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Leptin Signaling in the Hypothalamus during Chronic Central Leptin Infusion

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Using a rat model of chronic central leptin infusion in which neuropeptide Y neurons develop leptin resistance, we examined whether leptin signal transduction mechanism in the hypothalamus is altered during central leptin infusion. Adult male rats were infused chronically into the lateral cerebroventricle with leptin (160 ng/h) or vehicle via Alzet pumps for 16 d. In the leptin-infused group, the initial decrease in food intake was followed by a recovery to their preleptin levels by d 16, although food intake remained significantly lower than in artificial cerebrospinal fluid controls; and body weight gradually decreased reaching a nadir at d 11 and remained stabilized at lower level thereafter. Phosphorylated leptin receptor and phosphorylated signal transducer and activator of transcription-3 (p-STAT3) remained elevated in association with a sustained elevation in DNA-binding activity of STAT3 in the hypothalamus throughout 16-d period of leptin infusion. However, phosphorylated Janus kinase-2 was increased during the early part of leptin infusion but remained unaltered on d 16. Although hypothalamic suppressors of cytokine signaling-3 (SOCS3) mRNA levels were increased throughout leptin infusion, SOCS3 protein levels were increased only on d 16. This study demonstrates a sustained elevation in hypothalamic leptin receptor signaling through Janus kinase-STAT pathway despite an increased expression of SOCS3 during chronic central leptin infusion. We propose that an alteration in leptin signaling in the hypothalamus through pathways other than STAT3 and/or a defect in downstream of STAT3 signaling may be involved in food intake recovery seen after an initial decrease during chronic central leptin infusion. (*Endocrinology* 144: 3789–3798, 2003)

CUMULATIVE EVIDENCE SUGGESTS that leptin, a product of the obese gene (1), produced primarily in the adipose tissue, signals nutritional status to key regulatory centers in the hypothalamus and plays a major role in food intake and body weight regulation (2–4). Because human obesity, in the majority of cases, cannot be attributed to defects in leptin or its receptor (5–9) and because obese humans are hyperleptinemic (4, 10), it is suggested that obese individuals are, in general, leptin resistant. Obese humans, and rodents made obese by dietary manipulation, have elevated levels of circulating leptin but maintain a normal food intake (4, 10, 11). Thus, it is likely that an extended period of exposure of the brain, especially the hypothalamus, to a high level of leptin may result in the development of central leptin resistance. Available data from diet-induced obese (DIO) rodents, which may represent the form of obesity seen in most humans, such as in DIO Wistar rats (12), obese AY mice (13), DIO C57BL/6J mice (14), aged obese rats (15, 16), and DIO-prone Sprague Dawley rats (17) show resistance to central administration of leptin; suggesting an impairment in action of leptin at, or downstream of, leptin target sites. However, DIO AKR mice and New Zealand obese mice are resistant to peripheral but not central administration of leptin (11, 13) and the cerebrospinal fluid: Plasma leptin ratio is lower in obese individuals (18, 19), suggesting leptin transport into the brain may be one of the many defects in obesity (20).

Based on a mouse model (13), we recently developed a rat model of chronic central leptin infusion, in which an initial dramatic decrease in food intake is followed by a recovery, despite a continued leptin infusion, indicating the development of resistance to satiety action of this hormone (21). However, the neurobiology underlying the development of resistance to leptin's satiety action is mostly unknown, but understanding this phenomenon could shed some light on the mechanism of central leptin resistance in human obesity. Using this rat model, we recently demonstrated that neuropeptide Y (NPY) neurons develop leptin resistance within 2 wk of leptin infusion (21). Because leptin signaling in the hypothalamus is mediated through activation of leptin receptor and subsequent Janus-kinase 2 (JAK2)-signal transducer and activator of transcription 3 (STAT3) pathways of signal transduction (4, 22–24), it is conceivable that defects in any of the signaling components may underlie the development of resistance in NPY and other neuronal systems that in turn could lead to the recovery in food intake despite continuous leptin infusion.

Thus, to elucidate whether the recovery in food intake after an initial decrease and the development of leptin insensitivity in NPY neurons previously reported during chronic leptin infusion (21) is due to an alteration in leptin receptors and/or impaired leptin signal transduction in the hypothalamus, we examined 1) leptin receptor gene expression, 2) protein levels of leptin receptor, JAK2, phosphorylated-JAK2 (p-JAK2), STAT3, and phosphorylated STAT3 (p-STAT3), and 3) DNA-binding activity of p-STAT3 in the hypothalamus following chronic central leptin infusion. We also examined mRNA and protein levels of suppressors of cytokine

Abbreviations: aCSF, Artificial cerebrospinal fluid; DIO, diet-induced obese; JAK2, Janus-kinase 2; MBH, medial basal hypothalamus; NPY, neuropeptide Y; NT, neurotensin; PDE3B, phosphodiesterase 3B; PI3K, phosphotidylinositol-3 kinase; p-JAK2, phosphorylated-JAK2; POMC, proopiomelanocortin; p-STAT3, phosphorylated signal transducer and activator of transcription 3; SOCS3, suppressors of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3.

signaling 3 (SOCS3) in the hypothalamus because SOCS3 appears to be a critical part of a classic negative feedback loop that regulates cytokine signal transduction (25, 26). In addition, leptin treatment induces SOCS3 mRNA in the hypothalamus (27); and SOCS3 mRNA levels in the hypothalamus are increased in A(Y)/a mice (27), a murine obesity model characterized by hyperleptinemia and resistance to both central and peripheral leptin administration (13).

Materials and Methods

Animals

Adult male Sprague Dawley rats, weighing 250–300 g, obtained from Taconic Farms (Germantown, NY) were housed individually in a light- (lights on 0500–1900 h) and temperature (22 C)-controlled room with food (pelleted Purina rat chow, T.R. LAST, Gibsonia, PA) and water available *ad libitum*. After 7 d of acclimatization, rats were subjected to the following experiments, all of which were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Experiment 1: effects of chronic leptin infusion on hypothalamic leptin receptor and SOCS3 mRNA expression

Rats were implanted stereotaxically with 22-gauge osmotic pump connector cannulae (Plastic One, Roanoke, VA) into the lateral cerebroventricle under pentobarbital anesthesia as described previously (21). The lateral ventricle was then connected via Medical Vinyl tubing (size V4, Scientific Commodities, Inc., Lake Havasu City, AZ) to an artificial cerebrospinal fluid (aCSF, pH 7.4)-filled (13) Alzet osmotic pump (model 2002, DURECT Corp., Cupertino, CA) implanted sc in the back. After 7 d, aCSF pumps were replaced with new pumps to infuse either recombinant murine leptin (obtained from Dr. A. F. Parlow, National Hormone and Pituitary Program, NIDDK, Torrance, CA) at a dose of 160 ng/0.5 μl·h or aCSF vehicle for 16 d. In a preliminary study, we observed that an Alzet pump (model 2002), a 14-d pump, can be used safely for 16 d. In addition, in the present study, the pumps were filled with 235–240 μl of solution, and there was at least 35–40 μl of solution left in the pump at the end of infusion. Food intake and body weight were measured daily. Some aCSF-infused rats were allowed to eat the amount of food that was consumed on the day before by the leptin-infused group and served as the pair-fed group. Thus, pair feeding in aCSF-infused rats was started 1 d after the beginning of leptin infusion in the leptin group. In addition, the amount of food given to pair-fed rats was adjusted daily to their spillage during feeding. Rats were killed by decapitation between 0900 and 1200 h on d 2, 4, or 16 of infusion. Brains were removed immediately and the medial basal hypothalamus (MBH) were dissected out (21), frozen in liquid nitrogen, and kept at −80 C until processed for RNA extraction. The MBH tissue fragment was bounded rostrally by the posterior border of the optic chiasma, laterally by the lateral sulcus, and caudally by the mammillary bodies and cut to a depth of approximately 2 mm. Trunk blood was collected for glucose, insulin, and leptin determination. Epididymal fat was dissected out and weighed. To minimize the number of animals, we took the opportunity to use some (six to eight) of the MBH RNA samples that were available from our previous study (21).

