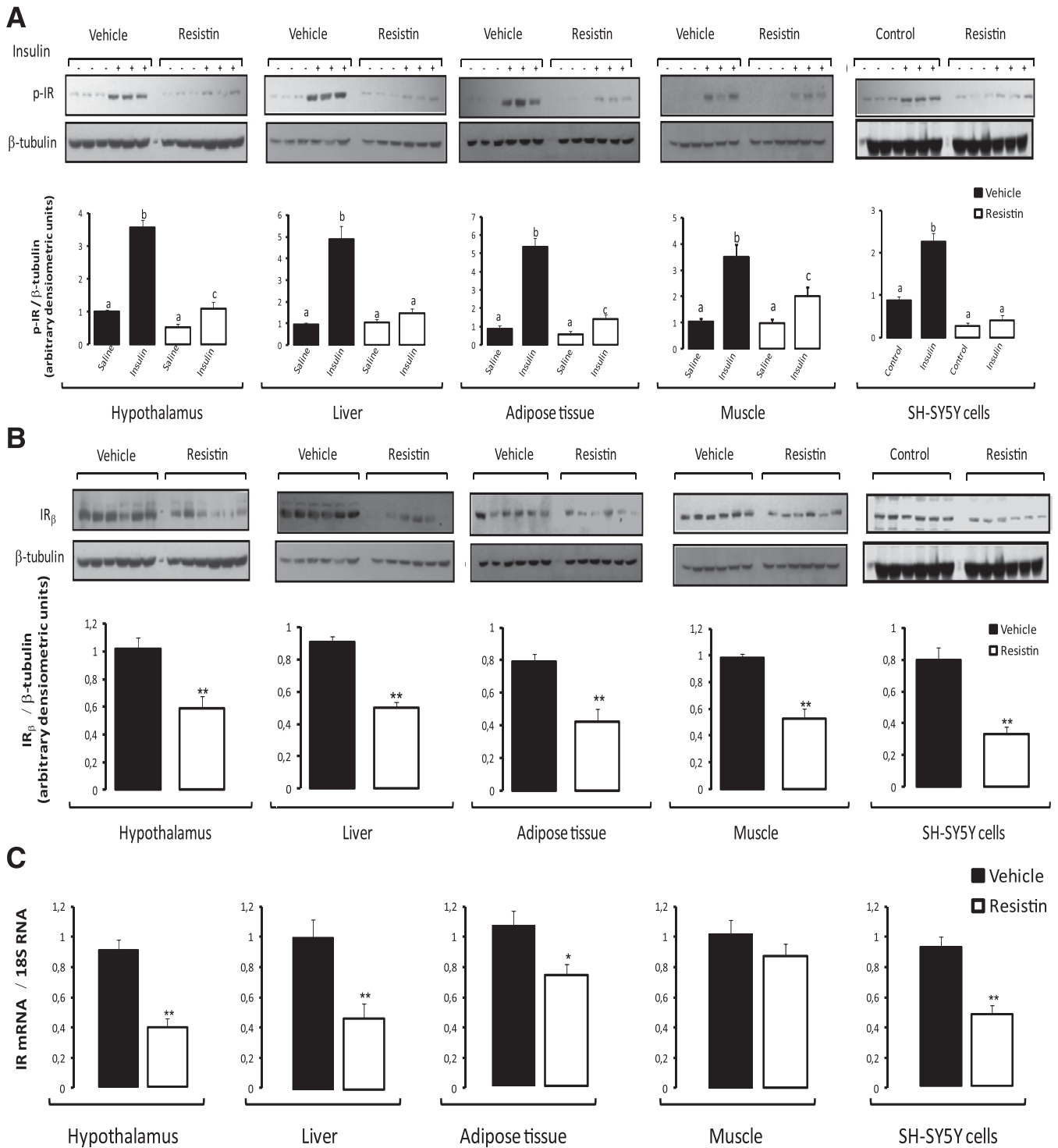


FIG. 3. Effect of chronic resistin treatment on IR phosphorylation and expression. Male Wistar rats received an ICV of vehicle or resistin (1.2 μ g/day) during a period of 2 weeks. At the end of the infusion period, vehicle and resistin-treated rats received IP human insulin (1 U/kg body weight) or saline bolus 30 min before euthanasia to test insulin sensitivity. Serum-deprived SH-SY5Y cells were incubated for 16 h with resistin (100 ng/mL) and then stimulated for 10 min with or without insulin (100 nmol/L). Protein lysates from hypothalamus, liver, muscle, adipose tissue, and SH-SY5Y cells were subjected to Western blot analysis. Membranes were probed sequentially with anti-*p*-IR (A) followed by anti-IR antibodies (B). The proteins on the blots were revealed by ECL and bands quantified by densitometry. The results are expressed as ratio of *p*-IR/ β -tubulin and IR/ β -tubulin. C: At the end of the experimental period of ICV resistin, SYBR Green real-time RT-PCR (Applied Biosystems) was conducted to measure IR mRNA levels in the hypothalamus and peripheral insulin-sensitive tissues. In SH-SY5Y cells, IR mRNA expression was measured after chronic resistin treatment (100 ng/mL) for 16 h. Results were normalized to 18S RNA. All data are means \pm SEM ($n = 3-6$ /group). ^{a,b,c} denote significant differences at $P < 0.05$ by ANOVA and Fisher post hoc test. * $P < 0.05$, ** $P < 0.01$ compared with vehicle.

