Original Article

Exercise Improves Insulin and Leptin Sensitivity in Hypothalamus of Wistar Rats

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Prolonged exercise of medium to high intensity is known to promote a substantial effect on the energy balance of rats. In male rats, moderately to severely intense programs lead to a reduction in food intake. However, the exact causes for the appetite-suppressive effects of exercise are not known. Here, we show that intracerebroventricular insulin or leptin infusion reduced food intake in exercised rats to a greater extent than that observed in control animals. Exercise was associated with a markedly increased phosphorylation/activity of several proteins involved in leptin and insulin signal transduction in the hypothalamus. The regulatory role of interleukin (IL)-6 in mediating the increase in leptin and insulin sensitivity in hypothalamus was also investigated. Treatment with insulin or leptin markedly reduced food intake in exercised rats that were pretreated with vehicle, although no increase in sensitivity to leptin- and insulin-induced anorexia after pretreatment with anti–IL-6 antibody was detected. The current study provides direct measurements of leptin and insulin signaling in the hypothalamus and documents increased sensitivity to these hormones in the hypothalamus of exercised rats in an IL-6–dependent manner. These findings provide support for the hypothesis that the appetite-suppressive actions of exercise may be mediated by the hypothalamus.
***Diabetes* 55:2554–2561, 2006**

Prolonged exercise of medium to high intensity is known to profoundly affect energy balance (1–3). Studies of individuals who have maintained significant weight loss for >1 year have demonstrated that dieters who achieve long-term success are often those who engage in regular and extensive exercise programs (4). Although the energy expenditure aspects of such exercise may contribute to the effects of weight maintenance, it has been suggested that even acute exercise may also contribute to the energy balance by altering appetite and reducing food intake. However, the mechanisms underlying the effects of exercise on food intake have not yet been identified.

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Received for publication 14 December 2005 and accepted in revised form 5 June 2006.

IL, interleukin; IRS, insulin receptor substrate; JAK, janus kinase; PI, phosphatidylinositol; STAT, signal transducer and activator of transcription.

DOI: 10.2337/db05-1622

The circulating peptide leptin is secreted predominantly by white adipose tissue and provides feedback information on the extent of the body's fat stores to hypothalamic leptin receptors (ObRs) that coordinate food intake and body weight homeostasis (5,6). Wild-type ObRs possess a number of signaling capabilities; these include activation of the janus kinase–signal transducer and activator of transcription (JAK-STAT) (7–11) and mitogen-activated protein kinase pathways and stimulation of tyrosine phosphorylation of insulin receptor substrate (IRS)-1, IRS-2, and phosphatidylinositol (PI) 3-kinase (10–13).

Insulin acts at the same hypothalamic areas as leptin to suppress feeding (6,14). The insulin receptor is a protein tyrosine kinase that is activated by insulin binding, undergoing rapid autophosphorylation and phosphorylating intracellular protein substrates, including IRS-1 and -2 (15,16). After tyrosine phosphorylation, the IRSs act as docking proteins for several SH2 (Src homology 2) domain–containing proteins, including PI 3-kinase, Grb2 (growth factor receptor-bound protein-2), SHP2 (src-homology phosphatase-2), Nck, and Fyn (17–21). PI 3-kinase activates two kinases: phosphoinositide-dependent protein kinase 1, which phosphorylates Akt on threonine 308, and a putative phosphoinositide-dependent protein kinase 2, which phosphorylates Akt on serine 473, leading to an increase in Akt kinase activity (22).

The level of circulating interleukin (IL)-6 increases dramatically in response to exercise (23), with IL-6 being produced by working muscle (24,25) and adipose tissue (26–28). IL-6 seems to have several important roles in metabolism, including induction of lipolysis (26,29) and enhancement of insulin sensitivity when injected into IL-6–deficient mice (30). Furthermore, it appears that centrally acting IL-6 plays a role in the regulation of appetite, energy expenditure, and body composition (30). Intracellular interactions between different signaling systems may enhance or counterregulate hormone actions. Thus, it is possible that the effects of acute exercise on central insulin and leptin sensitivity may be dependent on IL-6. The status of leptin and insulin signaling in hypothalamus has not previously been assessed in rats after acute exercise. We therefore examined hypothalamic JAK-STAT and IRS-1/2–PI 3-kinase signaling pathways as well as the role of IL-6 in insulin and leptin signaling in rats after acute exercise.

RESEARCH DESIGN AND METHODS

The reagents for SDS-PAGE and immunoblotting were from Bio-Rad. Tris, aprotinin, ATP, dithiothreitol, phenylmethylsulfonyl fluoride, Triton X-100, Tween 20, glycerol, and BSA (fraction V) were from Sigma (St. Louis, MO). Protein A-Sepharose 6MB, ^{125}I-protein A, and nitrocellulose paper (Hybond

ECL, 0.45 μm) were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). Sodium amobarbital (Amytal) and human recombinant insulin (Humulin R) were from Eli Lilly (Indianapolis, IN). Leptin was from Calbiochem (San Diego, CA). Ketamine hydrochloride was from Cristália (Itapira, Brazil). Antibodies to insulin receptor, IRS-1, IRS-2, Akt, JAK2, ObR, SOCS3 (suppressor of cytokine signaling 3), PTP1b (protein-tyrosine phosphatase 1b), STAT3, and IL-6 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Akt phosphoserine 473–specific and phosphothreonine 308–specific antibodies and the STAT3 phosphotyrosine 705–specific antibody were from New England Biolabs (Beverly, MA), and the antibody to the p85 subunit of PI 3-kinase was from Upstate Biotechnology (Lake Placid, NY). Routine reagents were purchased from Sigma, unless otherwise specified.

Animals and surgical procedure. Male Wistar rats (200–250 g) from the University of Campinas Central Animal Breeding Center were used in the experiments. All experiments involving animals were in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the ethics committee at the University of Campinas. Rats were maintained on a 12-h light/dark cycle and were provided free access to water and standard rodent chow before the exercise; they were randomly assigned to one of two groups: those exercised for 6 h or control rats. After an overnight fast, the rats were anesthetized with ketamine hydrochloride plus diazepan and positioned on a Stoelting stereotaxic apparatus. At 10 days before the exercise protocol, the catheter was implanted into the third ventricle as previously described (10). After a 1-week recovery period, catheter placement was confirmed by a positive drinking response after administration of angiotensin II (40 ng per 2 μl), and animals that did not drink 5 ml of water within 15 min after treatment were not included in the experiment.

Exercise protocol. Rats were acclimated to swimming for 10 min per day for 2 days. The swimming protocol was performed as previously described (31). The rats swam in groups of three in plastic barrels 45 cm in diameter that were filled to a depth of 50 cm, and the water temperature was maintained at 34–35°C. Animals performed two 3-h exercise bouts separated by one 45-min rest period.

Treatments and measurement of food intake. After the last bout of exercise, animals were injected (2-μl bolus injection i.c.v.) with either vehicle, insulin (Eli Lilly), or leptin (rat leptin from National Institutes of Health) at the doses indicated. Thereafter, standard chow was given, and food intake was determined by measuring the difference between the weight of chow given and the weight of chow at the end of a 12-h period. Similar studies were carried out after the last bout of exercise in rats that were initially intracerebroventricularly injected with anti–IL-6 (rabbit anti–IL-6 at the doses indicated; Santa Cruz Biotechnology) or vehicle and then, after 30 min, with insulin or leptin. In preliminary experiments, we determined plasma glucose levels in animals that received intracerebroventricular insulin infusion. Plasma glucose was not altered by third ventricle insulin or saline microinjection.