Experiment 2: effects of chronic leptin infusion on levels of leptin receptor, JAK2, p-JAK2, STAT3, p-STAT3, and SOCS3 protein and on DNA-binding activity of p-STAT3 in the hypothalamus

The rats were infused with leptin or aCSF as described in experiment 1. Control, pair-fed, and leptin-infused rats were killed on d 2, 4, or 16. The MBHs were quickly dissected out, frozen in liquid nitrogen, and kept at −80 C. The whole-cell extracts of the frozen MBHs were made using a high salt extraction buffer (28–30), containing 0.42 M NaCl, 20 mM HEPES (pH 7.9), 20 mM sodium fluoride, 1 mM trisodium orthovandate [Na_3VO_4 (ortho)], 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20% glycerol, and 2 mM phenylmethylsulfonyl fluoride. The MBHs were homogenized in plastic 1.5-ml microfuge tubes with handheld pistons moving up and down. The tubes containing homogenates were frozen (dry ice/ethanol bath) and thawed (37 C water bath) three times, followed by a spin for 10 min in a microfuge at 4 C. The supernatants were collected and protein concentrations were measured by a protein assay (Bio-Rad Laboratories, Hercules, CA) (31).

Ribonuclease protection assay

Total RNA was isolated from MBH, using RNAzol (RNA STAT 60, Tel-Test, Inc., Friendswood, TX) followed by precipitation with isopropanol and ethanol washes according to the manufacturer's instructions. The integrity of the RNA was checked by visualization of the ethidium bromide-stained 28S and 18S rRNA bands, and quantitation was performed by measuring absorbance at 260 nm.

A rat Ob-Rb (long-form, accession no. U52966)-specific cDNA fragment was synthesized from total rat hypothalamic RNA by RT-PCR (forward primer, 3′-TTTGACCACTCCAGATTCCACA-5′, reverse primer, 3′-TTTTCCCCGTGATTTTCTT-CAG-5′) and subcloned into a TA cloning vector (Invitrogen, Carlsbad, CA) and subsequently into the *Eco*R1 site of pBluescript (Stratagene, La Jolla, CA). A rat cDNA fragment common to all rat leptin receptor isoforms (Ob-Rtot, accession no. U53144) obtained by a *Bse*D1/*Bst*B1 digestion of the rat Ob-Rf cDNA (kindly provided by Dr. Roger Unger, University of Texas, Southwestern Medical Center at Dallas, Dallas, TX) was subcloned into pBluescript (Stratagene). A 356-bp cDNA fragment of rat SOCS3 (accession no. AF075383) was synthesized by RT-PCR from rat hypothalamic total RNA using a 20-mer forward primer 5′-CTT CAG CTC CAA GAG CGA GT-3′ (5′ position 89) and a 20-mer reverse primer 5′-GTT CCG TCG GTG GTA AAG AA-3′ (5′ position 444). This cDNA fragment was cloned in pBluescript SK+ plasmid and was used for SOCS3 cRNA probe preparation. A riboprobe generated from a plasmid containing a rat-specific β-actin cDNA fragment (Ambion Inc., Austin, TX) served as an internal control in all ribonuclease protection assays. [α-^{32}P]Uridine 5′-triphosphate-labeled antisense cRNA probes were synthesized using T3 (Ob-Rtot) or T7 (Ob-Rb, SOCS3, β-actin) RNA polymerase using a transcription kit (Ambion Inc.).

Ribonuclease protection assays were performed as previously described (32). Briefly, 6 μg MBH RNA, ^{32}P-labeled Ob-Rb, Ob-Rtot (200,000 cpm), and β-actin (20,000 cpm) cRNA probes, and 12 μg yeast tRNA (Roche Molecular Biochemicals, Indianapolis, IN) were allowed to hybridize in solution at 45 C overnight, followed by combined Rnase A and T1 digestion of nonhybridized probe at 32 C for 1 h. For SOCS3 mRNA, 4 μg MBH RNA, ^{32}P-labeled SOCS3 (200,000 cpm), and β-actin (20,000 cpm) cRNA probes and 14 μg yeast tRNA were allowed to hybridize. Stable hybrids were extracted with phenol-chloroform followed by ethanol precipitation and then separated on 6% polyacrylamide-8 M urea gels. The dried gels were exposed in a Molecular Imaging Screen-K (Bio-Rad Laboratories) for 6–40 h, and the image of each gel was acquired using a Molecular Imager FX (Bio-Rad Laboratories). The volume analysis of each band was performed using Quantity One software (Bio-Rad Laboratories). Ob-Rb, Ob-Rtot, and SOCS3 mRNA values were first normalized with β-actin mRNA levels, and then the values were expressed in relation to aCSF control.

Western blotting

Western blotting was done following standard procedures (30). Briefly, whole-cell extract proteins (25–50 μg) from the MBH were boiled 5 min and subjected to SDS-PAGE (12% gel for SOCS3 and 7% gel for all other proteins), followed by transfer of the resolved polypeptides to polyscreen polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA). Total amount of specific protein was measured by incubating with specific antibody followed by enhanced chemoluminescence as described by the manufacturer (NEN Life Science Products). The following antibodies were used for Western blotting: Ob-R (B-3, monoclonal, Sc-8391, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Ob-Rb (rabbit antihuman leptin receptor-long form, polyclonal, 4781-L, Linco Research, Inc., St. Charles, MO), p-Ob-Rb (tyr 1138) (polyclonal, Sc-16421, Santa Cruz Biotechnology), JAK2 (Upstate Biotechnology, Lake Placid, NY), p-JAK2 (Y 1007/1008, catalog no. 44–426, BioSource International, Camarillo, CA), STAT3 (K-15, polyclonal, Sc-483, Santa Cruz Biotechnology), p-STAT3 (B7, monoclonal, Sc-8059, Santa Cruz Biotechnology), and SOCS3 (M-20, polyclonal, Sc-7009, Santa Cruz

Biotechnology). To measure two or more proteins in the same blot, polyvinyl difluoride membranes were stripped in a soaking solution (1% SDS; 70 mM Tris-HCl, pH 6.8; and 0.1% β-mercaptoethanol) and then incubated with another antibody. The specificity of antibody was confirmed in two ways: first, on the basis of molecular weight of the protein expected using a specific antibody, and second, there were no bands seen when primary antibody was preincubated with blocking peptide (in case of p-Ob-Rb, p-JAK2, SOCS3, Ob-R) or by omitting secondary antibody (in case of Ob-Rb and JAK2) in Western blot assay. Immunoreactive bands were scanned and analyzed by NIH Image software. As an internal control, the membranes were immunoblotted with a monoclonal anti-β-actin antibody (Sigma, St. Louis, MO). The values for proteins were normalized with β-actin to account for variations in gel loading.

EMSA

DNA-binding capacity of the phosphorylated STAT3 protein was measured by EMSA (28–30, 33) using the high-affinity SIEm67 oligonucleotide as binding substrates (28). Double-stranded oligonucleotide probes were synthesized based on the published sequences (5′-CATT-TCCCGTAA-ATCAT-3′) with the addition of GATC at the 5′ termini to allow radiolabeling, after annealing, by Klenow fill-in reaction using ^{32}P-dATP (30). Twenty micrograms of whole-cell extract protein were incubated with ^{32}P-end-labeled duplex (100,000 cpm) probe and 1 μg poly(dI-dc) in 20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol. Resultant DNA-protein complexes were resolved in 5% native PAGE. The dried gels were exposed in a Molecular Imaging Screen (Bio-Rad Laboratories) for 10–12 h. The image of each gel was acquired by Personal Molecular Imager FX (Bio-Rad Laboratories). The volume analysis of each band obtained from EMSA was performed by Quantity One software (Bio-Rad Laboratories).

Leptin, insulin, and glucose determination

Plasma leptin was determined with a rat leptin RIA kit (Linco Research). This kit measures both rat and mouse leptin. Plasma leptin was also measured by a mouse RIA kit (Linco) to determine whether intracerebroventricular infusion of mouse recombinant leptin contributed to increased leptin levels. Because the mouse RIA kit measures approximately 50% of rat leptin, the ratio of the values from mouse and rat RIA kits should be greater in leptin-infused rats, compared with aCSF control rats if the increase in serum leptin was due to leakage from cerebral ventricle. Plasma insulin was measured with a rat insulin RIA kit (Linco). Plasma glucose was determined by the Trinder method (34) using a kit (Sigma).

Data analysis

All values are expressed as means ± SE. Because some (six to eight) of the data for leptin receptor mRNA and SOCS3 mRNA levels were obtained from MBH RNA samples of the animals that have been used previously (21), data for food intake, body weight, epididymal fat weight, plasma leptin, insulin, and glucose levels of those animals were included in data analysis. Statistical significance of differences in food intake and body weight were analyzed using repeated-measures one- or two-way ANOVA with *post hoc* testing using Student-Newman-Keuls multiple range test. All other data were analyzed by randomized one-way ANOVA followed by Student-Newman-Keuls multiple range test or an unpaired *t* test wherever necessary. The values for food intake, body weight, and hormones in experiments 1 and 2 were combined for statistical analysis. All statistical analyses were done using GB-Stat software for the Macintosh (Dynamic Microsystems, Inc., Silver Spring, MD). Comparisons with $P < 0.05$ were considered to be significant.