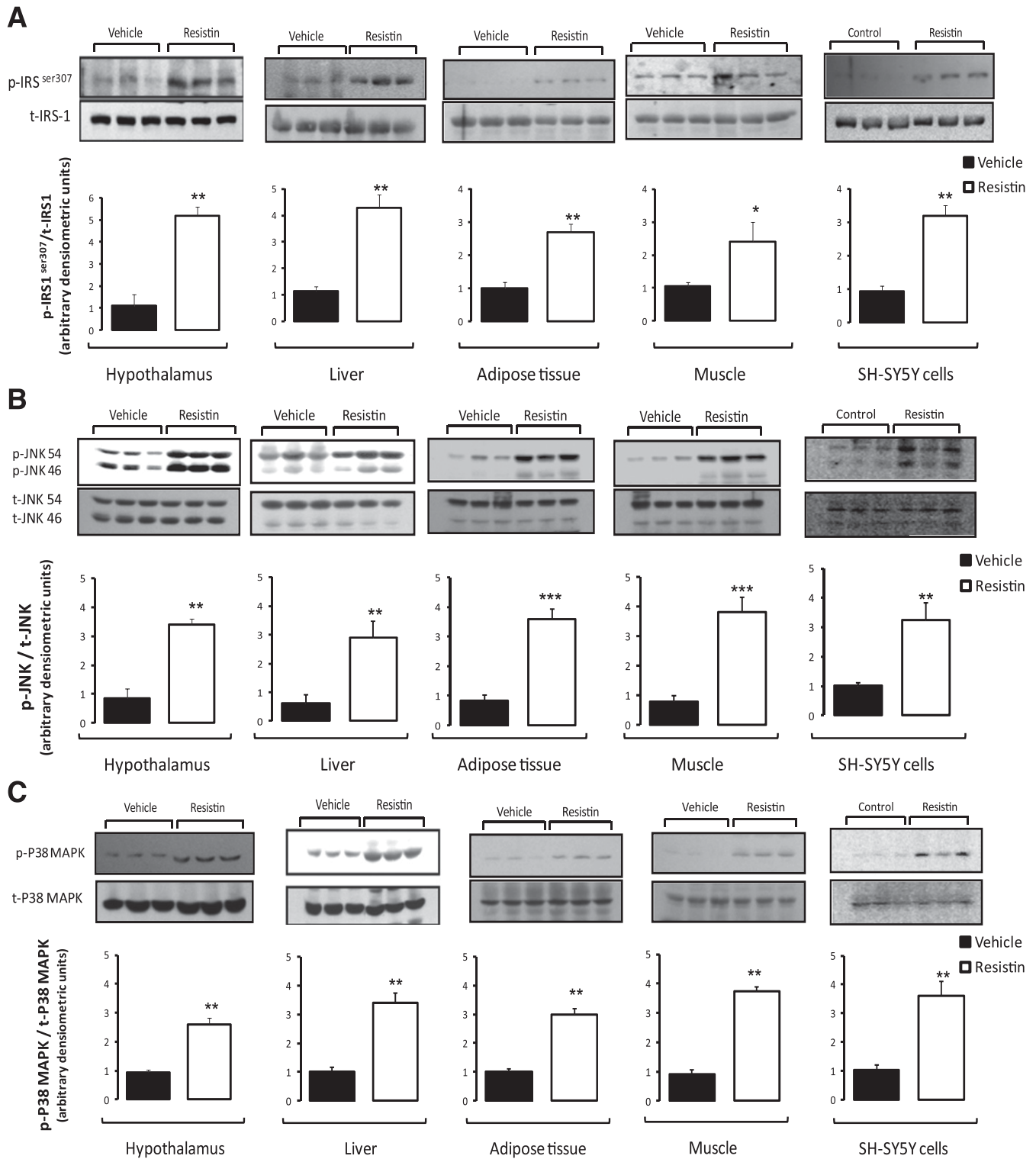


FIG. 4. Chronic resistin treatment increases IRS-1 serine³⁰⁷ phosphorylation and activates JNK and p38 MAPK. Male Wistar rats received an ICV of vehicle or resistin (1.2 μ g/day) during a period of 2 weeks. Serum-deprived SH-SY5Y cells were incubated for 16 h with resistin (100 ng/mL). Protein lysates from hypothalamus, liver, muscle, adipose tissue, and SH-SY5Y cells were subjected to Western blot analysis. Membranes were probed sequentially with anti-p-IRS-1 serine³⁰⁷ followed by anti-IRS-1 (A), anti-p-JNK(54/46) followed by anti-JNK (54/46) (B), and p-p38MAPK followed by p38MAPK (C). The proteins on the blots were revealed by ECL and bands quantified by densitometry. The results are expressed as ratio of p-IRS-1^{serine307}/total (t)-IRS-1, p-JNK(54/46)/t-JNK(54/46), and p-p38MAPK/t-p38MAPK. Data are means \pm SEM ($n = 3-6$ /group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared with vehicle.

