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Region-Specific Leptin Resistance within the Hypothalamus of Diet-Induced Obese Mice

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Leptin resistance in diet-induced obese (DIO) mice is characterized by elevated serum leptin and a decreased response to exogenous leptin and is caused by unknown defects in the central nervous system. Leptin normally acts on several brain nuclei, but a detailed description of leptin resistance within individual brain regions has not been reported. We first mapped leptin-responsive cells in brains from DIO mice using phospho-signal transducer and activator of transcription (P-STAT3) immunohistochemistry. After 16 wk of high-fat-diet feeding, leptin-activated P-STAT3 staining within the arcuate nucleus (ARC) was dramatically decreased. In contrast, other hypothalamic and extrahypothalamic nuclei remained leptin sensitive. Reduced leptin-induced P-STAT3 in the ARC could also be detected after 4 wk and as early as 6 d of a high-fat diet. To examine potential mechanisms for leptin-resistant STAT3 activation in the ARC of DIO mice, we measured mRNA levels of candidate signaling molecules in the leptin receptor-STAT3 pathway. We found that the level of suppressor of cytokine signaling 3 (SOCS-3), an inhibitor of leptin signaling, is specifically increased in the ARC of DIO mice. The study suggests that the ARC is selectively leptin resistant in DIO mice and that this may be caused by elevated suppressor of cytokine signaling 3 in this hypothalamic nucleus. Defects in leptin action in the ARC may play a role in the pathogenesis of leptin-resistant obesity. (*Endocrinology* 145: 4880–4889, 2004)

LEPTIN, A HORMONE SECRETED from white adipose tissue, is a major regulator of body weight and food intake (1). This effect of leptin is particularly evident in rodents and humans lacking a functional form of the protein, resulting in severe obesity and greatly increased appetite (2, 3). Equally impressive is the finding that treatment with recombinant leptin reverses the obese phenotype in leptin-deficient humans (4). In contrast, the majority of obese patients have high circulating leptin levels (5), even though a similar or lower concentration of leptin can cause a dramatic loss of body fat in normal mice and in humans lacking leptin (4, 6). This suggests the existence of leptin resistance in hyperleptinemic obese subjects. Indeed, administration of recombinant leptin to obese, hyperleptinemic subjects failed to decrease body weight and food intake (7). The cause of obesity and leptin resistance in most forms of human and rodent obesity is not understood.

The effect of leptin on food intake is mediated in part via leptin receptors present in the hypothalamus. Peripherally applied leptin in rodents induces a central signaling pathway that involves activation of signal transducer and activator of transcription 3 (STAT3) (8). The requirement of this pathway to prevent severe hyperphagia and obesity was recently demonstrated in mice specifically lacking the STAT3-binding site of the leptin receptor (9) and in mice with reduced levels of STAT3 proteins selectively in the central nervous system (CNS) (10). After binding to the long leptin receptor (ObRb), STAT3 becomes phosphorylated by Janus kinase 2 (JAK2) and acts in the nucleus to regulate transcription (11). El-Haschimi *et al.* (12) showed that in diet-induced obese (DIO) mice, a classical mouse model of leptin resistance and obesity, recombinant leptin completely failed to induce STAT3 activation in hypothalamic extracts, demonstrating severe leptin-resistant signaling in the hypothalamus of DIO mice. However, in contrast to leptin-deficient *ob/ob* mice that entirely lack signaling in the brain, DIO mice are not as obese, suggesting that intact or partial leptin signaling exists in some sites within the CNS of DIO mice. Because El-Haschimi *et al.* (12) used whole hypothalamic extracts, heterogeneity of resistance in neuronal populations within the hypothalamus, including the arcuate nucleus (ARC), the ventromedial hypothalamus (VMH), and dorsomedial hypothalamus (DMH), remains possible. Moreover, leptin resistance in brain regions located outside the hypothalamus was not examined.

The anorectic action of leptin occurs in part via the ARC where ObRb is highly expressed (13–15). Here leptin directly inhibits orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons and activates anorexigenic proopiomelanocortin (POMC) neurons (1, 16, 17). Anatomical studies of ObRb mRNA and of activated c-Fos in leptin-treated animals also reveal leptin-responsive sites beyond the ARC (15, 18). Consistent with these results, immunohistochemical (IHC) studies demonstrate rapid activation of STAT3 by leptin in hypothalamic and extrahypothalamic brain regions of rats and mice. These include the lateral hypothalamic area,

Abbreviations: AgRP, Agouti-related peptide; ARC, arcuate nucleus; BBB, blood-brain barrier; CNS, central nervous system; DIO, diet-induced obesity; DMH, dorsomedial hypothalamus; DR, dorsal raphe; HFD, high-fat diet; IHC, immunohistochemistry; LFD, low-fat diet; ME, median eminence; NTS, nucleus of the solitary tract; ObRb, long leptin receptor; POMC, proopiomelanocortin; PMN, premammilary nucleus; PVN, paraventricular nucleus of the hypothalamus; SOCS-3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; VMH, ventromedial nucleus of the hypothalamus.

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the paraventricular hypothalamus (PVN), and the premammilary nucleus (PMN) of the hypothalamus, the periaqueductal gray and the dorsal raphe (DR) in the midbrain, and the parabrachial nucleus and the nucleus of the solitary tract (NTS) in the brain stem (19–21). Although the specific function(s) of each nucleus in leptin action is yet largely unknown, injection of small doses of the hormone into the VMH, but not into the ARC, PVN, or DMH, stimulates the sympathetic nervous system (22). In addition, microinjection of leptin into the ARC (14), the VMH (14, 23), and the NTS (24), but not into the DR (23), inhibits food intake. Combined, these results suggest that distinct biological actions of leptin are mediated by different brain nuclei (22) but that overlapping or redundant functional sites also exist.

Decreased transport of leptin into the brain has been suggested as one mechanism causing central leptin resistance in obesity (25, 26). Supportive of this is the finding that the concentration of leptin in the cerebrospinal fluid from obese humans is not increased in proportion to their elevated serum leptin levels (27). On the other hand, central leptin gene therapy fails to overcome leptin resistance in DIO (28) and recent data suggest that the impaired blood-brain barrier (BBB) transport is acquired during development of obesity (29, 30). The latter data point to downstream signaling defects as possible primary causes of leptin resistance. Alterations at the level of the leptin receptor and at postreceptor steps have been examined to explain this, including downregulation of ObRb expression (12, 31, 32) and possible upregulation of negative signaling regulators such as suppressor of cytokine signaling 3 (SOCS-3) (12, 33–36). The results have, however, been variable and inconclusive, possibly because of a lack of detailed anatomical and quantitative analyses.