Results

Changes in food intake, body weight, epididymal fat weight, and circulating levels of leptin, insulin, and glucose following chronic leptin infusion

The changes in food intake and body weight during the 16-d infusion period in aCSF, pair-fed, and leptin-treated rats, presented in Fig. 1, were essentially similar as reported previously by us (21). Thus, in control aCSF rats, body weight progressively increased throughout the infusion period ($F_{16,272} = 177.31$; $P < 0.0001$) in association with a small but significant increase in food intake ($F_{16,272} = 3.68$; $P < 0.0001$). In leptin-infused rats, body weight gradually decreased to a nadir at d 11 and remained stabilized at a lower level thereafter ($F_{16,272} = 15.42$; $P < 0.0001$). Food intake showed an initial dramatic decrease followed by a recovery to the preleptin values by d 16, although it remained significantly ($P < 0.01$) lower than that of the aCSF control (Fig. 1). Specifically, comparison of food intake data before (d −2, −1, 0) and on d 16 of leptin infusion did not reveal any significant difference ($P = 0.08$). Pair-fed rats also showed a significant change in body weight during the 16-d period ($F_{16,272} = 28.75$; $P < 0.0001$) in that weight was significantly decreased during 2–11 d of infusion and then gradually increased beyond the levels seen on d 0.

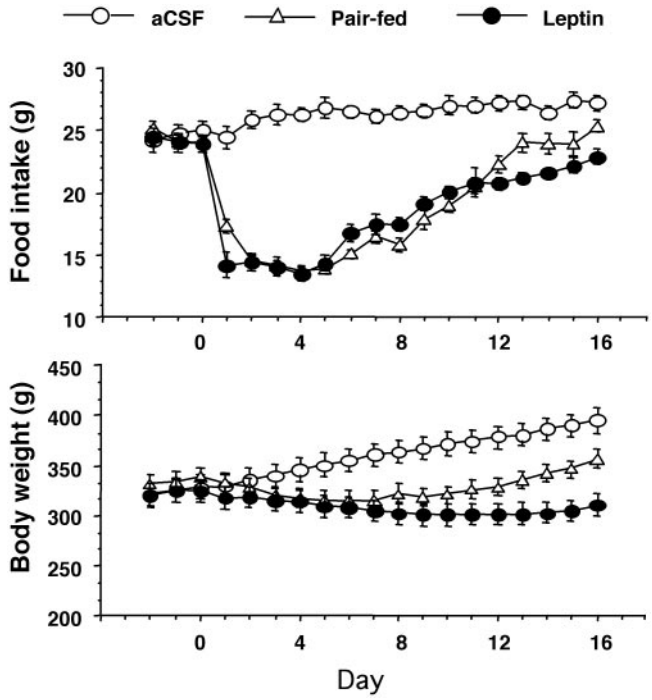

FIG. 1. Changes in food intake and body weight during central recombinant murine leptin (160 ng/0.5 μl·h) infusion for 16 d. Rats were infused into the lateral cerebroventricle with aCSF via Alzet osmotic minipump (0.5 μl/h) for 7 d before infusion with leptin or aCSF. One group of aCSF-infused rats was paired fed to that of leptin-infused group. Values represent the mean ± SEM of 16–17 animals.

As demonstrated previously (21), central leptin infusion increased plasma leptin levels by 8- to 10-fold on d 2 and 4, and by 3-fold on d 16 (Table 1). In addition the ratio of mouse to rat leptin immunoreactivity in the plasma as calculated from the values obtained with rat and mouse leptin RIA kits was significantly increased in the leptin-infused rats on d 2 (aCSF: 0.494 ± 0.024; leptin 0.742 ± 0.057; F (1,18) = 15.63, $P = 0.0009$), 4 (aCSF: 0.44 ± 0.015; leptin 0.643 ± 0.027; F (1,31) = 42.12, $P < 0.0001$), and 16 (aCSF: 0.489 ± 0.025; leptin 0.763 ± 0.022; F (1,31) = 65.74, $P < 0.0001$) of infusion, suggesting that centrally infused leptin leaked to the peripheral circulation. Plasma insulin levels were significantly decreased in the leptin infused rats, compared with that of

TABLE 1. Changes in epididymal fat weight and circulating levels of glucose, insulin, and leptin

Treatment and duration	n	Epididymal fat weight (g)	Glucose (mg/dl)	Insulin (ng/ml)	Leptin (ng/ml)
Day 2					
aCSF	11	3.12 ± 0.16	138 ± 5.87	1.72 ± 0.25^a	2.56 ± 0.33^a
PF	10	3.17 ± 0.09	143 ± 4.27	1.45 ± 0.19^a	1.81 ± 0.15^a
Leptin	11	3.47 ± 0.20	138 ± 3.61	0.89 ± 0.12^b	31.69 ± 2.91^b
Day 4					
aCSF	17	4.22 ± 0.27	137 ± 3.47^a	2.40 ± 0.19^a	3.05 ± 0.24^a
PF	16	3.64 ± 0.32	122 ± 3.34^b	0.96 ± 0.08^b	1.38 ± 0.07^b
Leptin	16	3.63 ± 0.40	123 ± 3.99^b	0.87 ± 0.14^b	23.81 ± 2.77^c
Day 16					
aCSF	17	5.18 ± 0.31^a	136 ± 5.08	2.29 ± 0.19^a	4.26 ± 0.50^a
PF	16	4.23 ± 0.27^b	142 ± 3.40	1.82 ± 0.20^b	3.12 ± 0.26^a
Leptin	17	0.81 ± 0.18^c	130 ± 4.80	0.34 ± 0.04^c	12.65 ± 2.05^b

PF, Pair-fed; n, number of animals. Values represent the mean ± SEM. Values with *different superscripts* are significantly different from each other.

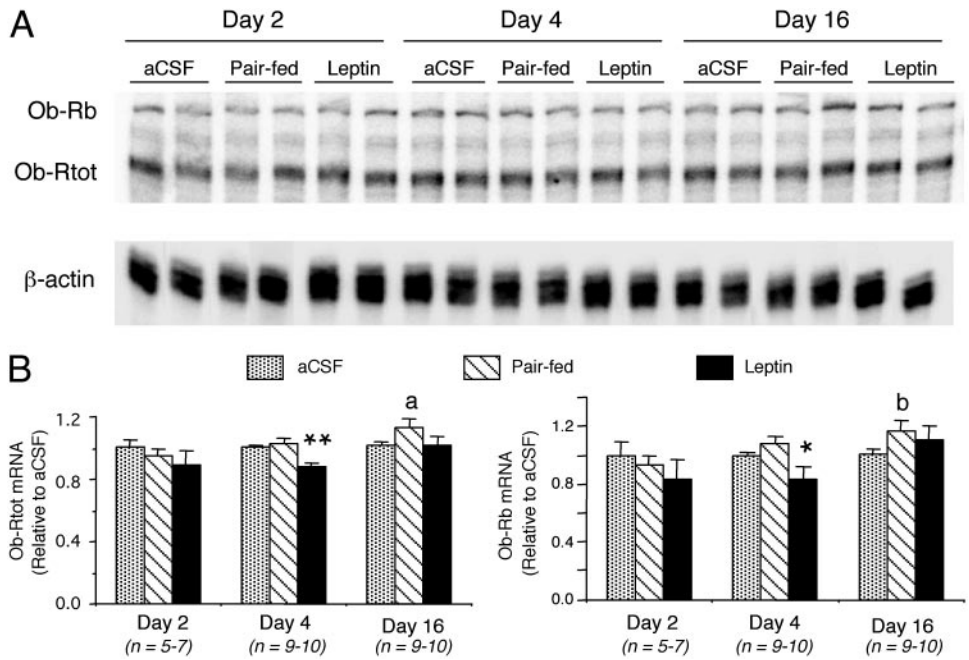

FIG. 2. Leptin receptor gene expression as determined by ribonuclease protection assay in the hypothalamus after 2, 4, or 16 d of leptin infusion. A, Representative phosphor images showing the level of the long-form leptin receptor mRNA (Ob-Rb), all leptin receptor isoform mRNA (Ob-Rtot), and β-actin mRNA in the MBH. B, Results obtained by phosphor imaging showing the changes in Ob-Rtot and Ob-Rb mRNA levels. The values were first normalized to β-actin mRNA levels and then expressed as relative to aCSF control. Values represent the mean ± SEM for the number of animals indicated in *parentheses*. *, $P < 0.05$ and **, $P < 0.01$ *vs.* all other groups on d 4; a, $P < 0.05$ *vs.* aCSF d 16; b, $P = 0.054$ *vs.* aCSF d 16.

aCSF-infused rats (Table 1). Plasma glucose levels remained mostly unchanged between the groups except on d 4 of infusion. There was no change in epididymal fat weight among leptin, pair-fed, and aCSF groups on d 2 and 4 of infusion. On d 16 of infusion, epididymal fat weight was decreased in the leptin-treated group by 84% and the pair-fed group by 19%, compared with that of aCSF control animals (Table 1).