Western blot analysis. Immediately after the last exercise bout, animals were treated with vehicle, insulin, or leptin, according to the protocols described in the preceding section, and then they were decapitated, and the hypothalami were removed at the time points indicated. The hypothalami were minced coarsely and homogenized immediately in solubilization buffer containing 100 mmol/l Tris (pH 7.6), 1% Triton X-100, 150 mmol/l NaCl, 0.1 mg aprotinin, 35 mg/ml phenylmethylsulfonyl fluoride, 10 mmol/l Na_3VO_4, 100 mmol/l NaF, 10 mmol/l $Na_4P_2O_7$, and 4 mmol/l EDTA, using a polytron PTA 20S generator (Model PT 10/35; Brinkmann Instruments, Westbury, NY) operated at maximum speed for 30 s and clarified by centrifugation. Equal amounts of protein were used for immunoprecipitation followed by Western blot analysis with the indicated antibodies and ^{125}I-protein A. ^{125}I-protein A bound to anti-peptide antibodies was detected by autoradiography, using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with Cronex Lightning Plus intensifying screens (DuPont, Wilmington, DE) at −80°C for 12–48 h. Band intensities were quantitated by optical densitometry (Scion Image software; ScionCorp, Frederick, MD) of the developed autoradiographs.

PI 3-kinase assay. Aliquots of supernatants containing equal amounts of protein were incubated overnight at 4°C, using antibodies against IRS-1 or -2, and the immunocomplexes were precipitated with a 50% solution of protein A-Sepharose 6MB. In vitro PI 3-kinase assays were performed as previously described (17). The ^{32}P-labeled 3-P-PI was quantitated using Scion Image software.

Statistical analysis. Where appropriate, the results are expressed as the means ± SE accompanied by the indicated number of rats used in experiments. Comparisons among groups were performed using parametric two-way ANOVA, where *F* ratios were significant; further comparisons were performed using the Bonferroni test.

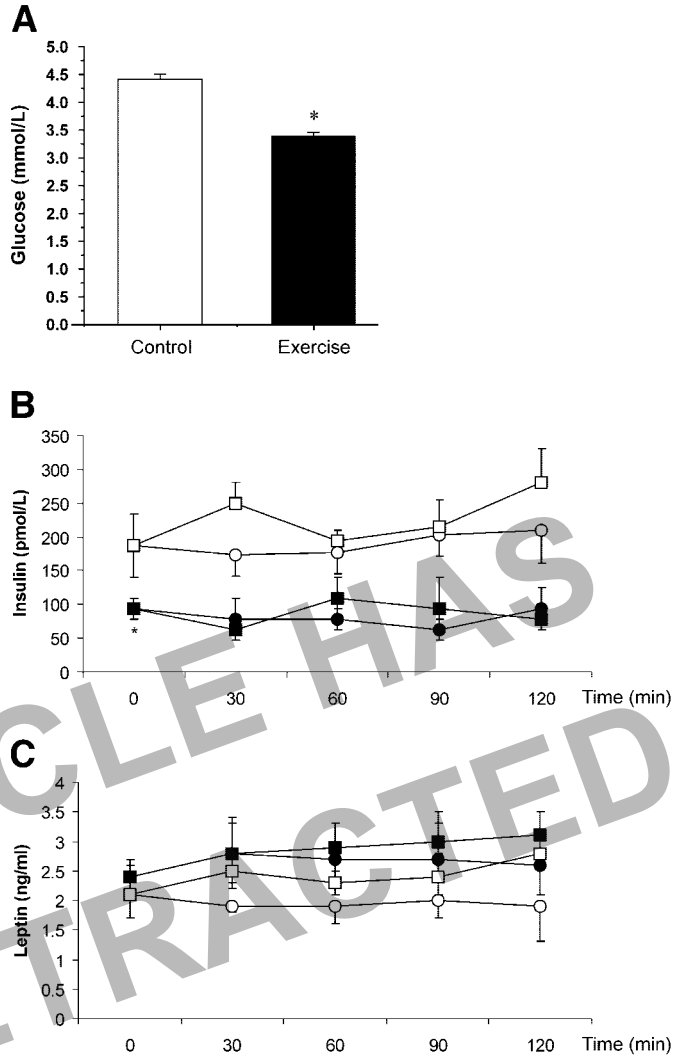

FIG. 1. Physiological characteristics of control and exercised rats. Effects of exercise on plasma glucose concentration (mmol/l) (*A*), plasma insulin concentration (pmol/l) (*B*), and plasma leptin concentration (ng/ml) (*C*). Data are the means ± SE, $n = 6$ animals per group. *$P < 0.01$ vs. control. □, control: insulin i.c.v.; ○, control: leptin i.c.v.; ■, exercise: insulin i.c.v.; ●, exercise: leptin i.c.v.

RESULTS

Physiological parameters measured in basal conditions after 6 h of exercise. The plasma glucose level was lower in the exercised group compared with the control group (3.39 vs. 4.41 mmol/l, respectively) (Fig. 1*A*), and the insulin levels were also lower (93.6 vs. 187.2 pmol/l) (Fig. 1*B*). Exercise did not, however, reduce plasma leptin (2.1 vs. 2.4 ng/ml) (Fig. 1*C*). As shown in Figs. 1*B* and *C*, insulinemia and leptinemia were not altered by third ventricle microinjection of insulin or leptin.

Intracerebroventricular leptin reduces food intake and activates the hypothalamic JAK-STAT pathway in exercised rats to a greater extent than in control animals. The effect of leptin (5, 10, and 20 μg), or its vehicle, on the control of food intake was studied by measuring the total food intake for 12 h after a session of exercise and a single intracerebroventricular injection of leptin or its vehicle. Leptin induced reductions in the 12-h food intake in both exercised and control rats in a concentration-dependent manner. In the exercised rats, leptin (5, 10, and 20 μg) reduced food intake by 38.5, 44.6, and