In this study, we systematically examined leptin-activated cells throughout the brains of mice during development of DIO. We found clear evidence that the ARC is a major site of leptin resistance. In contrast, other hypothalamic and extrahypothalamic sites appear leptin responsive, suggesting a state of selective leptin resistance in the brain of DIO mice. Further analyses show that the ARC is resistant to leptin very early in the development of DIO. Finally, SOCS-3 expression is increased specifically in the ARC. Taken together, the study suggests that abnormalities in postreceptor leptin signaling, specifically in the ARC, may play a role in the pathogenesis of DIO in mice.

Materials and Methods

Materials

Recombinant mouse leptin was from Dr. E. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases and The National Hormone and Pituitary Program, Torrance, CA). Supplies for IHC were purchased from Sigma Chemical Co. (St. Louis, MO) and the ABC Vectastain Elite kit was from Vector Laboratories (Burlingame, CA). Phosphospecific-(Y705)-STAT3 and STAT3 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA), goat antirabbit from Jackson ImmunoResearch Laboratories (West Grove, PA), and goat serum from Invitrogen Life Technologies, Inc. (Carlsbad, CA).

Animal housing

Male C57BL/6J mice, 3–4 wk of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals and procedures used were in accordance with the guidelines and approval of the Harvard Medical School and Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committees. All mice were housed individually. The low-fat diet (LFD) contained 17% fat (3.3 kcal/g; no. 8664, Harlan Teklad, Madison, WI) and the high-fat diet (HFD) contained 58% fat (5.6 kcal/g; no. D12331, Research Diets, New Brunswick, NJ).

Measurement of serum leptin

Blood serum was separated by centrifugation and samples stored at –20 C until further use. Double measurements of leptin were done with an ELISA (Chrystal Chem Inc., Downers Grove, IL).

Leptin stimulation and IHC

Mice were injected ip with leptin (5.0 mg/kg body weight) or vehicle (PBS) and anesthetized 30 min later with 7% chloral hydrate (7.5 μl/g body weight). Transcardiac perfusion with formalin, removal of the brain, postfixation, and cryoprotection were done as described earlier (21). Brains were cut in 25-μm coronal sections, collected in five series, and stored at −20 C until further use. P-STAT3 IHC was performed as described earlier (21). In brief, free-floating tissue sections were blocked in goat serum and then incubated with the P-STAT3 antibody (1:4000). Sections were washed and incubated with biotinylated antirabbit antibody (1:1000), followed by avidin-biotin-complex labeling. Signals were then developed with diaminobenzidine resulting in a blue-black precipitate. Pictures were taken under a bright-field microscope (Axioscope, Zeiss, Oberkochen, Germany) with a digital camera (Axiocam, Zeiss).

Cell counting and quantification

One of the five brain series obtained from each animal was subjected to P-STAT3 IHC as described above. Sections were first organized systematically in a rostral-to-caudal manner according to the mouse brain atlas (37). All sections in the series were then carefully examined by dark-field microscopy to assign positive cells to appropriate nuclei using locations of adjacent landmarks. All brain sections in the series that contained positive cells in each nucleus were analyzed. Cell counts were obtained from both sides of the brain in each section, and all P-STAT3-positive cells in each nucleus were counted irrespective of staining intensity. Except for weak baseline staining in the ARC, no P-STAT3-positive cells were detected in any brain nuclei of PBS-treated mice.

Microdissection

Animals were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine) before decapitation, and the brain was rapidly removed. Using a cooled mouse brain matrix with 1-mm section dividers (ASI Instruments Inc., Warren, MI), one sagittal cut was made to bisect the brain, followed by two cuts left and right of the bisectional cut to produce two 1-mm-thick sagittal sections left and right of the third ventricle. Landmarks like the fornix, optic tracts, and mammillary nuclei were used to dissect reproducible pieces of ARC-, VMH/DMH-, and PVN-enriched tissues. A sample of the cortex was also collected. The two tissue pieces (one from each hemisphere) from each brain region were finally combined and snap-frozen in liquid nitrogen and stored at –80 C until further use.

Western blotting

Microdissected tissues were homogenized in 100 μl RIPA lysis buffer (50 mM Tris, pH 7.4; 1% Nonidet P-40; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM Na_3VO_4, 1 mM NaF) using a Teflon potter in 1.5-ml tubes. Homogenates were shaken at 4 C for 30 min to allow complete lysis and then centrifuged for 15 min at 4 C and 23,000 × *g*, and supernatants were placed in a fresh tube. Protein concentrations were measured using a protein assay (DC protein assay, Bio-Rad, Hercules, CA). Twenty micrograms (ARC) or 30 μg (VMH/DMH) of protein lysates were loaded onto polyacrylamide gels (Criterion, 4–15% gradient Tris-HCl; Bio-Rad) followed by a transfer onto nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, NH) using transfer buffer (50 mM Tris, 20 mM glycine, 20% methanol). Membranes were blocked in 5% nonfat dried

milk (20 mM Tris, pH 7.4; 0.9% NaCl; 0.05% Tween 20) and incubated with anti-P-STAT3 antibody (1:1000) overnight at 4 C. Membranes were then washed and incubated with secondary antibodies (antirabbit-horseradish peroxidase coupled, 1:15,000; Bio-Rad), washed and developed by enhanced chemiluminescence (ECLplus, Amersham, Piscataway, NJ) and x-ray films (Amersham). For total STAT3, the same amount of protein lysate from each sample was separated by SDS-PAGE, and membranes were blotted with a rabbit STAT3 antibody (1:1000) and detected as describe above for the P-STAT3. Signal intensities were quantified using Scion Image software (Frederick, MD). Final scanning results were obtained by normalizing the P-STAT3 signal with the amount of STAT3 in the same sample.