Changes in OB-Rtot and Ob-Rb gene expression following chronic leptin infusion

On d 2 of leptin infusion, the levels of the long-form leptin receptor transcript, Ob-Rb mRNA, or the combined long- and short-form leptin receptor transcripts, Ob-Rtot mRNA, in the MBH did not change, compared with that in aCSF control and pair-fed groups (Fig. 2). On d 4 of leptin infusion, Ob-Rb and Ob-Rtot mRNA levels in the MBH were significantly decreased, compared with that in aCSF control (Ob-Rb: $F_{1,17} = 4.49$; $P = 0.0491$; Ob-Rtot: $F_{1,17} = 17.68$; $P = 0.0006$) and pair-fed (Ob-Rb: $F_{1,16} = 7.19$; $P = 0.0164$; Ob-Rtot: $F_{1,16} = 16.91$; $P = 0.0008$) groups (Fig. 2). In pair-fed rats, there was no change in either Ob-Rb or Ob-Rtot mRNA levels, compared with that of aCSF control. On d 16, although both Ob-Rb and Ob-Rtot mRNA levels were not different between aCSF and leptin groups (Fig. 2), they were significantly increased in pair-fed, compared with the aCSF group (Ob-Rb: $F_{1,17} = 5.61$; $P = 0.03$; Ob-Rtot: $F_{1,17} = 4.25$; $P = 0.05$).

Changes in protein concentrations of leptin receptor, JAK2, p-JAK2, STAT3, and p-STAT3 and in DNA-binding activity of p-STAT3 following chronic leptin infusion

Leptin receptor protein levels were examined using specific antibody to recognize total leptin receptor (Ob-R), long form of the leptin receptor (Ob-Rb), and phosphorylated (Tyr 1138) Ob-Rb (p-Ob-Rb). The p-Ob-Rb levels were significantly increased on d 2 ($F_{2,9} = 4.05$; $P = 0.05$), 4 ($F_{2,18} = 9.48$; $P = 0.0015$), and 16 ($F_{2,15} = 6.79$; $P = 0.008$) of leptin infusion (Fig. 3, A and B) without any change in total Ob-R or Ob-Rb levels in the hypothalamus (Fig. 4). On d 2 ($F_{2,6} = 36.01$; $P = 0.0005$) and 4 ($F_{2,15} = 11.87$; $P = 0.0008$) of infusion, p-JAK2

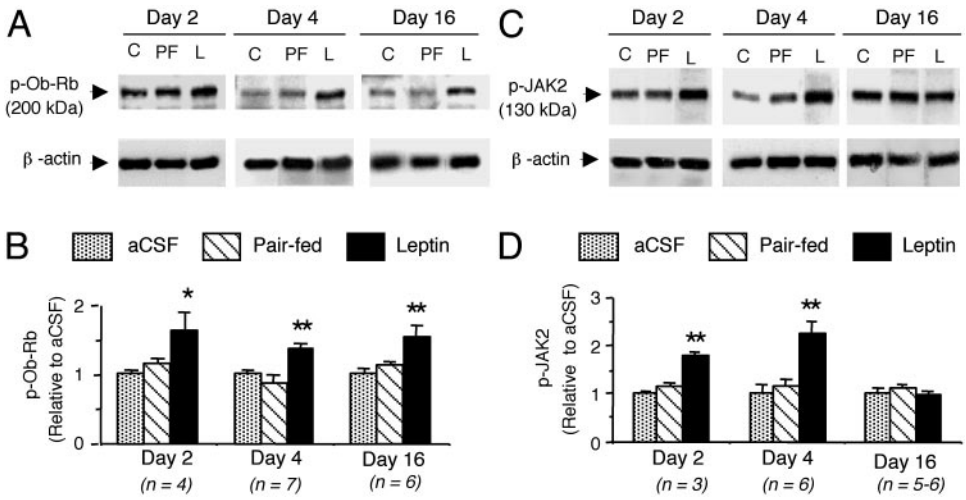

FIG. 3. Changes in phosphorylation of long form of the leptin receptor (Ob-Rb) and JAK2 in the hypothalamus following central leptin infusion. A, C, Western blot of p-Ob-Rb, p-JAK2, and β- actin in the MBH extracts. B, D, Densitometric analysis of the immunoreactive bands for p-Ob-Rb (B) and p-JAK2 (D). The values were first normalized to β-actin levels and then expressed as relative to corresponding aCSF control group. Values represent the mean ± SEM for the number of animals indicated in *parentheses*. *, $P < 0.05$ *vs.* aCSF and **, $P < 0.01$ *vs.* all other groups. C, aCSF control; PF, pair-fed; L, leptin.

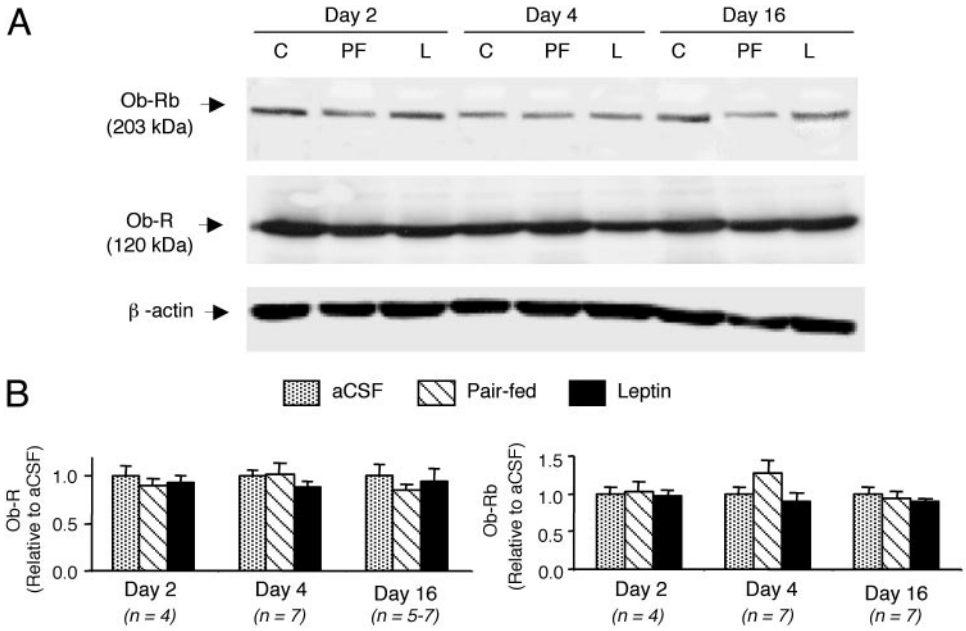

FIG. 4. Changes in protein levels of long form of the leptin receptor (Ob-Rb) and total leptin receptor (Ob-R) in the hypothalamus following central leptin infusion. A, Western blot of Ob-Rb, Ob-R, and β-actin in the MBH extracts. B, Densitometric analysis of the immunoreactive bands for Ob-R (*left panel*) and Ob-Rb (*right panel*). The values were first normalized to β-actin levels and then expressed as relative to corresponding aCSF control group. Values represent the mean ± SEM for the number of animals indicated in *parentheses*. C, aCSF control; PF, pair-fed; L, leptin.

levels were significantly increased in the leptin-treated group. On d 16, in contrast, p-JAK2 levels in the leptin-treated group did not differ from either aCSF or pair-fed groups (Fig. 3, C and D). There was no change in total JAK2 levels throughout 16 d of leptin infusion (data not shown).