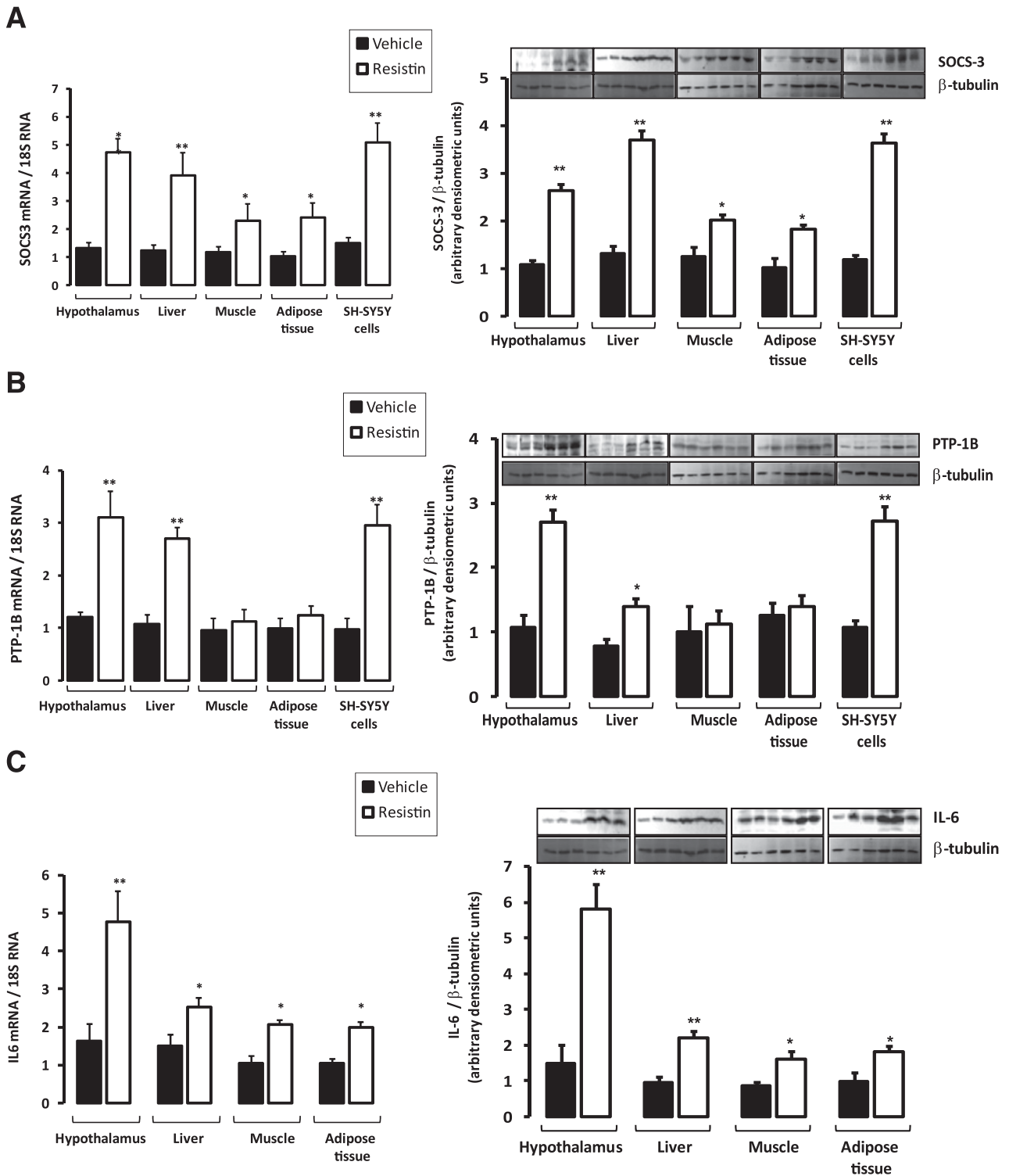


FIG. 5. Effect of chronic resistin treatment on SOCS-3, PTP-1B, and IL-6 expression. Male Wistar rats received an ICV of vehicle or resistin (1.2 μ g/day) during a period of 2 weeks. At the end of the infusion period, SOCS-3 (A), PTP-1B (B), and IL-6 (C) mRNA levels were evaluated by SYBR Green real-time RT-PCR (Applied Biosystems) in the hypothalamus and peripheral insulin-sensitive tissues. In SH-SY5Y cells, mRNA levels of SOCS-3 and PTP-1B were measured after chronic resistin treatment (100 ng/mL) for 16 h. Results were normalized to 18S RNA. For protein analysis, protein lysates from hypothalamus, liver, muscle, adipose tissue, and SH-SY5Y cells were subjected to Western blot analysis. Membranes were probed sequentially with anti-SOCS-3 (A), anti-PTP-1B (B), and anti-IL-6 (C). The proteins on the blots were revealed by ECL and bands quantified by densitometry. The results are expressed as ratio of SOCS-3/ β -tubulin, PTP-1B/ β -tubulin, and IL-6/ β -tubulin. Data are means \pm SEM ($n = 3-6$ /group). * $P < 0.05$, ** $P < 0.01$ compared with vehicle.

resistin to TLR4, TLR4 immunoprecipitates were analyzed by immunoblotting using anti-resistin antibodies. Only the 116-kDa band, corresponding to the resistin/TLR4 complex, was detected. Next, we investigated whether resistin binding to

TLR4 is attenuated by silencing of TLR4. Interestingly, TLR4 knockdown markedly reduced the intensity of the band representing resistin/TLR4 complex. In the absence of the cross-linker agent, protein band corresponding to resistin/TLR4