Semiquantitative RT-PCR

Total RNA was isolated from tissue blocks using RNA STAT-60 (Tel-Test Inc., Friendswood, TX). Half of 1 μg of total RNA was used for reversed transcription (RT-PCR kit, Clontech, Palo Alto, CA). PCR was performed using the following primers: SOCS-3a, 5′-accagcgccacttctcacg-3′; SOCS-3b, 5′gtggagcatcatactgatcc-3′; PTP1B-a, 5′-gactcgtcagtgcaggatca-3′; PTP1B-b, 5′-gcctgagcactttgaagacc-3′; ObRb-a, 5′-aaagagctccttctctgggtctcagagcac-3′; and ObRb-b, 5′-aaaaagcttctcaccagtcaaaagcacacc-3′. PCR was run in a volume of 25 μl using 5 μl cDNA. SOCS-3 was amplified using 32 cycles, and PTB1B and ObRb using 35 cycles. Products were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistical analyses

Data are presented as means ± SEM, and significance level was set at $P \leq 0.05$. Body weight and food intake data were analyzed by repeated-measurements ANOVA, and groups are compared by Fisher's projected least significant difference *post hoc* analysis or Student's *t* test as indicated. Analyses of cell counts, immunoblots, and RT-PCR data were done by Student's *t* tests.

Results

HFD induces obesity, hyperphagia, and hyperleptinemia in C57BL/6J mice

Here we used the established DIO model of obesity-prone C57BL/6J mice to study mechanisms of central leptin resistance. Young mice were fed a HFD or a LFD. In a first experiment, animals remained on diets for 16 wk (Fig. 1A). As expected, the HFD group rapidly separated their body weight from the LFD group, and significant differences could

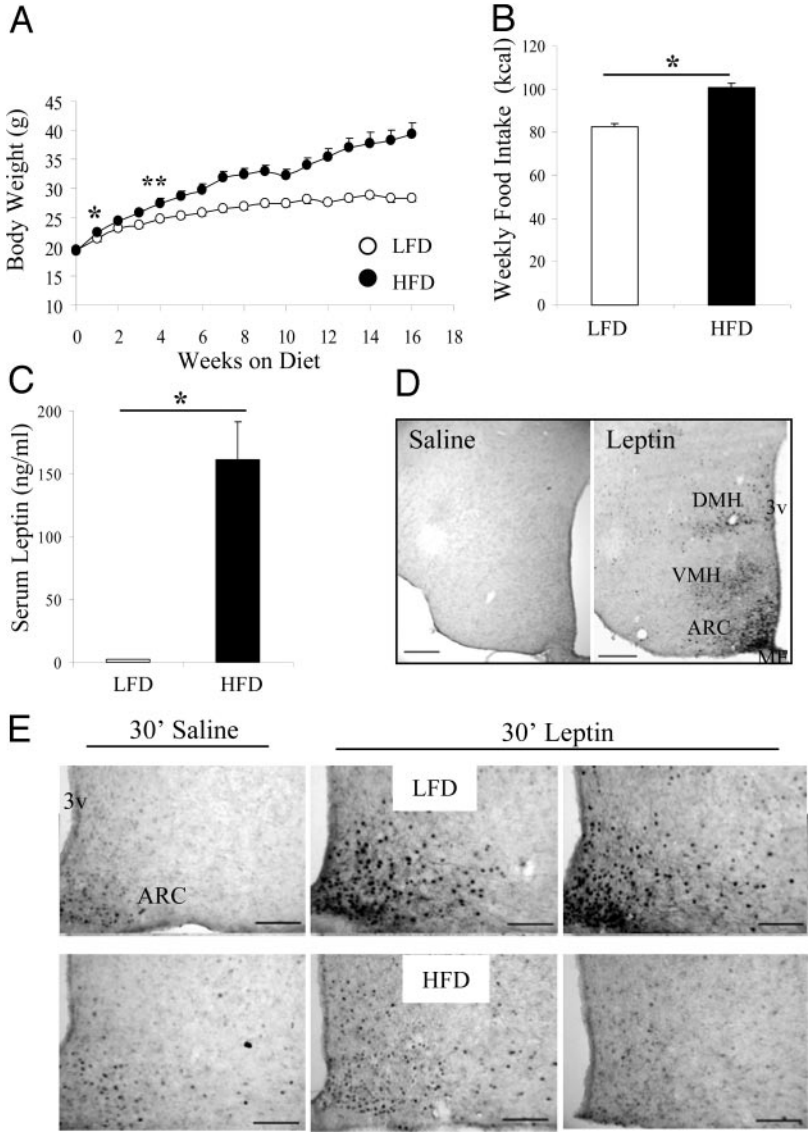

FIG. 1. Severe leptin resistance in the ARC of mice after 16 wk on HFD. A, Body-weight curves of C57BL6 mice given HFD or LFD. *, $P = 0.05$; **, $P < 0.01$ (n = 7–8 animals per group). B, Food intake during wk 12 of diets. *, $P < 0.02$ (n = 5–6). C, Average fasted serum leptin levels at 16 wk of diets. *, $P < 0.02$ (n = 2). Above data are means ± SEM. D, Representative microphotographs of P-STAT3 IHC of hypothalamic sections from a saline-treated (*left*) and a leptin-treated (*right*) lean mouse. Mice were injected with vehicle or leptin (5 mg/kg, ip) and killed 30 min later. Images are representative sections at Bregma –1.85 mm. *Scale bars*, 200 μm; 3v, third ventricle. E, High-magnification view of P-STAT3 IHC of sections from the ARC (Bregma level –1.8 mm) of LFD (*top*) and HFD (*bottom*) mice after a saline (*left*) or leptin injection (*middle* and *right*). Each panel represents one representative animal. *Scale bars*, 100 μm.

first be detected after 1 wk on the diets ($P = 0.05$) and become highly significant at 4 wk ($P < 0.01$). After 16 wk, HFD mice weighed approximately 10 g more than LFD animals. HFD mice had a significantly increased caloric intake (Fig. 1B) and dramatically increased serum leptin levels (Fig. 1C), demonstrating a state of leptin resistance.