The pSTAT3 (Tyr-705) levels in the hypothalamus were significantly increased on d 2 ($F_{2,8} = 18.71$; $P = 001$), 4 ($F_{2,18} = 22.24$; $P < 0.0001$), and 16 ($F_{2,17} = 34.52$; $P < 0.0001$) of leptin infusion (Fig. 5). In addition, p-STAT3 levels on d 16 were significantly increased ($P < 0.01$), compared with that on d 2 and 4 of leptin infusion (Fig. 5), and p-STAT3 levels on d 4 were significantly increased ($P < 0.05$) as compared with that on d 2 (Fig. 5). However, there was no change in total STAT3 protein levels throughout 16 d of leptin infusion. DNA-binding activity in the hypothalamic extracts showed a pattern similar to p-STAT3 levels in that the activity was significantly increased on d 2 ($F_{2,7} = 13.40$; $P = 0.004$), 4 ($F_{2,12} = 44.57$; $P < 0.0001$), and 16 ($F_{2,13} = 39.8$; $P < 0.0001$) of leptin infusion; and DNA-binding activity on d 16 was significantly increased ($P < 0.01$) as compared with that on d 2 and 4 of leptin infusion (Fig. 6). DNA-binding activity was specific for p-STAT3 because a supershift was observed in the EMSA when the samples were preincubated with p-STAT3 antibody (data not shown), as has been demonstrated previously (33).

Changes in SOCS3 mRNA and SCOS3 protein levels following chronic leptin infusion

Results obtained for SOCS3 mRNA levels are presented in Fig. 7. Leptin treatment resulted in an increase in SOCS3 mRNA levels on d 2 ($F_{2,15} = 7.52$; $P = 0.005$), 4 ($F_{2,23} = 11.50$; $P = 0.0003$), or 16 ($F_{2,26} = 20.25$; $P < 0.0001$) of infusion. In contrast, SOCS3 protein levels on d 2 and 4 of leptin infusion were not significantly different among aCSF, pair-fed, and leptin groups (Fig. 8). However, on d 16 of infusion, SOCS3 protein levels were increased in the leptin-treated group ($F_{2,13} = 5.629$; $P = 0.0173$).

Discussion

In agreement with our previous findings (21), the results of this study show that in leptin-infused rats, an initial marked decrease in food intake was followed by a recovery during the later period of leptin infusion. Although food intake in the leptin-infused rats was normalized to their preleptin levels, it was still lower, compared with that in aCSF control rats, on d 16 of infusion. In addition, despite normalization in food intake, body weight remained stabilized at a lower level throughout the leptin infusion. Although the mechanism by which the body weight is maintained at reduced level in leptin-infused rats is not clear, it could be due to increased energy expenditure (35, 36) or resetting of the body weight set point to low so that body weight can be maintained at a reduced level without any alteration in energy expenditure (13), or it could be a combination of both. As demonstrated previously by others (16) and us (21), centrally infused leptin was detected in the serum by immunoreactivity and contributed to increased circulating leptin. However, it remains to be determined whether central leptin infusion increases leptin secretion, which could also contribute to hyperleptinemia. Neverthe-

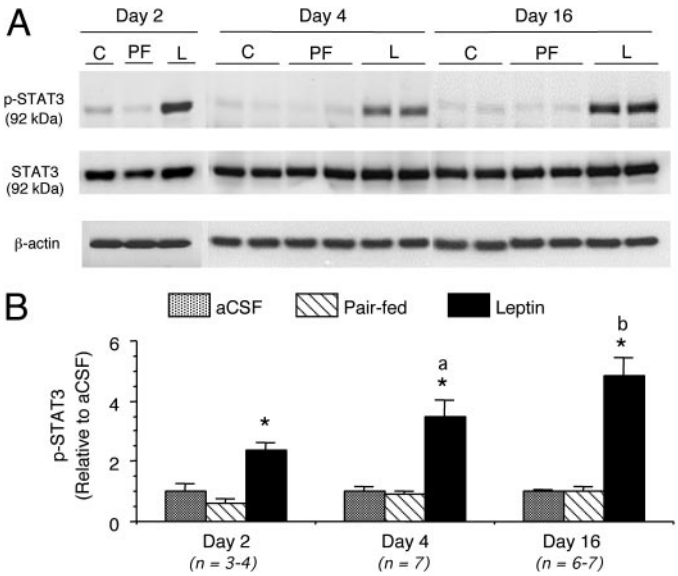

FIG. 5. Changes in phosphorylation of STAT3 in the hypothalamus following central leptin infusion. A, Western blot of STAT3, p-STAT3, and β-actin in the MBH extracts. B, Densitometric analysis of the immunoreactive bands for p-STAT3. The values were first normalized to β-actin levels and then expressed as relative to corresponding aCSF control group. Values represent the mean ± SEM for the number of animals indicated in *parentheses*. *, $P < 0.01$ *vs.* all other groups; a, $P < 0.05$ *vs.* d 2 leptin group; b, $P < 0.01$ *vs.* d 2 and d 4 leptin groups. C, aCSF control; PF, pair-fed; L, leptin.

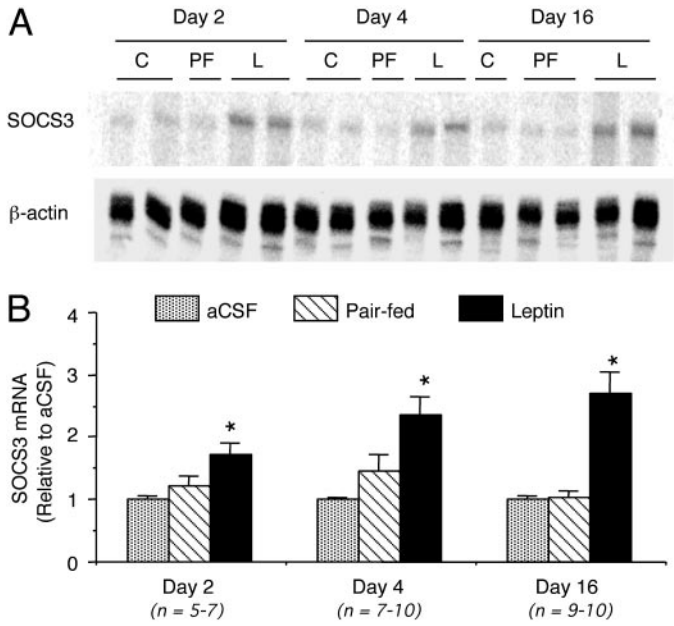

FIG. 7. SOCS3 gene expression as determined by ribonuclease protection assay in the hypothalamus after 2, 4, or 16 d of leptin infusion. A, Representative phosphor images showing the level of SOCS3 mRNA and β-actin mRNA in the MBH. B, Results obtained by phosphor imaging showing the changes in SOCS3 mRNA levels. The values were first normalized to β-actin mRNA and then expressed as relative to aCSF group. Values represent the mean ± SEM for the number of animals indicated in the *parentheses.* *, $P < 0.01$ *vs.* corresponding aCSF group. C, aCSF control; PF, pair-fed; L, leptin.

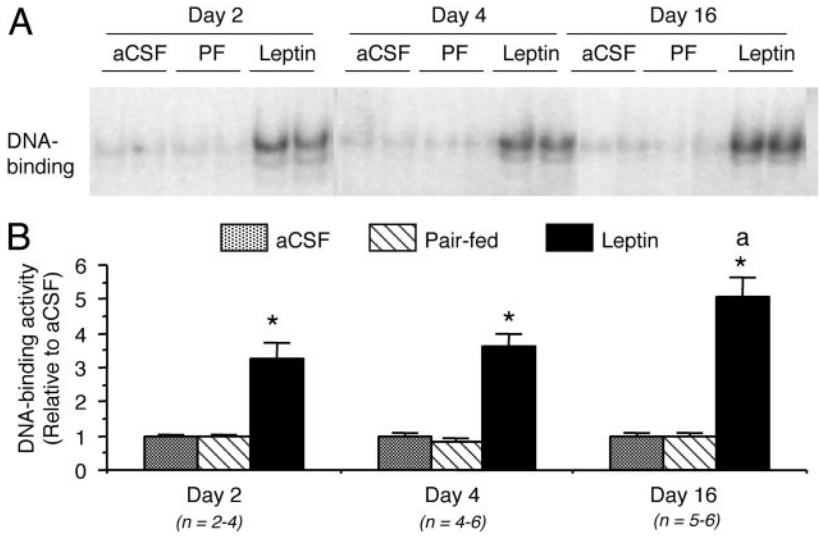

FIG. 6. Changes in DNA-binding activity of STAT3 in the hypothalamus following central leptin infusion. A, DNA-binding activity of STAT3 in the MBH extracts as determined by an EMSA using a ^{32}P-labeled M67-SIE oligonucleotide probe. DNA-binding activity was specific to p-STAT3 because a supershift did occur in the presence of p-STAT3 antibody (data not shown). B, Results obtained by phosphor imaging and expressed as relative to aCSF group. Values represent the mean ± SEM for the number of animals indicated in *parentheses*. *, $P < 0.01$ *vs.* all other groups; a, $P < 0.01$ *vs.* d 2 and d 4 leptin groups. PF, Pair-fed.