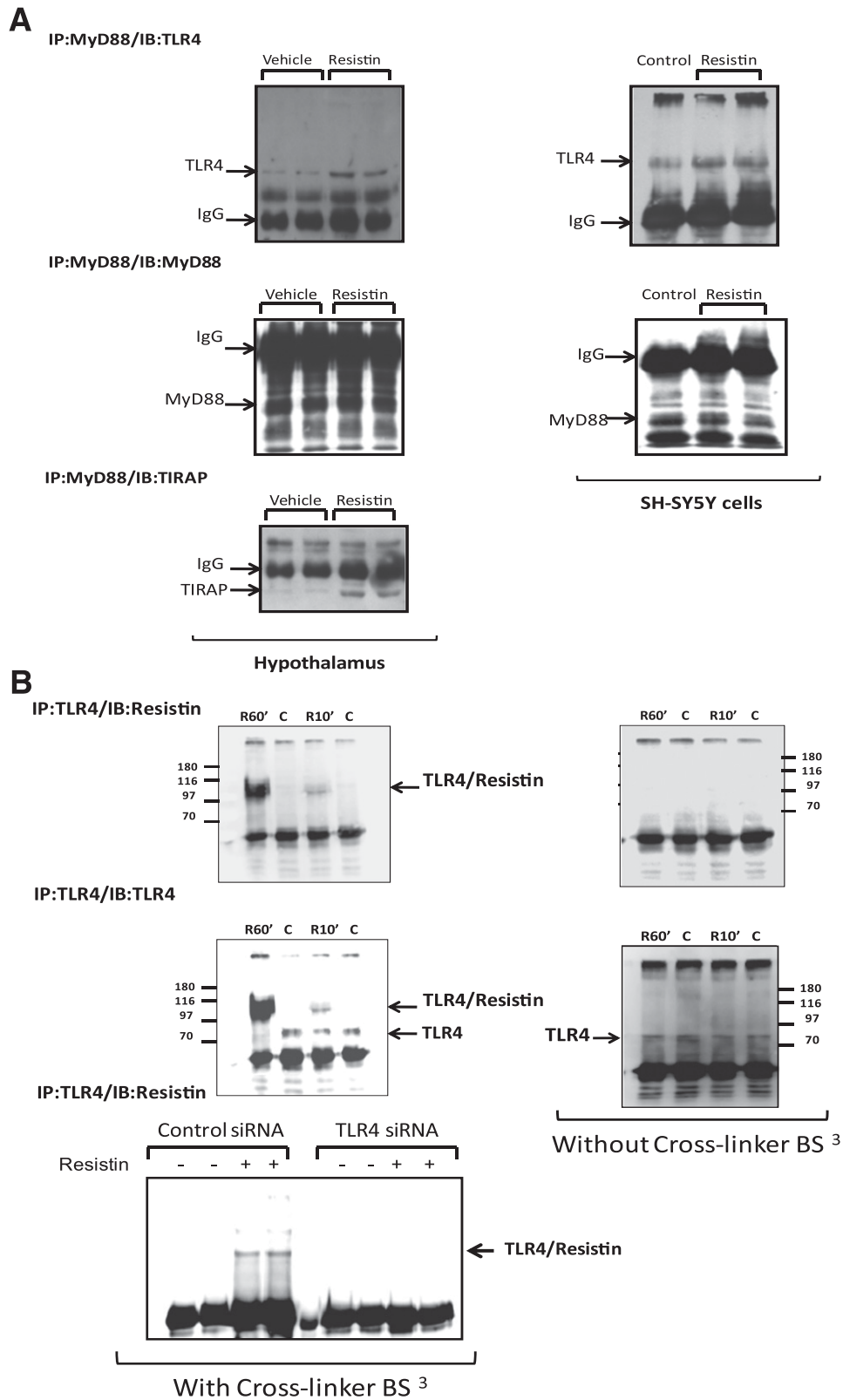


FIG. 6. Resistin binds to TLR4 and activates its downstream signaling molecules. *A:* Immunoprecipitation/immunoblot (IP/IB) analysis of the association of TLR4 with MyD88 and TIRAP in hypothalamic protein extracts obtained from rats treated intracerebroventricularly with resistin (1.2 $\mu\text{g/day}$) for 14 days and in protein lysates from SH-SY5Y cells treated with resistin (100 ng/mL) for 16 h. *B:* IP/IB analysis of the direct association of resistin with TLR4 in protein extracts from wild-type and TLR4-depleted SH-SY5Y cells treated with resistin (100 ng/mL) in the presence or absence of the cross-linker agent BS³.

complex was not detected (Fig. 6B), confirming that resistin specifically binds to TLR4 receptor in SH-SY5Y cells.

siRNA-mediated TLR4 knockdown impairs both resistin-induced inflammatory response and insulin resistance in SH-SY5Y human neuronal cells. To assess the role of the TLR4 on neuronal resistin signaling, we generated TLR4-depleted SH-SY5Y cells by siRNA-mediated knockdown, which induced a specific TLR4 protein decrease of 60% (Fig. 7A). As expected, the overexposure of wild-type SH-SY5Y cells to resistin markedly attenuated the phosphorylations of AKT and ERK1/2 in response to insulin. Interestingly, TLR4 knockdown restores the insulin-dependent phosphorylation of both AKT and ERK1/2 in resistin-treated cells (Fig. 7B). Furthermore, we showed that TLR4 deficiency significantly reduces the resistin-induced phosphorylation of JNK and p38 MAPK (Fig. 7C).

DISCUSSION

Resistin promotes both inflammation and insulin resistance, but its receptor has not yet been identified, and little is known about the molecular mechanisms mediating resistin effects particularly at the neuronal levels. In this study, we report that chronic resistin infusion in the lateral ventricle markedly affects both hypothalamic and peripheral insulin sensitivity. Central resistin treatment increased SOCS-3 and PTP-1B expression levels associated with reduced IR phosphorylation and expression. Importantly, we report for the first time, to our knowledge, the direct binding of resistin to TLR4 receptors *in vivo*, leading to the activation of proinflammatory pathways and promoting the insulin resistance. We characterize the potential mechanisms by which resistin induces insulin resistance in whole body.

Accumulating evidence indicates that resistin acts within the hypothalamus to modulate feeding and energy homeostasis (24–27). The resistin that reaches the hypothalamus could have local or systemic (adipose tissue secretion) origin. In this study, we show that resistin and TLR4 colocalized with POMC in the hypothalamus arcuate nucleus (Supplementary Fig. 1), which is in good agreement with a previous study reporting resistin immunoreactivity in mouse hypothalamus (22). Furthermore, peripheral resistin could also reach the hypothalamus through the blood–brain barrier, as evidenced by its presence in human cerebrospinal fluid (38).

Resistin exerts anorexigenic effects both by reducing the expression of orexigenic neuropeptides agouti-related protein and NPY and by increasing the expression of anorectic peptide cocaine and amphetamine-regulated transcript (26). In agreement with these findings, the current study demonstrates that chronic central resistin infusion reduces both food intake and body weight gain, with the upregulation of POMC and downregulation of NPY. Our results contrast with other studies that have reported that central resistin treatment increased hypothalamic NPY expression (27) and had no impact on POMC expression (26,27). This discrepancy could be explained by the animal models used and experimental conditions.

The involvement of resistin in the onset of insulin resistance has been previously evidenced (5,9,12). The overexpression of resistin impairs insulin action in insulin-sensitive tissues (14,16), whereas the reduction of circulating resistin restores insulin responsiveness (5,17–19). Recently, the expression of resistin has been reported in the hypothalamus (22), potentially linking energy homeostasis control to insulin responsiveness. Resistin is able to

activate hypothalamic neurons (23), and its ICV administration modulates both glucose and lipid metabolism (24–26) besides the impairment of insulin action in the liver (25). However, little is known about the effect of resistin on brain insulin sensitivity. In this study, we show that central chronic resistin infusion affects hypothalamic, muscle, liver, and adipose tissue insulin signaling. Agreeing with previous studies demonstrating the ability of central resistin to induce hepatic insulin resistance (25,27), we showed that ICV resistin dramatically reduced the insulin-dependent phosphorylation of Akt and ERK1/2 kinases in the liver. Peripheral resistin treatment has been also described to cause insulin resistance in skeletal muscle and adipose tissue (15,16,39). We also found that central resistin treatment impaired the insulin-stimulated phosphorylations of Akt and ERK1/2 in the muscle and adipose tissue. Taken together, these data clearly show that central resistin alters peripheral insulin sensitivity without significant changes in circulating resistin, most likely revealing the involvement of autonomic innervations of peripheral tissues including the liver, as previously suggested (27).