Characterization of leptin-responsive sites in the CNS of lean mice

Leptin-responsive sites in the CNS were detected by P-STAT3 IHC as described earlier in the rat (21). In Fig. 1D, presence of known leptin-responsive sites in the hypothalamus were confirmed in leptin-injected lean mice, as demonstrated by robust staining in the DMH, VMH, and ARC. Within the hypothalamus, dense staining in the PMN and scattered staining in the lateral hypothalamic area was also detected. In contrast to earlier studies in the rat (20, 21), no positive cells were found in the PVN. Outside the hypothalamus, we also confirmed the presence of leptin-induced P-STAT3 cells in the midbrain, specifically the DR, and in NTS of the brain stem (19, 21).

The ARC is selectively resistant to leptin in HFD mice

DIO in mice has earlier been associated with a dramatic loss of total hypothalamic STAT3 signaling (12), but potential regional differences within the hypothalamus were not examined. After 16 wk of diet, LFD or HFD mice received vehicle or recombinant leptin and brain sections subjected to P-STAT3 IHC. In contrast to LFD mice, HFD animals had a dramatic reduction in leptin-induced P-STAT3 staining in the ARC (Fig. 1E, *bottom*). As indicated by representative microphotographs, leptin-induced P-STAT3 was not altered in the VMH (Fig. 2A), the DMH (Fig. 2B), the PMN (Fig. 2C), or the NTS (Fig. 2D) of HFD mice. These data suggest that leptin action in the ARC, but not in other hypothalamic or extrahypothalamic leptin-responsive brain regions, is specifically impaired in DIO mice.

Reduced number of leptin-responsive cells in the ARC after 16 and 4 wk of HFD

To quantify the extent of leptin-resistant signaling in DIO animals, we counted leptin-responsive neurons in each brain region in LFD and HFD mice. Consistent with the images shown above, we found no differences in the number of P-STAT3-immunoreative cells in the VMH, DMH, PMN, or NTS (Fig. 3). Visual inspections of these regions suggested that the intensity of cellular P-STAT3 staining was also similar in the two leptin-treated groups. In contrast, counts of P-STAT3-positive neurons in the ARC of HFD mice showed a clear reduction by more than 30% compared with LFD animals ($P < 0.01$). It should be pointed out that this analysis includes all visible P-STAT3-positive nuclei and disregards differences in signal intensities. We therefore conclude that this analysis is likely an underestimate of the degree of leptin-resistant signaling in the ARC of DIO mice. The data indicate that the ARC is a selective site of leptin resistance in mice given a HDF for 16 wk. In a study of 4 wk, when increased body weight, energy intake, and hyperleptinemia is also present (Fig. 4, A-C), we also found a clear decrease in leptin-stimulated P-STAT3 immunoreactivity in the ARC

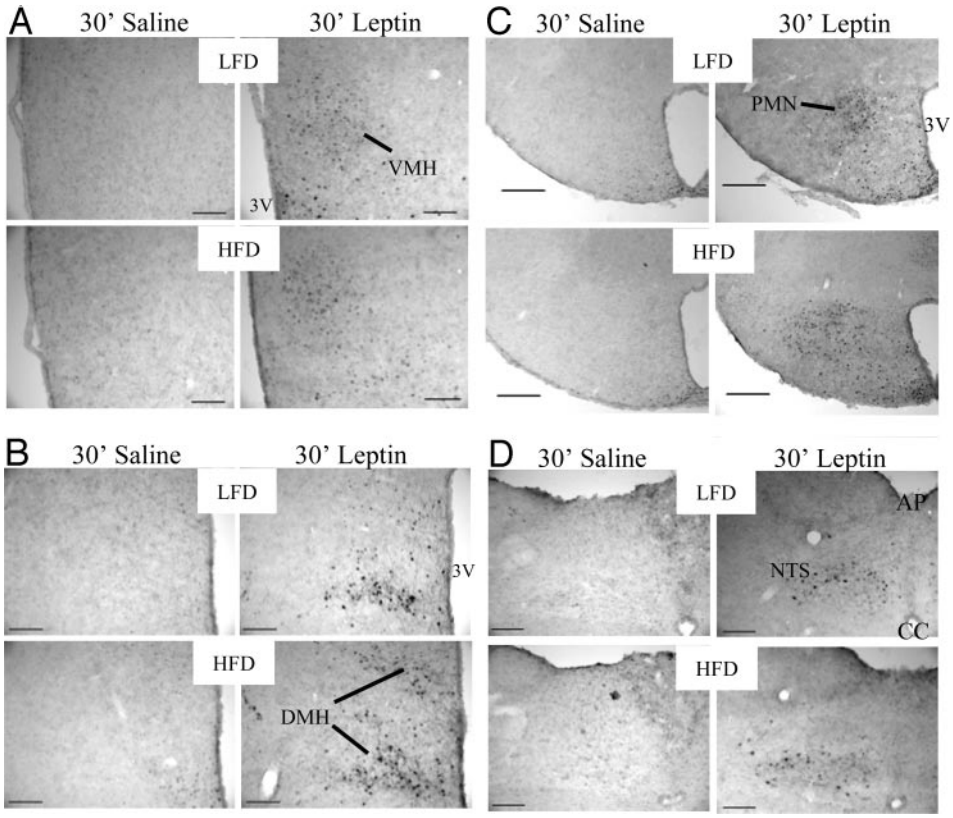

FIG. 2. Leptin-induced P-STAT3 staining is not reduced in other brain regions of HFD mice. Representative microphotographs of P-STAT3 IHC of sections from the VMH (Bregma level –1.4 mm) (A), DMH (Bregma −1.85 mm) (B), PMN (Bregma level –2.5 mm) (C), and NTS/DMV (Bregma 7.7 mm) (D) of LFD (*upper* panels) and HFD (*lower panels*) mice 30 min after saline (*left panels*) or leptin injection (*right panels*). Each panel represents one animal. Animals were on the diets for 16 wk. *Scale bars*, 100 μm; CC, central canal; AP, area postrema.