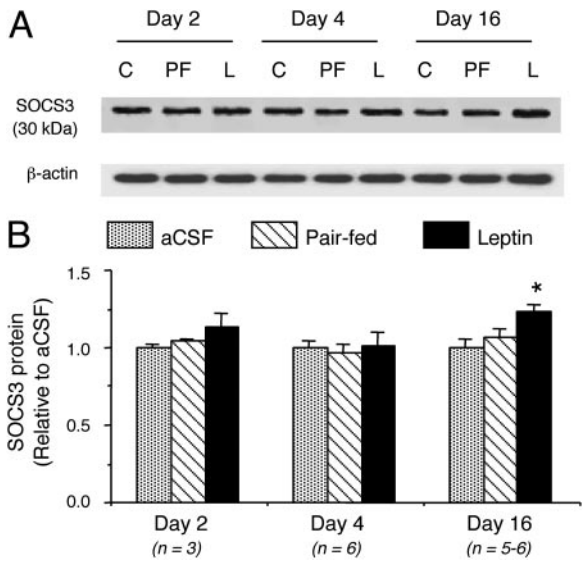

FIG. 8. SOCS3 protein levels as determined by Western blot assay in the hypothalamus after 2, 4, or 16 d of leptin infusion. A, Western blot of SOCS3 in the MBH extracts. B, Densitometric analysis of the immunoreactive bands for SOCS3. The values were first normalized to β-actin levels and then expressed as relative to aCSF group. Values represent the mean ± SEM for the number of animals indicated in the *parentheses*. *, $P < 0.05$ *vs.* all other groups. C, aCSF control; PF, pair-fed; L, leptin.

less, the direct inhibitory effect of leptin in lipogenesis and stimulation of lipolysis and fatty acid oxidation in adipocytes (37–39) may contribute to the reduced body weight following chronic leptin infusion. The present study also confirms our earlier report (21) that leptin infusion decreased plasma insulin levels, and this was not due to a reduced food intake because insulin levels on d 2 remained unaltered in the pair-fed group. The decreased circulating levels of insulin may be a direct effect of leptin on pancreatic β-cells (40). However, a central role of leptin in insulin secretion cannot be ruled out.

We previously demonstrated that hypothalamic NPY neurons develop resistance to chronic leptin infusion (21). In a preliminary study, we observed that proopiomelanocortin (POMC) and neurotensin (NT) producing neurons, which have been implicated to be involved in mediating anorectic action of leptin in the hypothalamus (41–43), also develop leptin resistance following chronic central leptin infusion (our unpublished observation). The present study addressed whether chronic leptin infusion altered leptin signal transduction mechanism in the hypothalamus. Among various mechanisms by which leptin exerts its effect, the participation of leptin receptor is often regarded as the first step in leptin signaling. Early recognition of leptin receptor as a member of the class 1 cytokine receptor superfamily (44) resulted in prompt identification of the JAK-STAT pathway as the major pathway of leptin signaling (22, 45–47). In the leptin-signaling cascade, leptin binding to the receptor initiates a sequence of events involving phosphorylation of JAK2, receptor, and STAT3. Phosphorylated STAT3 becomes dimerized and translocated to the nucleus in which it binds and regulates expression from target gene promoter (48). In the present study, we tested whether a defect in hypothalamic leptin receptor activity and associated signal transduction through JAK2-STAT3 pathway underlies the development of leptin resistance in NPY neurons (21) and other leptin-sensitive neurons (*e.g.* POMC, NT) following chronic central leptin infusion that could contribute to normalization in food intake after an initial decrease.

We assessed leptin receptor activity in the hypothalamus by evaluating the levels of receptor mRNA and receptor protein (phosphorylated and nonphosphorylated). Although there was a small (13%) but significant decrease in both OB-Rtot and Ob-Rb mRNA levels on d 4, there was no change in either OB-Rtot or Ob-Rb mRNA levels on d 16 of leptin infusion. However, leptin receptor protein levels for both long and short forms did not change throughout 16 d of leptin infusion, including d 4 when a small but significant reduction in mRNA levels was observed in leptin-infused rats. The reason for the discrepancy between Ob-R mRNA and protein levels on d 4 of leptin infusion was not examined but appears to be due to posttranscriptional events, such as increased transcriptional efficiency or decreased receptor protein degradation. Alternatively, the small change in receptor protein levels that might occur from a minimal decrease in mRNA expression could be beyond the sensitivity of the Western blot assay. In a recent study, chronic sc leptin infusion for 4 wk, which resulted in circulating leptin levels of about 160 ng/ml, decreased both Ob-Rb mRNA and leptin receptor protein levels in the rat hypothalamus (49). However, in the present study, both leptin receptor gene expression and leptin receptor protein levels remained unchanged following 2 wk of leptin infusion. The discrepancy in results could be due to route of administration, dose, and duration of leptin infusion.

Nevertheless, leptin receptor phosphorylation remained significantly elevated throughout 16 d of leptin infusion. In addition, because leptin receptor phosphorylation is dependent on leptin binding, our results suggest that leptin binding to its receptor remained intact during chronic leptin exposure to the hypothalamus. These results altogether suggest that leptin receptor activity in the hypothalamus is unimpaired and therefore cannot account for the normalization in food intake and the development of resistance in NPY neurons (21) during chronic leptin infusion. We also observed that the levels of phosphorylated JAK2 protein were increased during initial period (d 2 and 4) but not on d 16 of leptin infusion. In the leptin-signaling cascade, the binding of leptin to the receptor results in phosphorylation of JAK2. Phosphorylated JAK2, in turn, mediates phosphorylation at the specific receptor tyrosine residue, which then serves as a docking site for STAT3. Although leptin is reported to induce JAK2 phosphorylation in BaF3 cells transfected with Ob-Rb (47), a single iv injection of leptin, which induced STAT3 activation, failed to induce JAK2 phosphorylation in the rat hypothalamus (23). The present study, however, clearly shows an increased JAK2 phosphorylation in the hypothalamus following 2–4 d of continuous central leptin infusion. Although there was no increase in JAK2 phosphorylation on d 16 of leptin infusion and the reason behind this is not clear, it is possible that the sensitivity of our Western blot assay may not detect small increases in p-JAK2 levels that might have occurred during this period of leptin infusion.

The activation of STAT3, as defined by phosphorylation of STAT3 and subsequent induction of nuclear translocation of p-STAT3 (48), is one of the major events in intracellular signal transduction pathway of leptin. Our study shows that chronic central leptin infusion results in activation of STAT3 throughout the infusion period because both p-STAT3 levels and the DNA-binding activity of STAT3 were significantly increased in leptin-infused rats on d 2, 4, and 16 of infusion. In addition, STAT3 activation was higher on d 16, compared with that on d 2 or 4 of leptin infusion. These results strongly suggest that intracellular signal transduction through STAT3 activation remains intact in the presence of continuous exposure of the hypothalamus to leptin. Thus, normalization of food intake to the preleptin levels on d 16 of leptin infusion does not appear to be due to an impaired STAT3 pathway of leptin signaling. Although we cannot exclude possible involvement of other cytokines (50, 51), simultaneous increase in phosphorylated Ob-Rb protein and STAT3 activation during chronic central leptin infusion suggests that STAT3 signaling is due at least partly to leptin. Furthermore, although insulin stimulates STAT3 (52), the role of insulin is unlikely because chronic leptin infusion decreased circulating insulin levels. Although previous studies have shown leptin to increase thermogenesis (15, 35), it remains to be determined whether this was the case following chronic central leptin infusion and whether STAT3 activation underlies this response. If so, then it will explain some of the mechanisms behind the stabilization of body weight at lower level despite normalization in food intake following chronic leptin infusion. However, in a recent study, Scarpace *et al.* (53) reported resistance to anorectic and thermogenic responses to central leptin gene therapy in aged obese rats despite a persistent STAT3 activation.