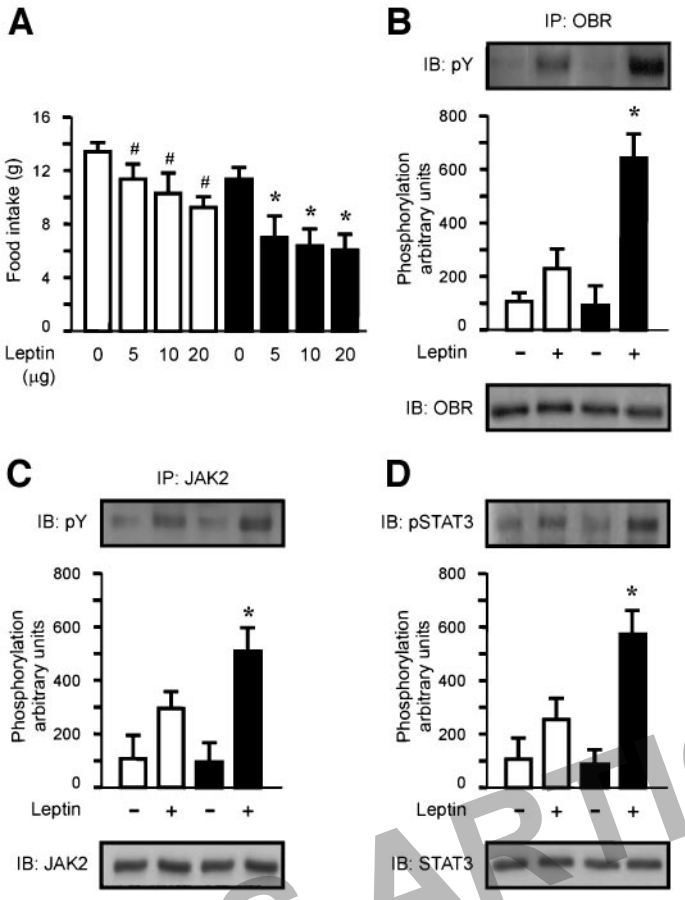

FIG. 2. Leptin inhibition of the 12-h cumulative food intake and leptin signaling in the hypothalamus of control and exercised rats. Vehicle (−) or leptin (+) was injected intracerebroventricularly after a 6-h session of exercise, and rats were immediately exposed to food for 12 h. Data are the means ± SE of 8–14 animals per group (*A*). At 15 min after the infusion, tissue extracts were immunoprecipitated (IP) with anti-ObR and anti-JAK2 and immunoblotted (IB) with anti-phosphotyrosine antibody (pY) (*B* and *C*, *upper panels*). Whole-tissue extracts were immunoblotted with pSTAT3 antibody (*D*, *upper panel*). Stripped membranes were reblotted with anti-ObR, anti-JAK2, and anti-STAT3 antibodies (*B–D*, *lower panels*). The results of scanning densitometry were expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 8$ animals per group. *$P < 0.05$, leptin control vs. leptin exercise; #$P < 0.05$, leptin control vs. control. □, control; ■, exercise.

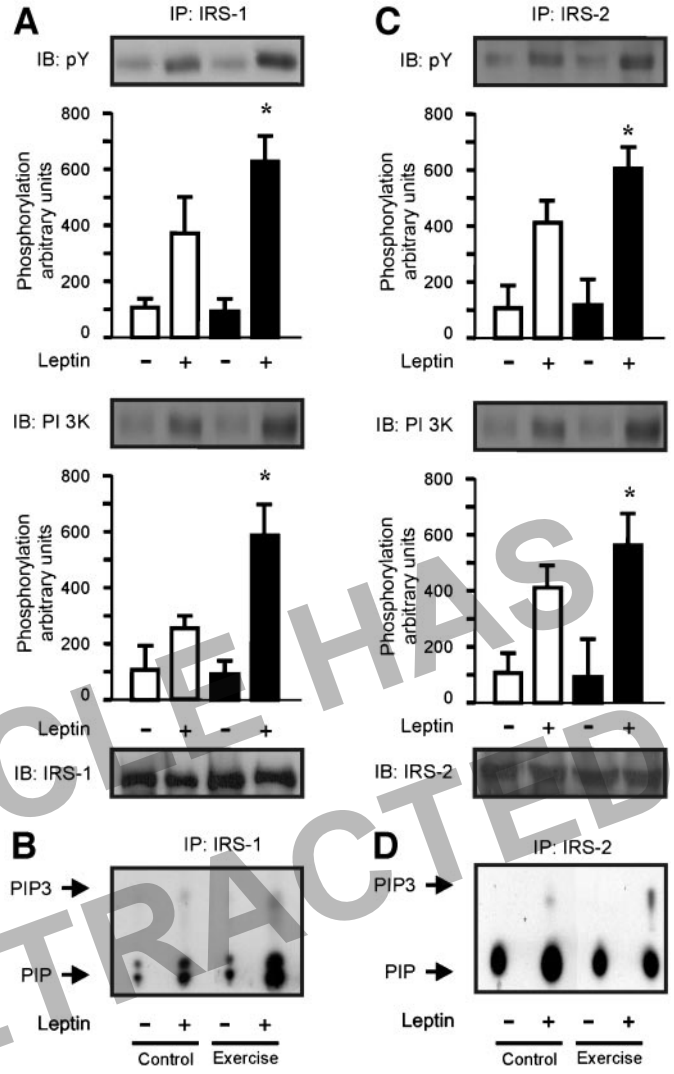

FIG. 3. Leptin signaling in the hypothalamus of control and exercised rats. Hypothalamus extracts from rats injected with vehicle (−) or leptin (+) were prepared as described in RESEARCH DESIGN AND METHODS. At 5 min after the infusion tissue, extracts were immunoprecipitated (IP) with anti–IRS-1 and anti–IRS-2 antibodies and immunoblotted (IB) with anti-phosphotyrosine (pY) (*A* and *C*, *upper panels*), anti–PI 3-kinase (*A* and *C*, *middle panels*), anti–IRS-1, and anti–IRS-2 antibodies (*A* and *C*, *lower panels*). PI 3-kinase assays were performed as described. Fluorographs show the silica thin-layer chromatography plates of IRS-1– or IRS-2–associated PI 3-kinase activity (*B* and *D*). The results of scanning densitometry were expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 6$ animals per group. *$P < 0.05$, leptin control vs. leptin exercise. □, control; ■, exercise. PIP, the migration position of PI 3–phosphate.

46.4%, respectively, whereas in the control group, these doses induced reductions of 15.7, 23.2, and 31.2%, indicating that leptin was much more effective in exercised rats (Fig. 2*A*).

To determine the effects of exercise on the early steps of the leptin signaling pathway, a dose of 10 μg was administered and the ObR and JAK2 tyrosine phosphorylation was assessed in the hypothalamus of exercised and control rats. Immunoprecipitation and Western blotting of hypothalamic extracts were performed using anti-ObR, anti-JAK2, and anti-phosphotyrosine antibodies. Leptin induced increases in ObR and JAK2 tyrosine phosphorylation levels in hypothalami from both control and exercised rats. In the exercised animals, leptin increased ObR and JAK2 tyrosine phosphorylation by 6.0- and 4.7-fold, respectively, compared with 2.1- and 2.7-fold increases in the hypothalami from control rats, representing increases in ObR tyrosine phosphorylation of 4.7- and 2.2-fold, respectively (Fig. 2*B* and *C*, *upper panels*). The same membranes used to detect tyrosine phosphorylation of ObR and JAK2 were reblotted with ObR and JAK2 antibodies, and, as expected, there were no changes in ObR and JAK2 protein expression (Fig. 2*B* and *C*, *lower panels*). Hypothalamic extracts from exercised and control rats that were stimulated with leptin (10 μg) were lysed and the proteins separated by SDS-PAGE gel and blotted with pSTAT3 antibodies. In the hypothalami from exercised animals, leptin increased STAT3 tyrosine phosphorylation by 5.3-fold compared with 2.4-fold increases in the hypothalami from control rats, representing 3.2-fold increases in STAT3 tyrosine phosphorylation (Fig. 2*D*, *upper panel*). No changes were observed in STAT3 protein expression (Fig. 2*D*, *lower panel*).