Interestingly, in the hypothalamus, chronic ICV resistin significantly increased the basal phosphorylation of both AKT and ERK1/2, reaching the level observed in control rats after insulin challenge. Because in peripheral tissues Akt and ERK1/2 basal phosphorylation in response to resistin ICV infusion was not augmented, we thus suggest that increased basal hypothalamic Akt and ERK1/2 phosphorylation is due to a direct effect of resistin. This has been also evidenced in SH-SY5Y cells following overexposure to resistin. Consequently, insulin was unable to further increase Akt and ERK phosphorylation in the hypothalamus of resistin ICV-treated animals that could be attributed to the downregulation of IR (Fig. 3). This finding discriminates between the direct effect of resistin as that observed in the hypothalamus from the indirect action driven probably by the vagus nerve as those observed in muscle, adipose tissue, and liver, leading in fine to a clear whole-body loss of insulin responsiveness. This is corroborated by the decreased IR expression and phosphorylation. Interestingly, in the hypothalamus, resistin treatment almost completely abolished the insulin-dependent phosphorylation of IR without changes of the basal phosphorylation, which contrast with the effect on Akt and ERK1/2 phosphorylation. This indicates that the effect of resistin on Akt and ERK1/2 phosphorylation is independent of IR signaling. These results contrast with previous reports documenting that resistin has no effect on insulin receptor content and phosphorylation (39,40). It is of note that these studies were performed in primary cell culture derived from liver and skeletal muscle, and the current study, to our knowledge, is the first investigation describing such resistin effects *in vivo*.

The alteration of insulin responsiveness in the hypothalamus and peripheral tissues also involves SOCS-3 and PTP-1B, considered as major negative regulators of insulin signaling. We showed the upregulation of these two components in studied tissues following central chronic resistin infusion. Indeed, SOCS-3 and PTP-1B act through multiple mechanisms. They inhibit insulin receptor activity and/or IRS-1/2 phosphorylation, altering then downstream targets such as phosphatidylinositol 3 kinase, Akt, and MAPK (reviewed in Refs. 41,42). Recently, resistin-induced hepatic insulin resistance has been recently attributed to the upregulation of SOCS-3 (19,26,43). Agreed with these findings, we found that central resistin increased hepatic

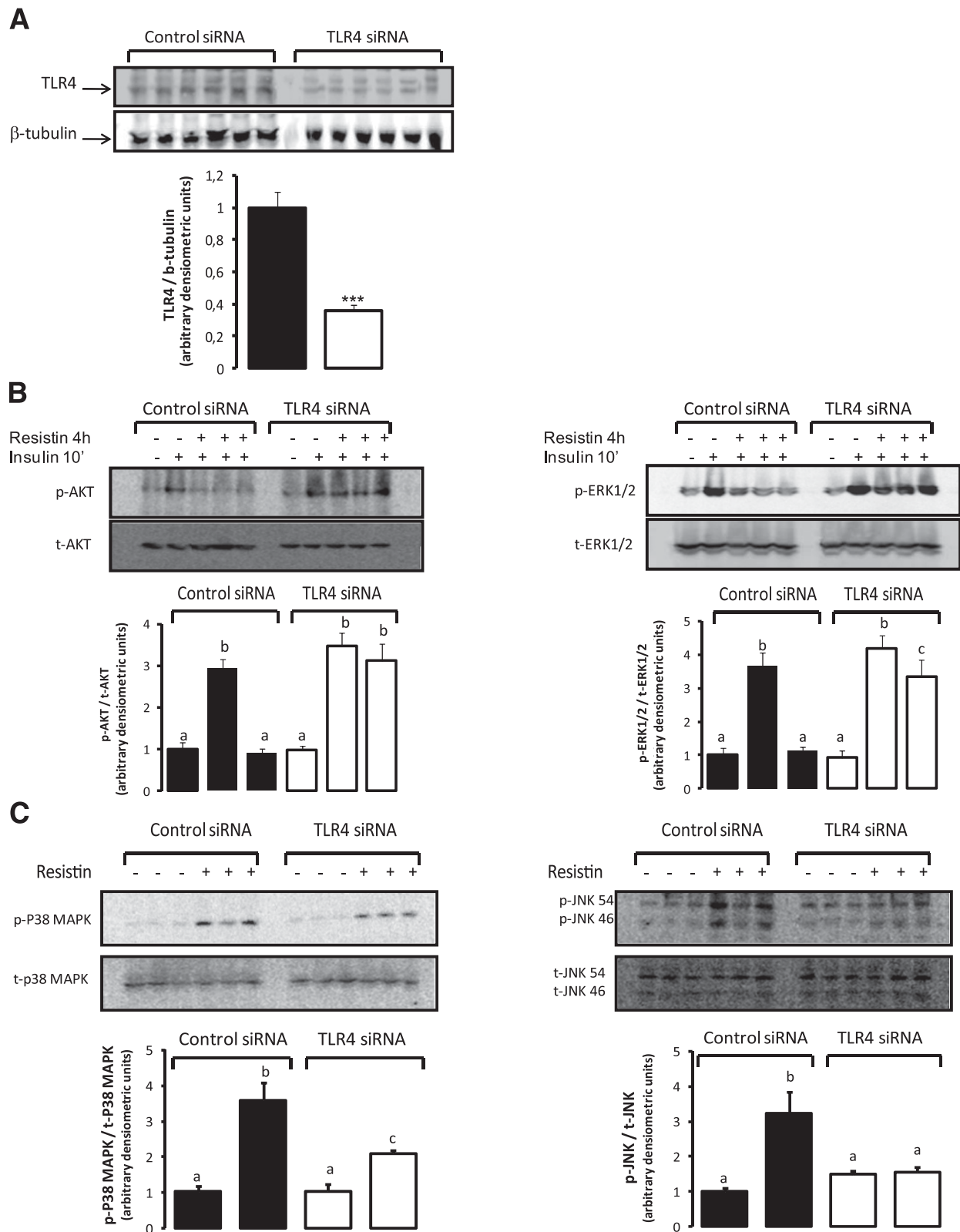


FIG. 7. siRNA-mediated TLR4 knockdown impairs resistin signaling in SH-SY5Y cells. SH-SY5Y cells were treated with control siRNA or specific siRNA duplexes targeting TLR4. Then, the protein lysates were subjected to Western blot analysis. **A:** Immunoblot analysis of TLR4 content in control and small interfering TLR4-treated cells. The results are expressed as the ratio of TLR4/β-tubulin. **B:** Immunoblot analysis of the phosphorylation of AKT and ERK1/2 by insulin (100 nmol/L for 10 min) in the presence or absence of resistin (100 ng/mL for 4 h) in SH-SY5Y cells titrated with control or TLR4 siRNA. **C:** Immunoblot analysis of the phosphorylation of JNK and P38 MAPK by resistin (100 ng/mL for 4 h) in SH-SY5Y cells treated with control siRNA or TLR4 siRNA. Results are expressed as ratio of p-P38MAPK/total (t)-P38MAPK and p-JNK (p-JNK54/46)/total JNK (t-JNK 54/46). All results are means ± SEM ($n = 3-6$ /group). *** $P < 0.001$ compared with control cells. ^{a,b,c} denote significant differences by ANOVA and Fisher post hoc test at $P < 0.05$.