SOCS3 has been established as a negative feedback regulator of leptin receptor signaling (27, 54) and many other cytokines including ciliary neurotrophic factor and IL-6 (55, 56). Because SOCS3 is thought to be involved in leptin resistance (27, 54), we examined SOCS3 mRNA and protein levels in the hypothalamus following chronic leptin infusion. We observed that SOCS3 mRNA levels were increased by approximately 2- to 3-fold on each day examined during leptin infusion. Although, we were unable to detect any change in SOCS3 protein levels during the initial period of leptin infusion, SOCS3 protein levels slightly increased on d 16 in leptin-infused rats. Although the reason behind this discrepancy between SOCS3 mRNA and protein levels is not clear, it appears that a posttranscriptional defect and/or an increased degradation of SOCS3 protein did occur on d 2–4 of leptin infusion. It is to be noted that in Chinese hamster ovary cells transfected with Ob-Rb and SOCS3 expression vectors, a significant increase in SOCS3 protein levels has been reported at 20 h of leptin treatment (54). Remarkably, despite an increased SOCS3 mRNA and protein levels, STAT3 remained activated on d 16. Because SOCS3 inhibition of leptin signaling is reported to be dependent on its binding to Ob-Rb (57) and JAK2 (58), it will be most interesting to address whether a defect in this mechanism may be responsible for a continued activation of JAK-STAT pathway throughout the leptin infusion. Also, in a recent study (59), recombinant adeno-associated virus-mediated central leptin gene therapy for 138 d in mildly obese 18-month-old male F-344X Brown Norway rats was associated with increased p-STAT3 levels and SOCS3 gene expression in the hypothalamus. Although *in vitro* and *in vivo* studies have postulated that SOCS3 might play an important role in leptin resistance (16, 27, 54, 60–62), it is not clear whether increased SOCS3 gene expression is responsible for the development of leptin resistance in NPY neurons (21) following leptin infusion. If it does, then it would be via a mechanism other than inhibition of STAT3 activation. Interestingly, in a recent study with diet-induced obese mice, hypothalamic SOCS3 mRNA levels remained unchanged despite a reduced STAT3 activation by leptin in these animals (63).

Our recent demonstration of the development of leptin resistance in NPY neurons (21) as well as in POMC and NT neurons (our unpublished observations) and the present finding of increased hypothalamic STAT3 activation during chronic leptin infusion raise an important question as to what are the mechanisms by which leptin target neurons, particularly NPY neurons, escape an intact STAT3 pathway of intracellular leptin signaling. Because STAT3 activation was examined in whole hypothalamic extracts, an alteration in STAT3 activation in specific hypothalamic sites or specific neurons is still possible and may contribute to develop leptin insensitivity. However, if the increased STAT3 activity observed in whole hypothalamic extract is due to an increase of STAT3 activity in specific leptin-sensitive neurons other than NPY neurons, it will require a remarkable increase in STAT3 activity in those neurons. On the other hand, if STAT3 transcriptional activity is impaired, it could explain leptin insensitivity despite an increased STAT3 activation in specific neurons. Recent evidence suggest that upon reaching the nucleus transcriptional activity of STAT proteins may be dependent on its interaction with other DNA-binding protein or coactivators (64). In this regard, it is noteworthy that STAT3 transcriptional activity can be regulated by other factors (coactivators), such as p300/CBP (cAMP response element-binding protein-binding protein) and steroid receptor coactivators 1 (NcoA/SRC1a) (65, 66). Although both CBP and SRC1 are localized in the hypothalamus (67, 68) and the role of these coactivators in hypothalamic STAT3 transcriptional activity is yet to be established, any alteration in these coactivators could compromise STAT3-mediated leptin action despite an activation of STAT3. These possibilities are currently under investigation in our laboratory.

Our recent evidence suggests that, besides the JAK2-STAT3 pathway, leptin action in the hypothalamus is mediated through an insulin-like signaling pathway involving stimulation of phosphotidylinositol-3 kinase (PI3K) and phosphodiesterase 3B (PDE3B), and reduction in cAMP levels (33). Others have also reported an activation of hypothalamic PI3K by leptin (69). Our additional evidence further suggested that a PI3K-PDE3B-cAMP pathway interacting with the JAK2-STAT3 pathway constitutes a critical component of leptin signaling in the hypothalamus (33). Thus, it will be extremely interesting whether PI3K-PDE3B-cAMP pathway of leptin signaling is altered during chronic leptin infusion, which might be responsible for the normalization in food intake and NPY expression following central leptin infusion. Current studies are underway to determine

whether this pathway of leptin signaling is defective following chronic central leptin infusion and in diet-induced obesity.

In summary, we have provided evidence suggesting that the JAK2-STAT3 pathway of leptin signaling in the hypothalamus is functioning normally in our model of chronic central leptin infusion. We suggest that normalization in food intake (and hypothalamic NPY expression, observed previously) during chronic leptin infusion must be due to other compensatory mechanisms and or an alteration in other pathways of leptin signaling in the hypothalamus.

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References

1. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM** 1994 Positional cloning of the mouse obese gene and its human homologue. Nature 372:425–432
2. **Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P** 1995 Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 269:546–549
3. **Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F** 1995 Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269:540–543
4. **Friedman JM, Halaas JL** 1998 Leptin and the regulation of body weight in mammals. Nature 395:763–770
5. **Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S** 1997 Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387:903–908
6. **Considine RV, Considine EL, Williams CJ, Nyce MR, Magosin SA, Bauer TL, Rosato EL, Colberg J, Caro JF** 1995 Evidence against either a premature stop codon or the absence of obese gene mRNA in human obesity. J Clin Invest 95:2986–2988
7. **Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bourneres P, Lebouc Y, Froguel P, Guy-Grand B** 1998 A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392:398–401
8. **Gotoda T, Manning BS, Goldstone AP, Imrie H, Evans AL, Strosberg AD, McKeigue PM, Scott J, Aitman TJ** 1997 Leptin receptor gene variation and obesity: lack of association in a white British male population. Hum Mol Genet 6:869–876
9. **Matsuoka N, Ogawa Y, Hosoda K, Matsuda J, Masuzaki T, Azuma N, Miyawaki T, Natsui K, Nishimura H, Yoshimasa Y, Nishi S, Thompson DB, Nakao K** 1997 Human leptin receptor gene in obese Japanese subjects: evidence against either obesity-causing mutations or association of sequence variants with obesity. Diabetologia 40:1204–1210
10. **Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF** 1996 Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334:292–295
11. **Van Heek M, Compton DS, France CF, Tedesco RP, Fawzi AB, Graziano MP, Sybertz EJ, Strader CD, Davis Jr HR** 1997 Diet-induced obese mice develop peripheral, but not central, resistance to leptin. J Clin Invest 99:385–390
12. **Widdowson PS, Upton R, Buckingham R, Arch J, Williams G** 1997 Inhibition of food response to intracerebroventricular injection of leptin is attenuated in rats with diet-induced obesity. Diabetes 46:1782–1785
13. **Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM** 1997 Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. Proc Natl Acad Sci USA 94:8878–8883
14. **Lin S, Thomas TC, Storlien LH, Huang XF** 2000 Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. Int J Obes Relat Metab Disord 24:639–646
15. **Shek EW, Scarpace PJ** 2000 Resistance to the anorexic and thermogenic effects of centrally administrated leptin in obese aged rats. Regul Peptide 92:65–71
16. **Scarpace PJ, Matheny M, Tumer N** 2001 Hypothalamic leptin resistance is associated with impaired leptin signal transduction in aged obese rats. Neuroscience 104:1111–1117
17. **Levin BE, Dunn-Meynell AA** 2002 Reduced central leptin sensitivity in rats with diet-induced obesity. Am J Physiol Regul Integr Comp Physiol 283:R941–R948
18. **Caro JF, Kolaczynski JW, Nyce MR, Ohannesian JP, Opentanova I, Goldman WH, Lynn RB, Zhang PL, Sinha MK, Considine RV** 1996 Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. Lancet 348:159–161
19. **Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte Jr D** 1996 Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. Nat Med 2:589–593
20. **Banks WA** 2001 Leptin transport across the blood-brain barrier: implications for the cause and treatment of obesity. Curr Pharm Des 7:125–133
21. **Sahu A** 2002 Resistance to the satiety action of leptin following chronic central leptin infusion is associated with the development of leptin resistance in neuropeptide Y neurones. J Neuroendocrinol 14:796–804
22. **Vaisse C, Halaas JL, Horvath CM, Darnell Jr JE, Stoffel M, Friedman JM** 1996 Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nat Genet 14:95–97
23. **McCowen KC, Chow JC, Smith RJ** 1998 Leptin signaling in the hypothalamus of normal rats *in vivo.* Endocrinology 139:4442–4447
24. **Hubschle T, Thom E, Watson A, Roth J, Klaus S, Meyerhof W** 2001 Leptin-induced nuclear translocation of STAT3 immunoreactivity in hypothalamic nuclei involved in body weight regulation. J Neurosci 21:2413–2424
25. **Nicholson SE, Hilton DJ** 1998 The SOCS proteins: a new family of negative regulators of signal transduction. J Leukocyte Biol 63:665–668
26. **Krebs DL, Hilton DJ** 2000 SOCS: physiological suppressors of cytokine signaling. J Cell Sci 113:2813–2819
27. **Bjørbæk C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS** 1998 Identification of SOCS-3 as a potential mediator of central leptin resistance. Mol Cell 1: 619–625
28. **Sadowski HB, Shuai K, Darnell Jr JE, Gilman MZ** 1993 A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science 261:1739–1744
29. **Wong P, Severns CW, Guyer NB, Wright TM** 1994 A unique palindromic element mediates IFN-γ induction of mig gene expression. Mol Cell Biol 14:914–922
30. **Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K** 1994 Current protocols in molecular biology. New York: John Wiley, Sons
31. **Bradford MM** 1976 A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Chem 72:248–254
32. **Sahu A** 2000 Quantification of NPY mRNA by ribonuclease protection assay. Methods Mol Biol 153:219–230
33. **Zhao AZ, Huan J-N, Gupta S, Pal R, Sahu A** 2002 A phosphatidylinositol 3-K-phosphodiesterase 3B-cyclic AMP pathway is involved in hypothalamic action of leptin on feeding. Nat Neurosci 5:727–728
34. **Trinder P** 1969 Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromagen. J Clin Pathol 22:158–161
35. **Scarpace PJ, Matheny M, Pollock BH, Tumer N** 1997 Leptin increases uncoupling protein expression and energy expenditure. Am J Physiol 273:E226–E230
36. **Qian H, Azain MJ, Compton MM, Hartzell DL, Hausman GJ, Baile CA** 1998 Brain administration of leptin causes deletion of adipocytes by apoptosis. Endocrinology 139:791–794
37. **Bai Y, Zhang S, Kim KS, Lee JK, Kim KH** 1996 Obese gene expression alters the ability of 30A5 preadipocytes to respond to lipogenic hormones. J Biol Chem 271:13939–13942
38. **Wang M-Y, Lee Y, Unger RH** 1999 Novel form of lipolysis induced by leptin. J Biol Chem 274:17541–17544
39. **Wang ZW, Zhou YT, Lee Y, Higa M, Kalra SP, Unger RH** 1999 Hyperleptinemia depletes fat from denervated fat tissue. Biochem Biophys Res Commun 260:653–657
40. **Kulkarni RN, Wang ZL, Wang RM, Hurley JD, Smith DM, Ghatei MA, Withers DJ, Gardiner JV, Bailey CJ, Bloom SR** 1997 Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, *in vivo,* in mice. J Clin Invest 100:2729–2736
41. **Sahu A** 1998 Evidence suggesting that galanin (GAL), melanin-concentrating hormone (MCH), neurotensin (NT), proopiomelanocortin (POMC) and neuropeptide Y (NPY) are targets of leptin signaling in the hypothalamus. Endocrinology 139:795–798