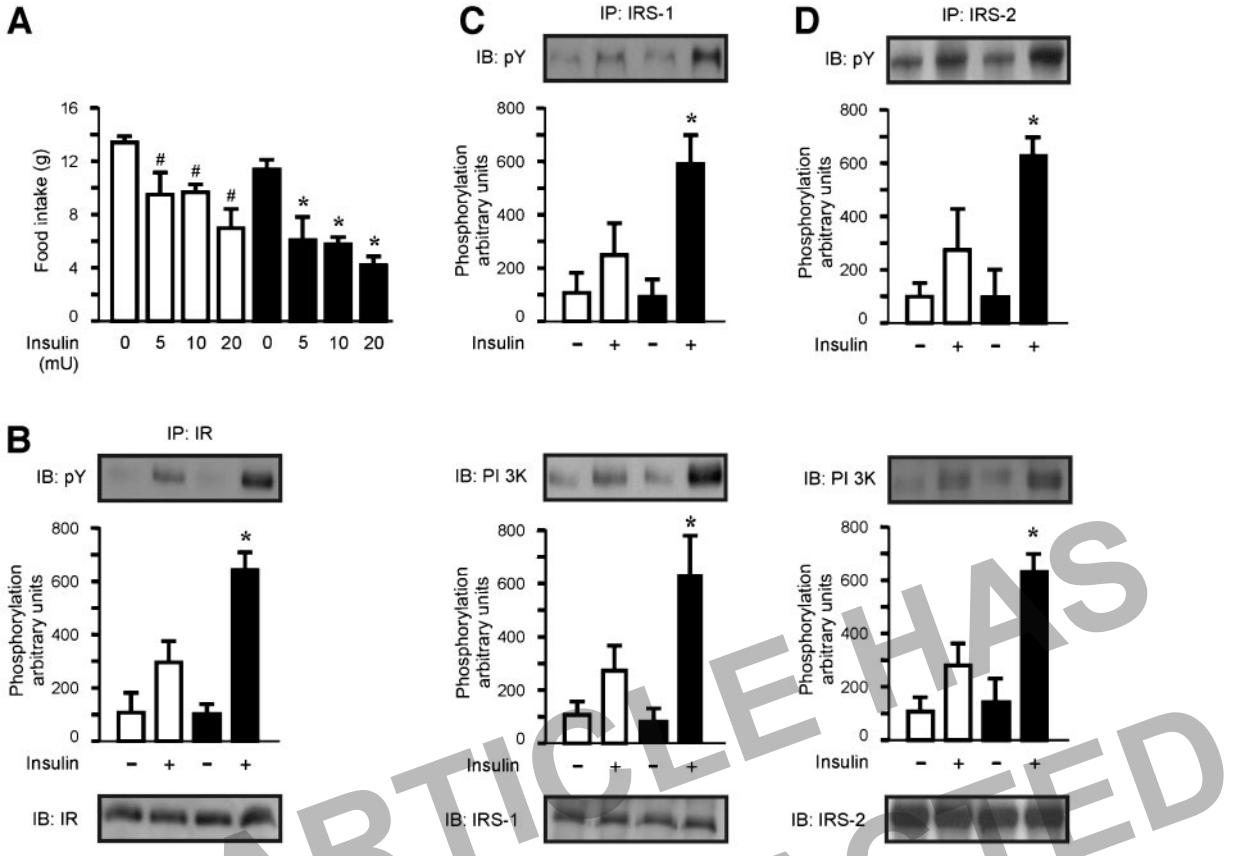

FIG. 4. Insulin inhibition of 12-h cumulative food intake and insulin signaling in the hypothalami of control and exercised rats. Vehicle or insulin was injected intracerebroventricularly after a session of 6-h exercise, and rats were immediately exposed to food for 12 h. Data are the means ± SE of 8–14 animals per group (*A*). At 15 min after the infusion, tissue extracts were immunoprecipitated (IP) with anti–insulin receptor antibody and immunoblotted (IB) with anti-phosphotyrosine antibody (pY) (*B*, *upper panel*) and anti–insulin receptor antibody (*B*, *lower panel*). Tissue extracts were also immunoprecipitated with anti–IRS-1 and anti–IRS-2 antibodies and immunoblotted with anti-phosphotyrosine (*C* and *D*, *upper panels*), anti–PI 3-kinase (*C* and *D*, *middle panels*), or anti–IRS-1 or anti–IRS-2 antibodies (*C* and *D*, *lower panels*). The results of scanning densitometry were expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 6$ animals per group. *$P < 0.05$, insulin control vs. insulin exercise; #$P < 0.05$, insulin control vs. control. □, control; ■, exercise.

Intracerebroventricular leptin activates the hypothalamic IRSs–PI 3-kinase pathway in exercised rats to a greater extent than in control animals. Immunoprecipitation and Western blotting of hypothalamic extracts were performed using anti–IRS-1, anti–IRS-2, and anti-phosphotyrosine antibodies. Leptin (10 μg) induced increases in IRS-1/2 tyrosine phosphorylation levels in hypothalami from both control and exercised rats. In the exercised animals, leptin increased IRS-1 and -2 tyrosine phosphorylation by 5.8- and 5.6-fold, respectively, compared with 3.4- and 3.8-fold increases in the hypothalami from control rats, representing increases in IRS-1 and -2 tyrosine phosphorylation of 2.0- and 1.6-fold, respectively (Fig. 3*A* and *C*, *upper panels*).

The same membranes used to detect tyrosine phosphorylation of IRS-1 and -2 were reblotted with antibodies against the p85 subunit of PI 3-kinase. The PI 3-kinase association with IRS-1 and -2 paralleled the changes in the phosphorylation of these proteins (Fig. 3*A* and *C*, *middle panels*). There were no changes in IRS-1 and -2 protein expressions (Fig. 3*A* and *C*, *lower panels*). To determine whether there was PI 3-kinase activity in IRS-1 and -2 immunoprecipitates, hypothalami were prepared and immunoprecipitated with anti–IRS-1 or anti–IRS-2 antibodies from both control and exercised rats. After treatment with leptin, there was an increase in PI 3-kinase activity associated with IRS-1 and -2. In the exercised animals, leptin increased PI 3-kinase activity associated with IRS-1 and -2 by 5.5- and 5.2-fold, respectively, compared with 2.4- and 3.8-fold increases in the hypothalami from control rats, representing increases in PI 3-kinase activity associated with IRS-1 and -2 of 2.0- and 1.6- fold, respectively (Fig. 3*B* and *D*).

Intracerebroventricular insulin reduces food intake and activates the hypothalamic IRS-1/2–PI 3-kinase pathway in exercised rats to a greater extent than in control animals. The effect of insulin, or its vehicle, on the control of food intake was studied by measuring total food intake for 12 h after a session of exercise and a single intracerebroventricular injection of insulin or its vehicle. Insulin induced reductions in 12-h food intake in both exercised and control rats in a concentration-dependent manner. In the exercised animals, insulin (5, 10, and 20 mU) reduced food intake by 46.4, 49.1, and 63.3%, respectively, whereas in the control group, these doses induced reductions of 29.2, 27.7, and 47.9%, indicating that insulin was much more effective in exercised rats (Fig. 4*A*).