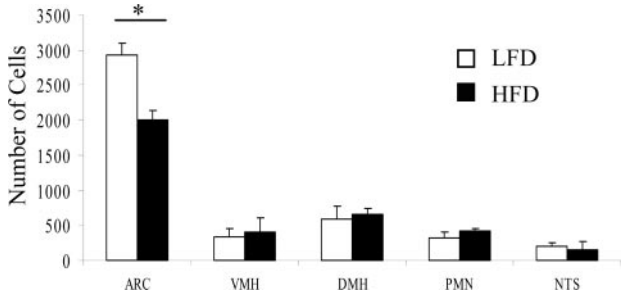

FIG. 3. The number of P-STAT3-positive cells is selectively reduced in the ARC of leptin-treated HFD mice. Shown are counts of P-STAT3-positive cells in different leptin target sites of LFD (n = 3) and HFD (n = 3) mice (16 wk of diet). One of five series from each animal was analyzed. *, $P < 0.01$.

of HFD animals (Fig. 4E). Consistent with visual analyses, counting of P-STAT3-positive cells in the ARC demonstrated an approximately 10% decrease ($P < 0.05$) in the obese animals (Fig. 4D). As discussed above, this is likely an underestimate of the actual leptin resistance. The fact that STAT3 phosphorylation is reduced in DIO mice after administration of a large pharmacological dose of leptin that leads to an increase in serum leptin levels by more than 100-fold (not shown) suggests that endogenous leptin signaling in the ARC of DIO mice is severely impaired.

The ARC of HDF mice is leptin resistant when increased body weight and energy intake first appears

To address the question of whether the observed leptin resistance in the ARC occurs at the onset of DIO or might be secondary to obesity, we investigated leptin responsiveness in HFD mice after only 6 d of diet when the difference in body weight is first detectable (Fig. 5A). A slight but significant increase in serum leptin levels was also present at this time (Fig. 5B). For better quantification of P-STAT3 in different hypothalamic areas we combined immunoblotting with a microdissection technique for the ARC and the VMH/DMH. To first test the quality of the tissue isolation method, several individual hypothalamic nuclei were obtained from two lean mice. We then performed RT-PCR analysis for POMC and AgRP mRNA, which both are markers for the ARC of the hypothalamus. As shown in Fig. 5C, POMC and AgRP mRNA were highly enriched in the ARC samples but were not detected in samples from the PVN or cortex. We then measured leptin-induced STAT3 phosphorylation in the ARC and VMH/DMH of HFD and LFD mice. As indicated in Fig. 5, D and E, leptin-induced P-STAT3 levels in the ARC were markedly reduced in HFD mice (~40%). In contrast, the VMH/DMH regions remained leptin sensitive, confirming the IHC findings above. These data suggest that leptin responsiveness is decreased in the ARC when obesity first occurs and is consistent with the possibility that leptin resistance in the ARC may play a role in the development of DIO.

SOCS-3 mRNA is increased in the ARC but not in the VMH/DMH of DIO mice

To investigate cellular mechanisms involved in development of leptin-resistant STAT3 phosphorylation in the ARC,

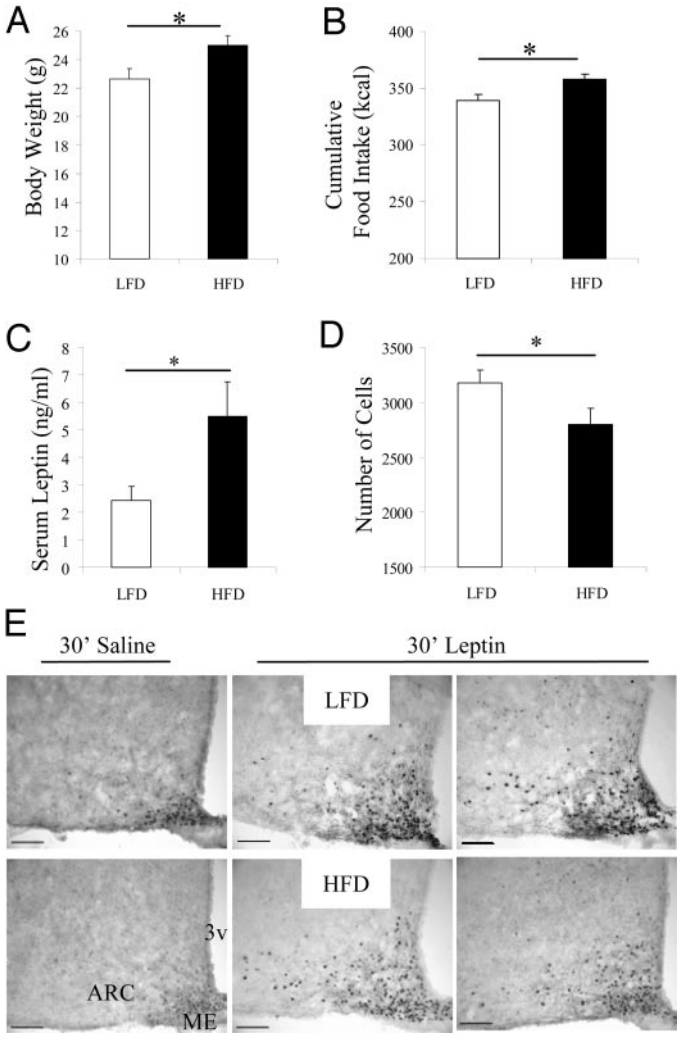

FIG. 4. Leptin resistance in the ARC of HFD mice after 4 wk of diet. A, Four-week body weights of LFD or HFD mice. *, $P < 0.02$ (n = 5–6 animals per group). B, Cumulative food intake during the 4 wk of diet. *, $P < 0.02$ (n = 5–6). C, Fasting serum leptin levels. *, $P < 0.05$. Data are means ± SEM. D, Shown are counts of P-STAT3-positive cells in the ARC of LFD and HFD (n = 3 per group). One of five series from each animal was analyzed. *, $P < 0.05$. E, Microphotographs of P-STAT3 IHC from saline (*left*) and leptin injected (*right*) LFD (*top*) and HFD (*bottom*) mice. Mice were administered leptin (5 mg/kg, ip) or vehicle and killed 30 min later. Images are representative matched sections at Bregma –1.8 mm, and each panel represents one animal. *Scale bars*, 200 μm.

we measured the mRNA of candidate genes such as SOCS-3 and PTP1B (phosphotyrosine phosphatase 1B) (38, 39), both known inhibitors of leptin signaling, as well as the leptin receptor (ObRb). The mRNA was quantified by RT-PCR using RNA isolated from microdissected ARC and VMH/DMH tissues from mice after 4 wk of LFD and HFD. There were no differences in the ARC or VMH/DMH in the mRNA of ObRb (Fig. 6B) or PTB1B (Fig. 6C) between the LFD and HFD groups. In contrast, SOCS-3 mRNA was significantly elevated in the ARC of HFD mice, whereas the VMH/DMH showed no differences (Fig. 6A). This interesting result is consistent with our finding of reduced leptin-induced STAT3 phosphorylation selectively in the ARC of HFD mice. Com-