SOCS-3 expression. We also showed a marked increase in the SOCS-3 levels in the hypothalamus, muscle, and adipose tissue following chronic resistin ICV treatment. Furthermore, we brought the first evidence for a resistin-dependent upregulation of PTP-1B, known as a promoter of insulin resistance, in the hypothalamus and liver (reviewed in Ref. 42). Similarly, the expression of SOCS-3 and PTP-1B was also enhanced in resistin-treated SH-SY5Y cells. Besides the impact on SOCS-3 and PTP-1B, we show that chronic resistin ICV treatment altered insulin signaling by increasing the serine phosphorylation of IRS-1 (S307), known to impair insulin signaling (44). Recent studies reported that resistin is able to modulate the serine phosphorylation of IRS1 in a cell model (45), but our finding is the first to report a resistin-dependent IRS-1 serine phosphorylation in a whole animal. Thus, the serine³⁰⁷ phosphorylation of IRS-1 appears to be another key candidate contributing to the resistin-dependent insulin resistance. To understand how resistin modulates IRS-1 serine³⁰⁷ phosphorylation, we investigated the effect of this adipokine on the activation of the serine kinases stress-activated protein kinase/JNK and p38, which are reported to phosphorylate IRS-1 on serine, leading to insulin resistance (46,47). In agreement with recent reports

demonstrating that resistin promotes the activation of JNK and p38MAPK in cellular models (45,48), the current study shows that central resistin treatment significantly increased the phosphorylation of JNK and p38MAPK both in the hypothalamus and peripheral insulin-sensitive tissues. The same results were obtained in resistin-treated SH-SY5Y cells. Taken together, these findings suggest that JNK and p38MAPK may mediate the inhibitory effects of central resistin on overall insulin signaling through the increase of the phosphorylation of IRS-1 on serine³⁰⁷ residue, leading to both neuronal and peripheral insulin resistance.

The upregulation of resistin has been associated to inflammatory state as reported in chronic liver diseases, lung diseases, and atherosclerosis plaques (5,6,9). Resistin also modulates the synthesis and secretion of key proinflammatory cytokines such as tumor necrosis factor- α , IL-6, and IL-12, known to inhibit insulin signal transduction (28,29). In agreement with these findings, we have shown that central resistin infusion markedly increases IL-6 expression in the hypothalamus and peripheral insulin-sensitive tissues promoting overall inflammation and insulin resistance. The molecular mechanisms by which resistin promotes inflammation are still unclear, but a recent