42. **Sahu A, Carraway R, Wang Y-P** 2001 Evidence that neurotensin mediates the central effect of leptin on food intake in rats. Brain Res 888:343–347
43. **Schwartz MW, Woods SC, Porte Jr D, Seeley RJ, Baskin DG** 2000 Central nervous system control of food intake. Nature 404:661–671
44. **Tartaglia LA** 1997 The leptin receptor. J Biol Chem 272:6093–6096
45. **Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC** 1996 Defective STAT signaling by the leptin receptor in diabetic mice. Proc Natl Acad Sci USA 93:6231–6235
46. **Bjorbaek C, Uotani S, da Silva B, Flier JS** 1997 Divergent signaling capacities of the long and short isoforms of the leptin receptor. J Biol Chem 272:32686–32695
47. **Ghilardi N, Skoda RC** 1997 The leptin receptor activates Janus kinase 2 and signals for proliferation in a factor-dependent cell line. Mol Endocrinol 11:393–399
48. **Darnell Jr JE** 1997 STATs and gene regulation. Science 277:1630–1635
49. **Martin RL, Perez E, He YJ, Dawson Jr R, Millard WJ** 2000 Leptin resistance is associated with hypothalamic leptin receptor mRNA and protein down-regulation. Metabolism 49:1479–1484
50. **Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L** 1998 Interleukin-6-type signaling through the gp130/Jak/STAT pathway. Biochem J 34:297–314
51. **Heim MH** 1999 The Jak-STAT pathway: cytokine signaling from the receptor to the nucleus. J Recept Signal Transduct Res 19:75–120
52. **Carvalheira JB, Siloto RM, Ignacchitti I, Brenelli SL, Carvalho CR, Leite A, Velloso LA, Gontijo JA, Saad MJ** 2001 Insulin modulates leptin-induced STAT3 activation in rat hypothalamus. FEBS Lett 500:119–124
53. **Scarpace PJ, Matheny M, Zhang Y, Tumer N, Frase CD, Shek EW, Hong B, Prima V, Zolotukhin S** 2002 Central leptin gene delivery evokes persistent leptin signal transduction in young and aged-obese rats but physiological responses become attenuated over time in aged-obese rats. Neuropharmacology 42:548–561
54. **Bjorbaek C, El-Haschimi K, Frantz JD, Flier JS** 1999 The role of SOCS-3 in leptin signaling and leptin resistance. J Biol Chem 274:30059–30065
55. **Bjorbaek C, Elmquist JK, El-Haschimi K, Kelly J, Ahima RS, Hileman S, Flier JS** 1999 Activation of SOCS-3 messenger ribonucleic acid in the hypothalamus by ciliary neurotrophic factor. Endocrinology 140:2035–2043
56. **Schmitz J, Weissenbach M, Haan S, Heinrich PC, Schaper F** 2000 SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. J Biol Chem 275:12848–12856
57. **Bjorbak C, Buchholz RM, Davis SM, Bates SH, Pierroz DD, Gu H, Neel BG, Myers Jr MG, Flier JS** 2001 The role of SHP-2 in MAPK activation by leptin receptors. J Biol Chem 276:4747–4755
58. **Banks AS, Davis SM, Bates SH, Myers Jr MG** 2000 Activation of downstream signals by the long form of the leptin receptor. J Biol Chem 275:14563–14572
59. **Scarpace PJ, Matheny M, Zhang Y, Shek EW, Prima V, Zolotukhin S, Tumer N** 2002 Leptin-induced leptin resistance reveals separate roles for the anorexic and thermogenic responses in weight maintenance. Endocrinology 143:3026–3035
60. **Scarpace PJ, Matheny M, Shek EW** 2000 Impaired leptin signal transduction with age related obesity. Neuropharmacology 39:1872–1879
61. **Wang ZW, Pan WT, Lee Y, Kakuma T, Zhou YT, Unger RH** 2001 The role of leptin resistance in the lipid abnormalities of aging. FASEB J 15:108–114
62. **Peralta S, Carrascosa JM, Gallardo N, Ros M, Arribas C** 2002 Ageing increases SOCS-3 expression in rat hypothalamus: effects of food restriction. Biochem Biophys Res Commun 296:425–428
63. **El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS** 2000 Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. J Clin Invest 105:1827–1832
64. **Levy DE, Darnell Jr JE** 2002 STATS: transcriptional control and biological impact. Nat Rev Mol Cell Biol 3:651–662
65. **Gingras S, Simard J, Groner B, Pfitzner E** 1999 p300/CBP is required for transcriptional induction by interleukin-4 and interacts with Stat6. Nucleic Acids Res 27:2722–2729
66. **Ray S, Sherman CT, Lu M, Brasier AR** 2002 Angiotensinogen gene expression is dependent on signal transducer and activator of transcription 3-mediated p300/cAMP response element binding protein-binding protein coactivator recruitment and histone acetyltransferase activity. Mol Endocrinol 16:824–836
67. **Meijer OC, Steenbergen PJ, De Kloet ER** 2000 Differential expression and regional distribution of steroid receptor coactivators SRC-1 and SRC-2 in brain and pituitary. Endocrinology 141:2192–2199
68. **Stromberg H, Svensson SP, Hermanson O** 1999 Distribution of CREB-binding protein immunoreactivity in the adult rat brain. Brain Res 818:510–514
69. **Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers Jr MG, Schwartz MW** 2001 Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature 413:794–795