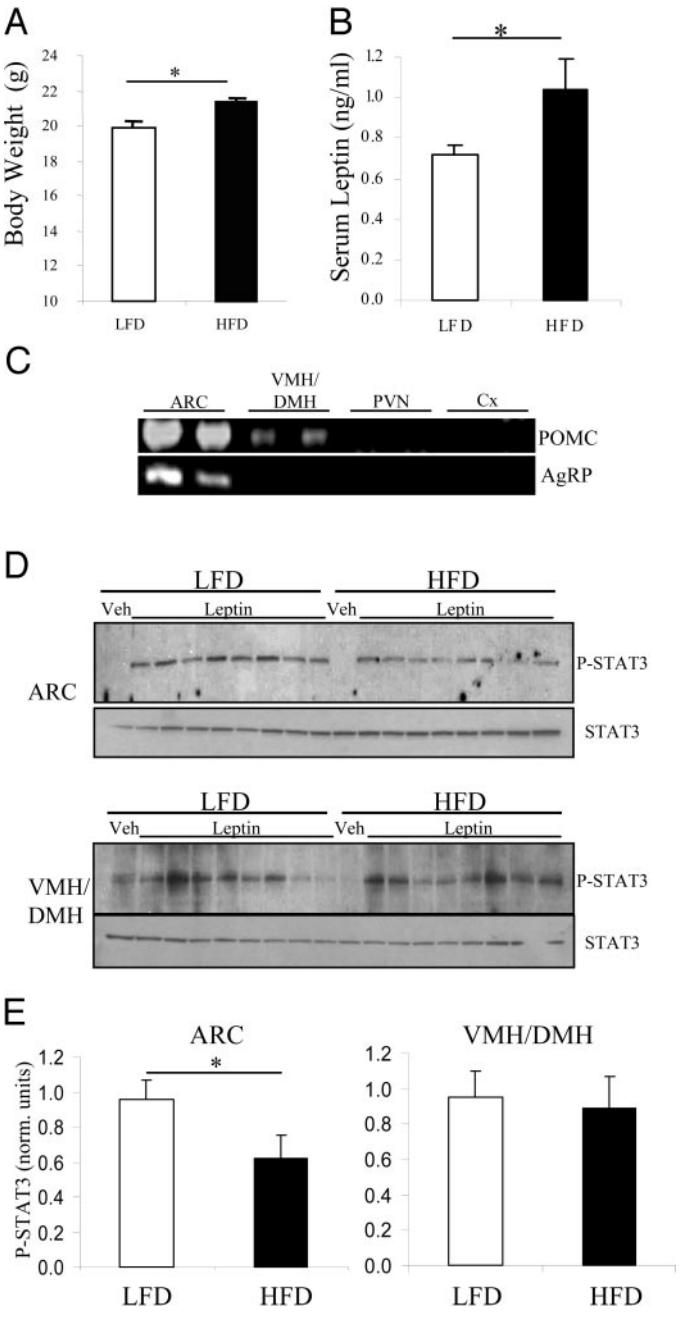

FIG. 5. Leptin-induced STAT3 phosphorylation is reduced in the ARC, but not in the VMH/DMH, after 6 d of HFD feeding. A, Body weights of mice given LFD or HFD for 6 d. *, $P < 0.002$ (n = 13 animals per group). B, Fasted serum leptin levels at 6 d of diets. *, $P < 0.03$ (n = 5) C, Microdissection of the ARC. Several areas of the brain were microdissected from two lean mice, and the insolated mRNA subjected to RT-PCR for POMC and AgRP mRNA as described in *Materials and Methods*. Cx, Cortex. D, Mice were given HFD or LFD for 6 d (from A) and then injected with vehicle (Veh) or leptin (5 mg/kg) and killed 60 min later. Shown are Western blots of P-STAT3 and total STAT3 in microdissected ARC and VMH/DMH tissue. Equal amounts of lysate were run on two separate gels and blotted for P-STAT3 and STAT3, respectively. Each lane represents one animal. E, Quantification of data from D. Levels of leptin-stimulated P-STAT3 in each sample from the LFD and HFD groups were normalized to the level of total STAT3 in the same tissue sample. Data are means ± SEM; *, $P < 0.04$.

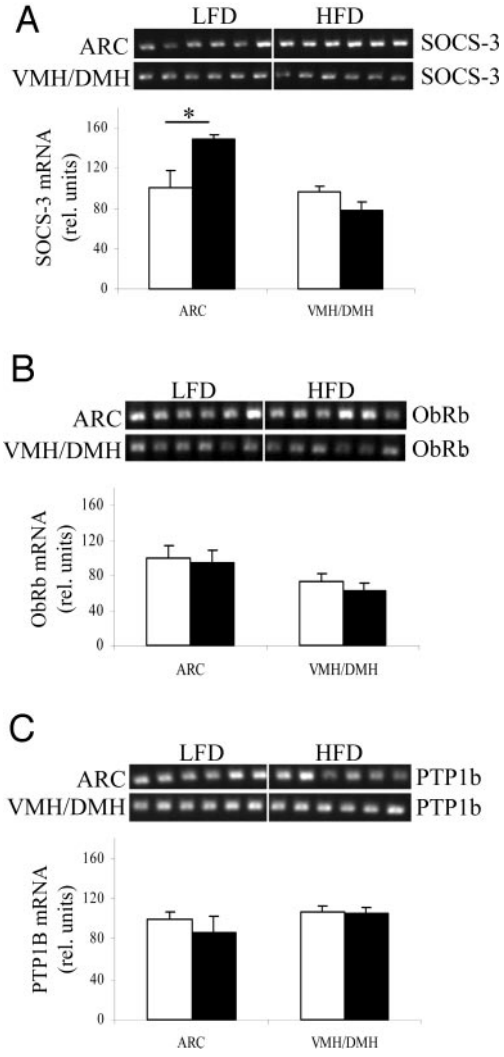

FIG. 6. SOCS-3 mRNA, but not ObRb and PTP1B mRNA, is specifically increased in the ARC of DIO mice. Mice were given LDF or HFD for 4 wk. Shown is semiquantitative RT-PCR for SOCS-3 (A), ObRb (B), and PTP1B (C) of mRNA isolated from microdissected ARC and VMH/DMH tissues from LFD and HFD mice. *, $P < 0.02$. Data are means ± SEM (n = 6 per group). *White bars* depict results from the LFD group and *black bars* the HFD group.

bined, these data suggest that increased SOCS-3 levels may cause defective leptin signaling in this brain region.