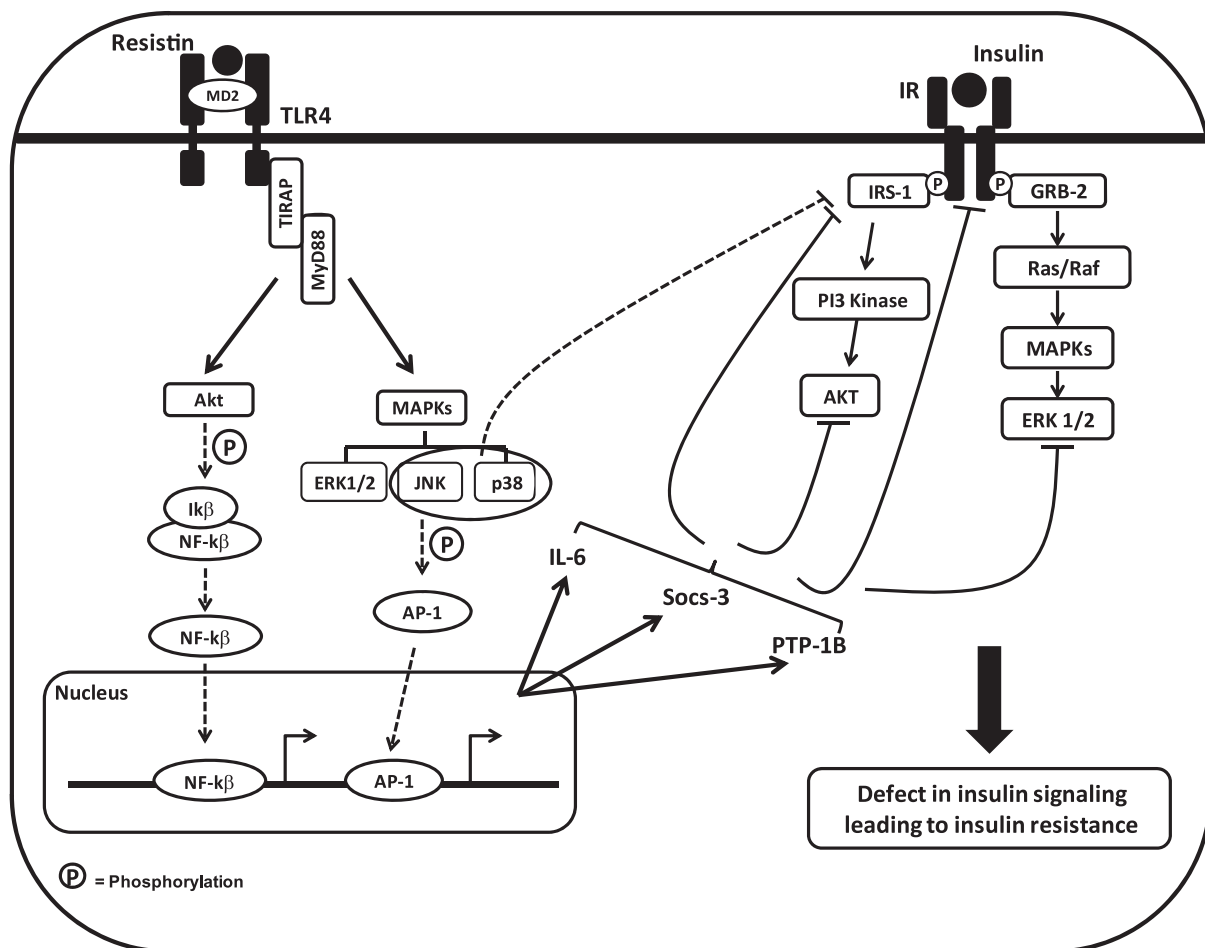


FIG. 8. Summary model. This figure summarizes the signaling pathways involved in resistin-induced insulin resistance at the neuronal levels. Resistin binding to TLR4 initiates TLR4 signaling pathways (Akt, ERK1/2, JNK, and p38 MAPK) through the recruitment of the adaptor molecules TIRAP and MyD88. Downstream signaling pathways are subsequently triggered, upregulating IL-6, SOCS-3, and PTP-1B, most likely through the activation of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein 1 (AP-1). The upregulation of SOCS-3, PTP-1B, and IL-6 combined to the activation on JNK and p38 MAPK predispose to the insulin resistance. GRB-2, growth factor receptor-bound 2; PI3, phosphatidylinositol 3.

report indicated that resistin could compete with LPS for TLR4. In fact, LPS binds to the heterodimer TLR4 and myeloid differentiation factor 2 (MD-2) to initiate TLR4 signaling (49). Additionally, other investigations reported the contribution of TLR4 activation in the pathogenesis of obesity and insulin resistance. Saturated fatty acids activate both hypothalamic and peripheral TLR4 signaling promoting proinflammatory cytokine production and endoplasmic reticulum stress (30–32). Conversely, TLR4 loss-of-function mutation or pharmacological inhibition of this receptor prevents saturated fatty acid-induced inflammation and insulin resistance (30,31,33). More recently, an original study has demonstrated in human epithelial cells that resistin competes with LPS for binding to TLR4, revealing more direct evidence for the involvement of TLR4 in the proinflammatory effects of resistin (34). However, the mechanism by which resistin acts in the hypothalamus and key insulin-dependent tissues was not yet identified. In this study and based on these previous observations, we postulate that resistin may act through the TLR4 receptor to promote the impairment of insulin signaling and responsiveness. Our results permit the dissection of resistin-signaling pathways in the hypothalamus and SH-SY5Y neuronal cells. We show that chronic ICV resistin treatment increases the association of TLR4 with MyD88 and TIRAP, two adaptors critically involved in TLR4 signal transduction in the hypothalamus (Fig. 8). Furthermore, we demonstrate, using cross-linking experiments, that resistin is able to bind directly to TLR4 receptor in SH-SY5Y human neuronal cells. The cross-link of resistin to TLR4 reveals a band with an apparent molecular weight of ~116 kDa that may correspond to a complex involving TLR4 (70 kDa), resistin (12.5 kDa), and MD-2 (20 kDa) as that described for LPS interaction with the TLR4/MD-2 complex. The role of TLR4 in resistin action at the neuronal level has been further investigated by the invalidation of TLR4 expression using siRNA, and we show a marked reduction of resistin binding to TLR4 and the resistin-induced activation of JNK and p38MAPK pathways, preventing the onset of insulin resistance.

Our findings bring new insight into the mechanisms involved in resistin-dependent insulin resistance at the level of the whole animal and identify the TLR4/MD-2 complex as the binding site for resistin in the hypothalamus.

ACKNOWLEDGMENTS

P.D.L. is affiliated with Shenandoah Biotechnology, Inc., Warwick, Pennsylvania. No other potential conflicts of interest relevant to this article were reported.

Y.B. and M.T. researched data and wrote the manuscript. A.G. contributed to research data and discussion. P.D.L., D.C., and H.O.H. contributed to research data. L.R. contributed to research data. M.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Claire-Marie Vacher, University Paris-Sud, Unité Mixte de Recherche (UMR) 8195, Orsay, France, for scientific advice and technical guidance concerning immunohistochemistry; Alain Aubourg, University Paris-Sud, UMR 8195, for technical advice; Charlotte Benoit, University Paris-Sud, UMR 8195, for contribution to enzyme-linked immunosorbent assay; and Flavien Berthou, University Paris-Sud, UMR 8195, for guidance concerning ICV experiments.