To determine the effects of exercise on the early steps of the insulin signaling pathway, insulin receptor, IRS-1, and IRS-2 tyrosine phosphorylation was assessed in the hypothalamus of trained and control rats. Immunoprecipitation and Western blotting of hypothalamic extracts were performed using anti–insulin receptor, anti–IRS-1, anti–IRS-2, and anti-phosphotyrosine antibodies. Insulin (10 mU) in-

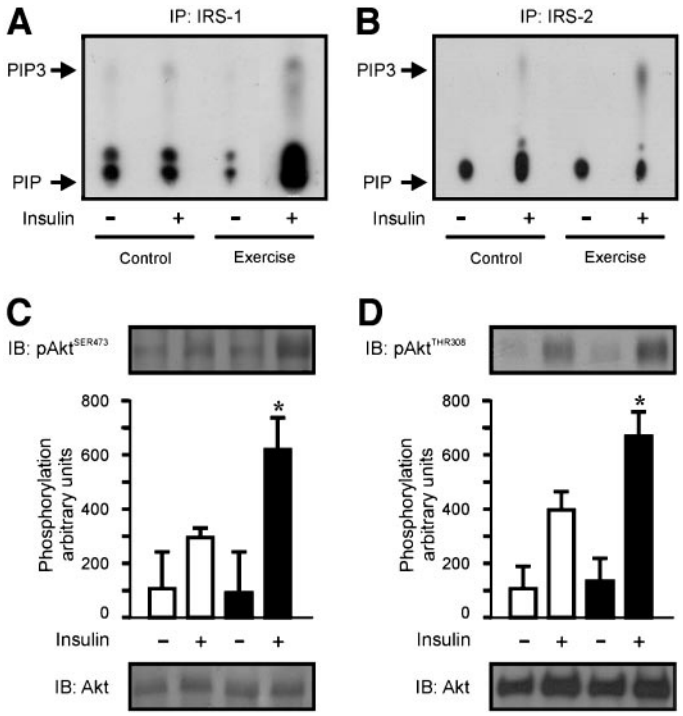

FIG. 5. Insulin signaling in the hypothalami of control and exercised rats. Hypothalami from rats injected with vehicle (−) or insulin (+) were prepared 15 min after the infusion, as described in RESEARCH DESIGN AND METHODS. Fluorographs show the silica thin-layer chromatography plates of IRS-1– or IRS-2–associated PI 3-kinase activity. At 15 min after the infusion, whole-tissue extracts were immunoblotted (IB) with anti–pAkt serine 473 (*C*, *upper panel*) or anti–pAkt threonine 308 (*D*, *upper panel*) and with anti-Akt antibodies (*C* and *D*, *lower panels*). The results of scanning densitometry were expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 6$ animals per group. *$P < 0.05$, insulin control vs. insulin exercise. □, control; ■, exercise. PIP, the migration position of PI 3–phosphate (*A* and *B*).

duced increases in insulin receptor, IRS-1, and IRS-2 tyrosine phosphorylation levels in the hypothalamus of both control and exercised rats. In the exercised animals, insulin increased insulin receptor, IRS-1, and IRS-2 tyrosine phosphorylation by 6.0-, 5.5-, and 5.8-fold, respectively, compared with 2.7-, 2.3-, and 2.5-fold increases in the hypothalami from control rats, representing increases in insulin receptor and IRS-1/2 tyrosine phosphorylation of 3.0-, 3.6-, and 3.2-fold, respectively (Fig. 4*B*, *C*, and *D*, *upper panels*). The same membranes used to detect tyrosine phosphorylation of IRS-1/2 were reblotted with antibodies against the p85 subunit of PI 3-kinase. The PI 3-kinase association to IRS-1 and -2 paralleled the changes in phosphorylation of these proteins (Fig. 4*C* and *D*, *middle panels*).

To determine whether there was PI 3-kinase activity in the IRS-1/2 immunoprecipitates, hypothalami from control and exercised rats treated with insulin (10 mU), after a session of exercise, were prepared and immunoprecipitated with anti–IRS-1 or anti–IRS-2 antibodies. After treatment with insulin, there was an increase in PI 3-kinase activity associated with IRS-1 and -2. In the exercised animals, insulin increased PI 3-kinase activity associated with IRS-1 and -2 by 5.8- and 6.0-fold, respectively, compared with 2.5- and 2.6-fold increases in the hypothalami from control rats, representing increases in PI 3-kinase activity associated with IRS-1 and -2 of 2.0- and 1.6-fold, respectively (Fig. 5*A* and *B*).

Hypothalamic extracts from exercised and control rats that were stimulated with insulin (10 mU) were lysed and the proteins separated by SDS-PAGE on gel and blotted with pAkt antibodies. In the hypothalami from exercised animals, insulin increased Akt serine 473 and Akt threonine 308 phosphorylation by 5.7- and 6.0-fold, respectively, compared with 2.8- and 3.7-fold increases in the hypothalami from control rats, representing increases in Akt serine phosphorylation of 2.7-fold (Fig. 5*C*, *upper panel*). No changes were observed in Akt protein expression (Fig. 5*C*, *lower panel*).

Role of IL-6 in anorectic response to leptin and insulin. IL-6 expression was detected in control animals; however, a 2.8-fold increase was observed in exercised animals (Fig. 6*A*). We tested whether the inhibitory effects of leptin and insulin on food intake depend on IL-6 by intracerebroventricular infusion of anti–IL-6 into exercised rats. Treatment with leptin or insulin markedly reduced 12-h food intake in exercised rats pretreated with vehicle, although pretreatment with anti–IL-6 blocked exercise-induced leptin (Fig. 6*B*) and insulin (Fig. 6*C*) sensitivity in a concentration-dependent manner, respectively (Fig. 6*B* and *C*). Consistent with the increase in leptin sensitivity, JAK2 (Fig. 6*D*) and STAT3 (Fig. 6*E*) phosphorylation were induced by exercise and reversed by anti–IL-6 in accordance with the control of food intake. Insulin induced a significant increase in insulin receptor (Fig. 6*F*), Akt serine 473 (Fig. 6*G*), and threonine (Fig. 6*H*) phosphorylation in the hypothalamus of exercised rats pretreated with vehicle. In animals pretreated with anti–IL-6, the effect of exercise on insulin signaling was reversed in a concentration-dependent manner.

DISCUSSION

Exercise training has multiple effects on metabolism and gene expression (31). However, little is known about the mechanisms by which exercise leads to reduced appetite. Here, we provide evidence for a molecular mechanism to account for increased insulin and leptin action in hypothalamus after exercise. Intracerebroventricular insulin or leptin infusion in doses that did not alter insulinemia or leptinemia reduced food intake in exercised rats to a greater extent than that observed in control animals. Exercise was associated with a marked increase in the phosphorylation/activity of several proteins involved in leptin and insulin signal transduction in hypothalamus. In addition, we investigated the regulatory role of IL-6 in mediating the increase in leptin and insulin sensitivity in hypothalamus. Treatment with insulin or leptin markedly reduced food intake in exercised rats that were pretreated with vehicle, although no increase in sensitivity to leptin- and insulin-induced anorexia was detected after pretreatment with anti–IL-6 antibody. Increased leptin and insulin action in the brain may thus contribute to the modulation of energy homeostasis in exercised rats.