Discussion

Leptin resistance, defined as the presence of hyperleptinemia and a diminished response to the signaling or weight-reducing effects of recombinant leptin, exists in the majority of obese rodents (12, 25). Despite recent progress in the understanding of leptin signaling circuits in the brain, molecular mechanisms underlying the development of leptin resistance are still unknown.

One of the most widely used models of leptin resistance and human obesity is DIO in mice and rats. We have earlier reported that DIO mice are completely devoid of detectable STAT3 DNA-binding activity in the hypothalamus in response to peripheral injections of recombinant leptin (12).

Despite this severe leptin resistance, DIO mice become much less obese than *ob/ob* mice that completely lack leptin signaling. Because leptin acts on several brain nuclei both within and outside the hypothalamus (21), we hypothesized that some sites within the CNS may remain leptin sensitive in DIO mice.

Using first a sensitive IHC assay, we demonstrate here that the ARC of the hypothalamus has markedly reduced leptin signaling in mice given a HFD for 16 wk. In contrast, other hypothalamic or extrahypothalamic sites remain relatively sensitive to leptin-induced STAT3 phosphorylation. The apparent discrepancy between this finding and our earlier study where total hypothalamic leptin-dependent signaling was nearly ablated in DIO mice is likely because of the superior sensitivity of the IHC assay compared with the earlier gel-shift assay that was performed using whole hypothalamic extracts. On the other hand, based on cell counts of leptin-responsive cells, it is clear that the ARC accounts for the majority (~75%) of leptin-responsive cells in the hypothalamus. Thus, the marked suppression of signaling in the ARC would be consistent with our earlier finding that the hypothalamus as a whole has impaired leptin responsiveness in DIO mice (12).

To explore whether the defective signaling in the ARC seen after 16 wk of DIO might be detectable earlier during development of obesity, therefore supporting a possible primary defect, we measured responses to leptin in mice after 4 wk and 6 d on the HFD. At 4 wk, the number of leptin-induced P-STAT3 cells in the ARC was also diminished in the HFD group, although to a lesser degree (~10%) compared with 16 wk (~30%). Moreover, after only 6 d of HFD, when increased body weight is first clearly detectable, activation of STAT3 in the ARC is already impaired. This was determined by Western blotting of microdissected tissues and showed an approximate 40% reduction in STAT3 phosphorylation. This significant impairment is likely because the Western blotting analysis, compared with counting of positive cells by IHC, also measures partial decreases in STAT3 phosphorylation in individual cells. Because the stimulation of STAT3 phosphorylation is markedly attenuated in response to a large pharmacological dose of leptin after only 6 d of HFD, it is likely that the arcuate nucleus is severely leptin resistant to endogenous leptin and that this resistance is present even before the onset of obesity.

Based on the early onset of leptin-resistant STAT3 signaling in the ARC, we speculate that this region-specific defect in leptin action may play a causal role in the development of DIO, albeit this hypothesis clearly needs to be tested in direct studies. However, a significant body of evidence points to the ARC as a key site of leptin action to influence energy intake, thus indirectly supporting the hypothesis. For example, it has been shown that microinjection of small doses of leptin directly into the ARC result in reduced energy intake (14). Furthermore, chemical ablation of the ARC causes severe hyperphagia and obesity (40). Moreover, genetic studies demonstrate that 1) gene therapy delivering *ObRb* viruses into the ARC of rats that lack functional leptin receptors improves energy balance (41) and that 2) STAT3 in the brain is critical for leptin action to regulate energy homeostasis (9, 10). To ultimately prove that reduced leptin action selectively in the ARC is required for the development of DIO, studies of mice with genetically altered STAT3, SOCS-3, or ObRb expression specifically in this nucleus are required.

Our data suggest that leptin resistance in the ARC could be important for development of DIO, but the underlying mechanism causing decreased activation of STAT3 has remained unresolved. The finding that SOCS-3 is increased specifically in the ARC, but not in other regions of the hypothalamus, is exciting and opens the possibility of a causal relationship because we have earlier demonstrated that SOCS-3 is a potent inhibitor of leptin signaling (42). Other studies have not found elevated SOCS-3 in the hypothalamus in DIO animals (12, 43). This may relate to our data showing that the increase in SOCS-3 appears to be restricted to the ARC and that earlier studies have used whole hypothalamic tissue, which may have prevented detection of changes in the subregion. In further direct support of the hypothesis of a causal relationship between increased SOCS-3 and reduced leptin-stimulated STAT3 phosphorylation in obese mice are our recent data from studies of mice that are heterozygous for a *SOCS-3* gene deletion (44). SOCS-3 +/–mice exhibit enhanced activation of hypothalamic STAT3 phosphorylation by leptin. Moreover, these mice have increase sensitivity to the weight-reducing effects of leptin and are resistant to development of DIO. Thus, only a 50% reduction of SOCS-3 expression appears sufficient to both increase leptin action and to decrease sensitivity to a HFD. Furthermore, whereas heterozygous overexpression of ObRb selectively in neurons of *db/db* mice partly corrects several aspects of leptin-receptor deficiency, mice that are homozygous for the same leptin receptor transgene have almost full restoration of all defects, including food intake, fat accumulation, diabetes, and fertility (45). Combined, these results clearly suggest that small changes in central leptin receptor signaling results in significant alterations in regulation of energy balance and supports our hypothesis that only an approximately 50% increase of SOCS-3 in the ARC leads to reduced ObRb-STAT3 signaling and extra weight gain in HFD-fed mice.