REFERENCES

- Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr Rev* 1999;20:68–100
- Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 2000;404:661–671
- Kahn BB, Flier JS. Obesity and insulin resistance. *J Clin Invest* 2000;106:473–481
- Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. *J Clin Invest* 2003;112:1785–1788 DOI: 10.1172/JCI200320514
- Steppan CM, Bailey ST, Bhat S, et al. The hormone resistin links obesity to diabetes. *Nature* 2001;409:307–312
- Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol* 2010;72:219–246
- Savage DB, Sewter CP, Klenk ES, et al. Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes* 2001;50:2199–2202
- Patel L, Buckels AC, Kinghorn IJ, et al. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. *Biochem Biophys Res Commun* 2003;300:472–476
- Mojiminiyi OA, Abdella NA. Associations of resistin with inflammation and insulin resistance in patients with type 2 diabetes mellitus. *Scand J Clin Lab Invest* 2007;67:215–225
- McTernan PG, Kusminski CM, Kumar S. Resistin. *Curr Opin Lipidol* 2006;17:170–175
- Holcomb IN, Kabakoff RC, Chan B, et al. FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *EMBO J* 2000;19:4046–4055
- Reilly MP, Lehrke M, Wolfe ML, Rohatgi A, Lazar MA, Rader DJ. Resistin is an inflammatory marker of atherosclerosis in humans. *Circulation* 2005;111:932–939
- Nogueiras R, Gualillo O, Caminos JE, Casanueva FF, Diéguez C. Regulation of resistin by gonadal, thyroid hormone, and nutritional status. *Obes Res* 2003;11:408–414
- Rajala MW, Obici S, Scherer PE, Rossetti L. Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. *J Clin Invest* 2003;111:225–230
- Pravenec M, Kazdová L, Landa V, et al. Transgenic and recombinant resistin impair skeletal muscle glucose metabolism in the spontaneously hypertensive rat. *J Biol Chem* 2003;278:45209–45215
- Satoh H, Nguyen MT, Miles PD, Imamura T, Usui I, Olefsky JM. Adenovirus-mediated chronic “hyper-resistinemia” leads to in vivo insulin resistance in normal rats. *J Clin Invest* 2004;114:224–231
- Muse ED, Obici S, Bhanot S, et al. Role of resistin in diet-induced hepatic insulin resistance. *J Clin Invest* 2004;114:232–239
- Banerjee RR, Rangwala SM, Shapiro JS, et al. Regulation of fasted blood glucose by resistin. *Science* 2004;303:1195–1198
- Qi Y, Nie Z, Lee Y-S, et al. Loss of resistin improves glucose homeostasis in leptin deficiency. *Diabetes* 2006;55:3083–3090
- Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BL, Murphy LJ. Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. *Eur J Endocrinol* 2003;149:331–335
- Vendrell J, Broch M, Vilarrasa N, et al. Resistin, adiponectin, ghrelin, leptin, and proinflammatory cytokines: relationships in obesity. *Obes Res* 2004;12:962–971
- Wilkinson M, Wilkinson D, Wiesner G, Morash B, Ur E. Hypothalamic resistin immunoreactivity is reduced by obesity in the mouse: co-localization with alpha-melanostimulating hormone. *Neuroendocrinology* 2005;81:19–30
- Brunetti L, Orlando G, Recinella L, Michelotto B, Ferrante C, Vacca M. Resistin, but not adiponectin, inhibits dopamine and norepinephrine release in the hypothalamus. *Eur J Pharmacol* 2004;493:41–44
- Tovar S, Nogueiras R, Tung LY, et al. Central administration of resistin promotes short-term satiety in rats. *Eur J Endocrinol* 2005;153:R1–R5
- Muse ED, Lam TK, Scherer PE, Rossetti L. Hypothalamic resistin induces hepatic insulin resistance. *J Clin Invest* 2007;117:1670–1678
- Vázquez MJ, González CR, Varela L, et al. Central resistin regulates hypothalamic and peripheral lipid metabolism in a nutritional-dependent fashion. *Endocrinology* 2008;149:4534–4543
- Singhal NS, Lazar MA, Ahima RS. Central resistin induces hepatic insulin resistance via neuropeptide Y. *J Neurosci* 2007;27:12924–12932
- Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ. Human resistin stimulates the proinflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway. *Biochem Biophys Res Commun* 2005;334:1092–1101

29. Bokarewa M, Nagaev I, Dahlberg L, Smith U, Tarkowski A. Resistin, an adipokine with potent proinflammatory properties. *J Immunol* 2005;174:5789–5795
30. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* 2007;56:1986–1998
31. Shi H, Kokoeba MV, Inouye K, Tzamelis I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 2006;116:3015–3025
32. Milanski M, Degasperi G, Coope A, et al. Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. *J Neurosci* 2009;29:359–370
33. Saberi M, Woods NB, de Luca C, et al. Hematopoietic cell-specific deletion of toll-like receptor 4 ameliorates hepatic and adipose tissue insulin resistance in high-fat-fed mice. *Cell Metab* 2009;10:419–429
34. Tarkowski A, Bjersing J, Shestakov A, Bokarewa MI. Resistin competes with lipopolysaccharide for binding to toll-like receptor 4. *J Cell Mol Med* 2010;14(6B):1419–1431
35. Daquinag AC, Zhang Y, Amaya-Manzanares F, Simmons PJ, Kolonin MG. An isoform of decorin is a resistin receptor on the surface of adipose progenitor cells. *Cell Stem Cell* 2011;9:74–86
36. Sánchez-Solana B, Laborda J, Baladrón V. Mouse resistin modulates adipogenesis and glucose uptake in 3T3-L1 preadipocytes through the ROR1 receptor. *Mol Endocrinol* 2012;26:110–127
37. Boström EA, Svensson M, Andersson S, et al. Resistin and insulin/insulin-like growth factor signaling in rheumatoid arthritis. *Arthritis Rheum* 2011;63:2894–2904
38. Kos K, Harte AL, da Silva NF, et al. Adiponectin and resistin in human cerebrospinal fluid and expression of adiponectin receptors in the human hypothalamus. *J Clin Endocrinol Metab* 2007;92:1129–1136
39. Palanivel R, Maida A, Liu Y, Sweeney G. Regulation of insulin signaling, glucose uptake and metabolism in rat skeletal muscle cells upon prolonged exposure to resistin. *Diabetologia* 2006;49:183–190
40. Yang Y, Xiao M, Mao Y, et al. Resistin and insulin resistance in hepatocytes: resistin disturbs glycogen metabolism at the protein level. *Biomed Pharmacother* 2009;63:366–374
41. Howard JK, Flier JS. Attenuation of leptin and insulin signaling by SOCS proteins. *Trends Endocrinol Metab* 2006;17:365–371
42. Koren S, Fantus IG. Inhibition of the protein tyrosine phosphatase PTP1B: potential therapy for obesity, insulin resistance and type-2 diabetes mellitus. *Best Pract Res Clin Endocrinol Metab* 2007;21:621–640
43. Steppan CM, Wang J, Whiteman EL, Birnbaum MJ, Lazar MA. Activation of SOCS-3 by resistin. *Mol Cell Biol* 2005;25:1569–1575
44. Gual P, Le Marchand-Brustel Y, Tanti JF. Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie* 2005;87:99–109
45. Kang S, Chemaly ER, Hajjar RJ, Lebeche D. Resistin promotes cardiac hypertrophy via the AMP-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) and c-Jun N-terminal kinase/insulin receptor substrate 1 (JNK/IRS1) pathways. *J Biol Chem* 2011;286:18465–18473
46. Aguirre V, Uchida T, Yenush L, Davis R, White MF. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 2000;275:9047–9054
47. Hemi R, Yochananov Y, Barhod E, et al. p38 mitogen-activated protein kinase-dependent transactivation of ErbB receptor family: a novel common mechanism for stress-induced IRS-1 serine phosphorylation and insulin resistance. *Diabetes* 2011;60:1134–1145
48. Shen YH, Zhang L, Gan Y, et al. Up-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome ten) mediates p38 MAPK stress signal-induced inhibition of insulin signaling. A cross-talk between stress signaling and insulin signaling in resistin-treated human endothelial cells. *J Biol Chem* 2006;281:7727–7736
49. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 2009;458:1191–1195