Despite a recent publication showing that the 12-week wheel exercise protocol reduced the expression of ObRb mRNA in the arcuate nucleus (32), our data demonstrate that after a session of exercise, there were no changes in the expression of hypothalamic proteins involved in leptin and insulin signal transduction. However, the phosphorylation status of these proteins was deeply modified. Exercise led to an increase in leptin- and insulin-stimulated ObR/JAK2 and insulin receptor tyrosine phosphorylation, respectively. The next step in leptin and insulin signaling may involve the tyrosine phosphorylation of IRS-1 and -2.

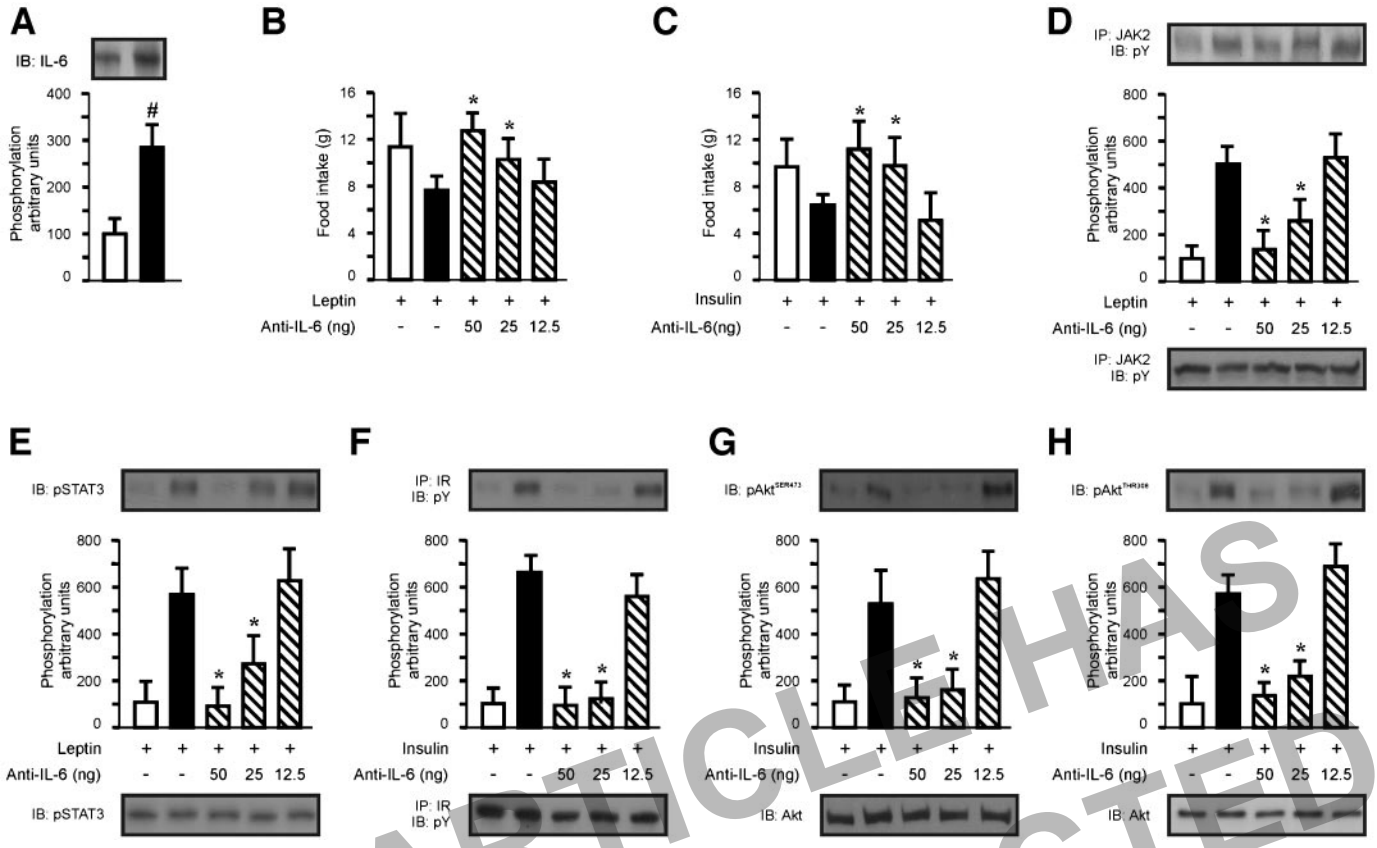

FIG. 6. Blockade of leptin and insulin-induced inhibition of food intake by anti–IL-6. Hypothalami from rats were prepared as described in RESEARCH DESIGN AND METHODS. Tissue extracts from control and exercised rats were immunoblotted with anti–IL-6 antibody (*A*). Leptin and insulin were injected intracerebroventricularly in control rats, exercised rats, and exercised rats pretreated with anti–IL-6 at the doses indicated, and the animals were immediately exposed to food for 12 h. Data are the means ± SE of 8–14 animals per group (*B* and *C*). Tissue extracts from control rats, exercised rats, and exercised rats pretreated with anti–IL-6 were treated with leptin and immunoprecipitated (IP) with anti-JAK2 antibody and immunoblotted (IB) with anti-phosphotyrosine antibody (*D*, *upper panel*) and with anti-JAK2 (*D*, *lower panel*). Whole-tissue extracts were immunoblotted with anti–phospho STAT3 antibody (*E*, *lower panel*) and with anti-STAT3 (*E*, *lower panel*). Hypothalamus tissue extracts from control, exercised, and exercised rats pretreated with anti–IL-6 were treated with insulin and immunoprecipitated with anti–insulin receptor antibody and immunoblotted with anti-phosphotyrosine antibody (*F*, *upper panel*) and with anti–insulin receptor (*F*, *lower panel*). Whole-tissue extracts were immunoblotted with anti–phosphoserine 473 and anti–threonine 308 Akt antibody (*G* and *H*, *lower panels*) and with anti-Akt (*G* and *H*, *lower panels*). The results of scanning densitometry are expressed as arbitrary units. Columns and bars represent the means ± SE, $n = 8$ animals per group. #$P < 0.05$, exercise vs. control; *$P < 0.05$, exercise + anti–IL-6 vs. exercise. □, control; ■, exercise; ▨, exercise + anti–IL-6.

As shown above, the amounts of IRS-1 and -2 were unchanged in the hypothalamus of exercised rats. In contrast, the phosphorylation of IRS-1 and -2 after stimulation with leptin or insulin increased in those rats compared with control animals. Because IRS-1 and -2 are the main molecules linking leptin and insulin signaling to PI 3-kinase activity, we examined the leptin- and insulin-induced association of IRS-1 and -2 with the p85 subunit of PI 3-kinase and found it to be increased in the hypothalamus of exercised rats. After IRS-1– or IRS-2–PI 3-kinase association, PI 3-kinase is activated and may in turn activate Akt, a serine kinase with pleiotropic actions in several tissues (33). The activation of Akt-1/protein kinase B is accompanied by an increase in its serine and threonine phosphorylation (22). Thus, the increase in the association between IRS-1 or IRS-2 and PI 3-kinase, and the increase in PI 3-kinase activity after leptin or insulin infusion in the hypothalamus of exercised rats, may play a role in the increased responsiveness to leptin and insulin in these animals.