Although we speculate that increased SOCS-3 results in decreased STAT3 phosphorylation by leptin in the ARC of DIO mice, this is based on the premise that SOCS-3 is indeed elevated in leptin-responsive neurons, which needs to be demonstrated. But if true, because SOCS-3 inhibits Janus kinase 2 (JAK2), a tyrosine kinase believed to be required for all known leptin-dependent signaling pathways (11), this might suggest that leptin's regulation of signaling proteins like ERK, insulin-receptor substrate, and phosphatidylinositol 3-kinase are also impaired in the ARC of DIO mice. Interestingly, SOCS-3 is also an inhibitor of insulin signaling (46–48), and insulin has been reported to require signaling proteins and pathways in the CNS that overlap with those of leptin (*e.g.* phosphatidylinositol 3-kinase) to influence energy balance (49). Because insulin may act on specific cells in the ARC that are also leptin responsive (50, 51), it is possible that the ARC of DIO mice is also resistant to insulin. Additional testing of this hypothesis is warranted and may be relevant in the pathogenesis of insulin-resistant type II diabetes, which is often associated with overweight.

The reason for the selective leptin resistance in the ARC, but not of other brain regions, is unclear. One interesting

feature of the ARC compared with the other regions examined here is its close proximity to the median eminence (ME), a circumventricular organ (52, 53). This at least raises the possibility that neurons in the ARC are not fully protected from all circulating factors by the BBB compared with other brain regions. We hypothesize that factor(s) present in the circulation of HFD-fed mice may gain access to the ARC via the ME and negatively influence leptin signaling in cells located in this nucleus. If such factor(s) are not easily transported across the BBB this might explain why other brain regions remain leptin sensitive in DIO mice. Alternatively, because there are clearly neurons in the brain that are protected by the BBB but also have terminal segments located in the ME, it is possible that segments of neurons in the ARC that extend into the ME have specific properties that makes them uniquely sensitive to certain circulating factors. Additional studies are needed to examine the cause of this selective leptin resistance in the ARC of DIO mice.

The chemical characteristics of leptin-resistant neurons in the ARC of DIO mice are unknown. However, specific neurons exist within the ARC that express leptin receptors, including neuropeptide Y, AgRP, POMC, and galanin-like-peptide neurons (1, 54). Based on the known anatomical location of these cells in the ARC of mice, the present data suggest that a significant proportion of these cells are resistant to leptin in DIO mice. Indeed, genetic deletion of the leptin receptor in POMC neurons demonstrates that leptin signaling in these cells is required for normal regulation of body weight homeostasis (54). Direct double-labeling studies of brain slices are, however, required to fully determine the chemical nature of all leptin-resistant cells in the ARC of DIO mice. Some, but not all, mRNA studies do indicate that these ARC neurons have diminished leptin responsiveness in DIO mice (34, 55).

Data show that DIO mice respond to leptin given intracerebroventricularly, but not peripherally, suggesting that transport of leptin into the brain is defective in obesity (12, 25). This is consistent with direct measurements demonstrating reduced whole-brain uptake in obese rodents (26). Other data suggest that defective signaling, rather than reduced leptin transport, precedes development of obesity (30). It is relatively clear that overtly obese mice do have reduced whole-brain leptin transport (56), but the importance of this result is unresolved. We did not detect reduced STAT3 activation by leptin in most brain regions after 4 or 16 wk of DIO, arguing against a global defect in leptin transport. The reason for this discrepancy is unclear but may relate to the fact that we have used pharmacological doses of leptin, and it cannot be ruled out that a lower amount of recombinant leptin would result in reduction of stimulated STAT3 phosphorylation in some brain regions of DIO mice in addition to the ARC. Although our study has not directly assessed the issue of reduced transport *vs.* signaling in DIO mice, the result showing an early impairment in STAT3 activation selectively in the ARC support the possibility of a signaling defect rather than a general deficiency in leptin transport across the BBB.

Interestingly, because some CNS sites retain leptin responsiveness in DIO mice, it is possible that a HFD induces region-specific leptin resistance in the CNS that allows some aspects of leptin action to be unaffected, although other processes are attenuated. For example, the high levels of leptin in obese mice do not result in suppression of appetite as would be expected in lean mice given the same level of circulating leptin. However, the leptin signals required for regulating autonomic function seems relatively intact in DIO mice. Hyperleptinemia could even contribute to increased sympathetic activity and arterial pressure in the obese state where there is resistance to the metabolic actions of leptin (57, 58). We speculate that selective leptin resistance to the anorexigenic actions of leptin may have been advantageous during evolution, thus allowing for rapid accumulation of energy at times of excess food availability and at the same time maintaining other critical leptin-dependent functions.

Recently, Ladyman *et al.* (59) reported that pregnancy in rats, a state of hyperleptinemia and hyperphagia, is associated with impaired responsiveness to the appetite-reducing effects of leptin given intracerebroventricularly, thus suggesting the presence of leptin resistance in the brain. Interestingly, using Western blotting, they found regional differences in leptin-inducible STAT3 phosphorylation between nonpregnant and pregnant animals, namely suppressed activation in the ARC and the VMH, but not in the DMH or lateral hypothalamus. In addition, selectively bred obesity-prone rats on a chow diet have reduced STAT3 activation in the ARC, VMH, and DMH, but not in the NTS, in response to peripherally administered leptin (30). The latter was examined using P-STAT3 IHC followed by counting of P-STAT3-immunoreactive cells. Thus, in additional models of obesity, region-specific leptin resistance can be detected. Moreover, both studies found that the ARC was resistant to leptin, which combined with our data suggest a key role of this nucleus in development of leptin-resistant obesity. However, different results were detected in the VMH and the DMH between the two studies and compared with the present study. The reason for this is unclear but may be because of variability in the microdissections or scoring of STAT3-positive cells. Alternatively, the different results could relate to the fact that different models were used.

In conclusion, these data show that leptin resistance at the level of STAT3 activation in brains of DIO mice is not global. Rather, leptin signaling in the ARC is severely decreased, whereas several other sites retain leptin responsiveness. This defect in leptin signaling may involve increased expression of SOCS-3. We speculate that defects in leptin signaling in neurons within the ARC may play a role in the pathogenesis of leptin-resistant obesity. Increasing leptin signaling in these cells may therefore ameliorate or prevent DIO in mice and human obesity. Important issues that remain include identification of the signal that stimulates SOCS-3 in the ARC of DIO mice. Furthermore, the mechanism by which the ARC specifically becomes leptin resistant in DIO has yet to be determined.

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