Selective impairment of leptin and insulin signaling through the PI 3-kinase pathway in the hypothalamus could be pathophysiologically important in the development of obesity. Recent studies have shown that activation of the PI 3-kinase pathway could be involved in the anorexigenic effect of insulin or leptin (14,34,35). Our findings, in a model of exercise training, are relevant because insulin-induced tyrosine phosphorylation of IRSs and PI 3-kinase activity are reduced in the hypothalamus of different animal models of obesity (14,36,37). Thus, exercise training may be one therapeutic strategy to restore impaired leptin and insulin signal transduction in the hypothalamus of obese individuals.

In addition to the increased insulin and leptin sensitivity observed in the PI 3-kinase pathway, our data also provide evidence that there is an increase in leptin sensitivity in the JAK2/STAT3 pathway. Leptin activation of STAT3 requires the leptin receptor, which associates with and activates JAK2 in a ligand-dependent manner (8,9,12,38). One potential mediator of increased STAT3 activation in the hypothalamus of exercised rats is expression of SOCS-3, a suppressor of cytokine signaling. Forced expression of SOCS-3 in mammalian cells antagonizes leptin signaling, probably by binding and antagonizing JAK activity (39). Using Western blotting, we examined the expression of SOCS-3 in hypothalami of exercised rats. No significant differences were found between the two groups (data not shown). In addition, we found no significant

difference in hypothalamic protein-tyrosine phosphatase 1b expression and its association with JAK2 and ObR between the two groups (data not shown). Thus, a molecular basis for the observed increase in leptin's ability to activate STAT3 signaling after 1 day of exercise remains to be determined.

Perhaps the most striking finding was the reversal of exercise-induced increased hypothalamic insulin and leptin sensitivity by blocking the action of IL-6 action. These data are in accordance with earlier studies demonstrating that IL-6 treatment enhances energy expenditure in both rodents and humans (30,40–42). It has been previously shown that IL-6 treatment stimulates energy expenditure at the level of the brain in rodents (30,41,43), and it might be assumed that endogenous IL-6 also acts on the brain during exercise. The IL-6 exerting this effect during exercise could be produced by the brain itself, which has been shown to have increased IL-6 production during exercise (44). Alternatively, the large quantities of endocrine IL-6 produced from working skeletal muscle (45) might reach appropriate sites in the brain (23,46,47).

Numerous biological responses of different cell types are induced by IL-6, which activates STAT3 and Ras–extracellular signal-regulated kinase-1/2 via JAKS, and the balance of activation of both pathways is considered to direct the cell fate in response to IL-6 (48). The cross talk of signals mediated by a cytokine and growth factor has been previously reported in the case of the phosphorylation of tyrosine kinase receptors by the growth hormone–activated JAK2 (49). This suggests that the IL-6–induced activation of JAK2 is involved in the activation of insulin and leptin receptor–mediated signals in rat hypothalamus. Conversely, it has been reported that the PI 3-kinase and Akt pathways may be activated via gp130 (the glycoprotein of 130 kDa) recruitment of adaptor molecules to create binding sites to the SH2 domain of the p85 subunit of PI 3-kinase (50).

In the current study, using an in vivo approach, we saw a synergistic effect of IL-6 on the insulin-stimulated tyrosine phosphorylation of IRS-1 and on the serine phosphorylation of protein kinase B/Akt in rat hypothalamus. These results are clearly different from the findings of Senn et al. (51) in HepG2 cells and may indicate that the induction of SOCS-3 by IL-6 either follows a different time course in hepatocytes or that the major effect of IL-6 is exerted through other mechanisms, such as the transcriptional regulation identified in the current work. However, the recent finding (52) that a high IL-6 infusion for 2 h in rats did not reduce the insulin effect during a euglycemic clamp clearly supports the theory that any acute inhibitory effects of IL-6, mediated through a transient activation of SOCS-3, are of less importance for whole-body insulin sensitivity. Similar results have recently been reported in humans (53). Because IL-6 has been shown both to interfere with insulin-signaling pathways in the liver and adipocytes in an inhibitory manner and to reduce insulin-stimulated glycogen synthesis in hepatocytes, the cross talk of IL-6 with insulin-signaling pathways appears to be tissue specific.

The current study provides direct measurements of leptin and insulin signaling in the hypothalamus, and it documents increased sensitivity to these hormones in the hypothalamus of exercised rats in an IL-6–dependent manner. These findings provide support for the hypothesis that exercise could have appetite-suppressive actions mediated by the hypothalamus.

ACKNOWLEDGMENTS

These studies were supported by grants from Fundacão de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

We are thank Dr. Nicola Conran for English language editing.

REFERENCES

1. Richard D, Lachance P, Deshaies Y: Effects of exercise-rest cycles on energy balance in rats. *Am J Physiol* 256:R886–R891, 1989
2. Richard D, Rivest S: The role of exercise in thermogenesis and energy balance. *Can J Physiol Pharmacol* 67:402–409, 1989
3. Rivest S, Landry J, Richard D: Effect of exercise training on energy balance of orchidectomized rats. *Am J Physiol* 257:R550–R555, 1989
4. Wing RR, Hill JO: Successful weight loss maintenance. *Annu Rev Nutr* 21:323–341, 2001
5. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM: Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 269:543–546, 1995
6. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P: Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science* 269:546–549, 1995
7. Vaisse C, Halaas JL, Horvath CM, Darnell JE Jr, Stoffel M, Friedman JM: Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat Genet* 14:95–97, 1996
8. Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC: Defective STAT signaling by the leptin receptor in diabetic mice. *Proc Natl Acad Sci U S A* 93:6231–6235, 1996
9. Ghilardi N, Skoda RC: The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. *Mol Endocrinol* 11:393–399, 1997
10. Carvalheira JB, Siloto RM, Ignacchitti I, Brenelli SL, Carvalho CR, Leite A, Velloso LA, Gontijo JA, Saad MJ: Insulin modulates leptin-induced STAT3 activation in rat hypothalamus. *FEBS Lett* 500:119–124, 2001
11. Carvalheira JB, Ribeiro EB, Folli F, Velloso LA, Saad MJ: Interaction between leptin and insulin signaling pathways differentially affects JAK-STAT and PI 3-kinase-mediated signaling in rat liver. *Biol Chem* 384:151–159, 2003
12. Bjorbaek C, Uotani S, da Silva B, Flier JS: Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J Biol Chem* 272:32686–32695, 1997
13. Kellerer M, Koch M, Metzinger E, Mushack J, Capp E, Haring HU: Leptin activates PI-3 kinase in C2C12 myotubes via janus kinase-2 (JAK-2) and insulin receptor substrate-2 (IRS-2) dependent pathways. *Diabetologia* 40:1358–1362, 1997
14. Carvalheira JB, Ribeiro EB, Araujo EP, Guimaraes RB, Telles MM, Torsoni M, Gontijo JA, Velloso LA, Saad MJ: Selective impairment of insulin signalling in the hypothalamus of obese Zucker rats. *Diabetologia* 46:1629–1640, 2003
15. Sun XJ, Rothenberg P, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF: Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* 352:73–77, 1991
16. Lavan BE, Lane WS, Lienhard GE: The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J Biol Chem* 272:11439–11443, 1997
17. Folli F, Saad MJ, Backer JM, Kahn CR: Insulin stimulation of phosphatidylinositol 3-kinase activity and association with insulin receptor substrate 1 in liver and muscle of the intact rat. *J Biol Chem* 267:22171–22177, 1992
18. Saad MJ, Folli F, Kahn JA, Kahn CR: Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *J Clin Invest* 92:2065–2072, 1993
19. Kuhne MR, Pawson T, Lienhard GE, Feng GS: The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp. *J Biol Chem* 268:11479–11481, 1993
20. Skolnik EY, Lee CH, Batzer A, Vicentini LM, Zhou M, Daly R, Myers MJ Jr, Backer JM, Ullrich A, White MF, et al.: The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. *EMBO J* 12:1929–1936, 1993
21. Yamauchi K, Milarski KL, Saltiel AR, Pessin JE: Protein-tyrosine-phosphatase SHPTP2 is a required positive effector for insulin downstream signaling. *Proc Natl Acad Sci U S A* 92:664–668, 1995
22. Kobayashi T, Cohen P: Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is

mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *Biochem J* 339:319–328, 1999
23. Pedersen BK, Hoffman-Goetz L: Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev* 80:1055–1081, 2000
24. Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B, Klarlund Pedersen B: Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J Physiol* 529:237–242, 2000
25. Penkowa M, Keller C, Keller P, Jauffred S, Pedersen BK: Immunohistochemical detection of interleukin-6 in human skeletal muscle fibers following exercise. *FASEB J* 17:2166–2168, 2003
26. Lyngso D, Simonsen L, Bulow J: Metabolic effects of interleukin-6 in human splanchnic and adipose tissue. *J Physiol* 543:379–386, 2002
27. Keller C, Keller P, Marshal S, Pedersen BK: IL-6 gene expression in human adipose tissue in response to exercise: effect of carbohydrate ingestion. *J Physiol* 550:927–931, 2003
28. Keller P, Keller C, Carey AL, Jauffred S, Fischer CP, Steensberg A, Pedersen BK: Interleukin-6 production by contracting human skeletal muscle: autocrine regulation by IL-6. *Biochem Biophys Res Commun* 310:550–554, 2003
29. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, et al.: Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1:1155–1161, 1995
30. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson SL, Ohlsson C, Jansson JO: Interleukin-6-deficient mice develop mature-onset obesity. *Nat Med* 8:75–79, 2002
31. Chibalin AV, Yu M, Ryder JW, Song XM, Galuska D, Krook A, Wallberg-Henriksson H, Zierath JR: Exercise-induced changes in expression and activity of proteins involved in insulin signal transduction in skeletal muscle: differential effects on insulin-receptor substrates 1 and 2. *Proc Natl Acad Sci U S A* 97:38–43, 2000
32. Kimura M, Tateishi N, Shiota T, Yoshie F, Yamauchi H, Suzuki M, Shibasaki T: Long-term exercise down-regulates leptin receptor mRNA in the arcuate nucleus. *Neuroreport* 15:713–716, 2004
33. Franke TF, Kaplan DR, Cantley LC, Toker A: Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 275:665–668, 1997
34. Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG Jr, Schwartz MW: Intracellular signalling: key enzyme in leptin-induced anorexia. *Nature* 413:794–795, 2001
35. Niswender KD, Morrison CD, Clegg DJ, Olson R, Baskin DG, Myers MG Jr, Seeley RJ, Schwartz MW: Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. *Diabetes* 52:227–231, 2003
36. De Souza CT, Araujo EP, Bordin S, Ashimine R, Zollner RL, Boschero AC, Saad MJ, Velloso LA: Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus. *Endocrinology* 146:4192–4199, 2005
37. Prada PO, Zecchin HG, Gasparetti AL, Torsoni MA, Ueno M, Hirata AE, Corezola do Amaral ME, Hoer NF, Boschero AC, Saad MJ: Western diet modulates insulin signaling, c-Jun N-terminal kinase activity, and insulin receptor substrate-1ser307 phosphorylation in a tissue-specific fashion. *Endocrinology* 146:1576–1587, 2005
38. Baumann H, Morella KK, White DW, Dembski M, Bailon PS, Kim H, Lai CF, Tartaglia LA: The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proc Natl Acad Sci U S A* 93:8374–8378, 1996
39. Bjorbaek C, El-Haschimi K, Frantz JD, Flier JS: The role of SOCS-3 in leptin signaling and leptin resistance. *J Biol Chem* 274:30059–30065, 1999
40. Stouthard JM, Romijn JA, Van der Poll T, Endert E, Klein S, Bakker PJ, Veenhof CH, Sauerwein HP: Endocrinologic and metabolic effects of interleukin-6 in humans. *Am J Physiol* 268:E813–E819, 1995
41. Rothwell NJ, Busbridge NJ, Lefeuvre RA, Hardwick AJ, Gauldie J, Hopkins SJ: Interleukin-6 is a centrally acting endogenous pyrogen in the rat. *Can J Physiol Pharmacol* 69:1465–1469, 1991
42. Tsigos C, Papanicolaou DA, Defensor R, Mitsiadis CS, Kyrou I, Chrousos GP: Dose effects of recombinant human interleukin-6 on pituitary hormone secretion and energy expenditure. *Neuroendocrinology* 66:54–62, 1997
43. Li G, Klein RL, Matheny M, King MA, Meyer EM, Scarpace PJ: Induction of uncoupling protein 1 by central interleukin-6 gene delivery is dependent on sympathetic innervation of brown adipose tissue and underlies one mechanism of body weight reduction in rats. *Neuroscience* 115:879–889, 2002
44. Nybo L, Nielsen B, Pedersen BK, Moller K, Secher NH: Interleukin-6 release from the human brain during prolonged exercise. *J Physiol* 542:991–995, 2002
45. Ostrowski K, Rohde T, Zacho M, Asp S, Pedersen BK: Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J Physiol* 508:949–953, 1998
46. Pedersen BK, Steensberg A, Schjerling P: Muscle-derived interleukin-6: possible biological effects. *J Physiol* 536:329–337, 2001
47. Febbraio MA, Pedersen BK: Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *FASEB J* 16:1335–1347, 2002
48. Ohtani T, Ishihara K, Atsumi T, Nishida K, Kaneko Y, Miyata T, Itoh S, Narimatsu M, Maeda H, Fukada T, Itoh M, Okano H, Hibi M, Hirano T: Dissection of signaling cascades through gp130 in vivo: reciprocal roles for STAT3- and SHP2-mediated signals in immune responses. *Immunity* 12:95–105, 2000
49. Yamauchi T, Ueki K, Tobe K, Tamemoto H, Sekine N, Wada M, Honjo M, Takahashi M, Takahashi T, Hirai H, Tushima T, Akanuma Y, Fujita T, Komuro I, Yazaki Y, Kadowaki T: Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. *Nature* 390:91–96, 1997
50. Hideshima T, Nakamura N, Chauhan D, Anderson KC: Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. *Oncogene* 20:5991–6000, 2001
51. Senn JJ, Klover PJ, Nowak IA, Mooney RA: Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes* 51:3391–3399, 2002
52. Rotter Sopasakis V, Larsson BM, Johansson A, Holmang A, Smith U: Short-term infusion of interleukin-6 does not induce insulin resistance in vivo or impair insulin signalling in rats. *Diabetologia* 47:1879–1887, 2004
53. Steensberg A, Fischer CP, Sacchetti M, Keller C, Osada T, Schjerling P, van Hall G, Febbraio MA, Pedersen BK: Acute interleukin-6 administration does not impair muscle glucose uptake or whole-body glucose disposal in healthy humans. *J Physiol* 548:631–638, 2003