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Leptin Resistance of Adipocytes in Obesity: Role of Suppressors of Cytokine Signaling

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Liver-derived hyperleptinemia induced in normal rats by adenovirus-induced gene transfer causes rapid disappearance of body fat, whereas the endogenous adipocyte-derived hyperleptinemia of obesity does not. Here we induce liver-derived hyperleptinemia in rats with adipocyte-derived hyperleptinemia of acquired obesity caused by ventromedial hypothalamus lesioning (VMH rats) or by feeding 60% fat (DIO rats). Liver-derived hyperleptinemia in obese rats caused only a 5–7% loss of body weight, compared to a 13% loss in normoleptinemic lean animals; but in actual grams of weight lost there was no significant difference between obese and lean groups, suggesting that a subset of cells remain leptin-sensitive in obesity. mRNA and protein of a putative leptin-resistance factor, suppressor of cytokine signaling (SOCS)-1 or -3, were both increased in white adipose tissues (WAT) of VMH and DIO rats. Since transgenic overexpression of SOCS-3 in islets reduced the lipopenic effect of leptin by 75%, we conclude that the increased expression of SOCS-1 and -3 in WAT of rats with acquired obesity could have blocked leptin's lipopenic action in the leptin-resistant WAT population. © 2000 Academic Press

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The fact that obesity is sustained despite high plasma levels of leptin implies that leptin resistance is present in the common forms of the disorder (1). Since leptin has lipopenic activity in adipocytes and other tissues (2–4), it is remarkable that the adipocytes of obese animals can accumulate so much triacylglycerol (TG) despite the extremely high local (interstitial fluid) concentrations of the hormone that must surround these hypersecreting cells. There being no known abnormality in the leptin receptor of rats with diet-induced obesity (DIO), any resistance to leptin action is presumed to be at a postreceptor level. Several candidate postreceptor inhibitors of leptin action have already been identified. One of these, SH2-containing phosphatase 2 (SHP-2), inhibits STAT-3-mediated gene induction by dephosphorylating activated OB-R (5). A second inhibitor, named "protein inhibitor of activated STAT" (PIAS3), blocks the DNA-binding activity of STAT-3 (6). Finally, "suppressor of cytokine signaling" (SOCS) proteins (6) bind via their SH2 domain to activated Janus kinase (JAK) molecules and thus inhibit them from phosphorylating STAT-3. SOCS proteins are of particular interest because they may be part of a negative feedback loop that restricts the activity of the cytokine class of ligands (7), of which leptin is a member. The demonstration of an increase in SOCS-3 mRNA in the hypothalamus of *ob*/*ob* mice receiving leptin by peripheral administration (8) raises the possibility that the endogenous hyperleptinemia of acquired obesity also induces SOCS-mediated resistance to certain of its extrahypothalamic actions.

In striking contrast to the hyperleptinemia of obese rats, adenovirus-induced overexpression of the transgene in the liver (9) causes the disappearance of all detectable fat within 6 days and the phenotype of the fat-depleted adipocytes reverts to one resembling a preadipocyte (4). The mRNAs of leptin and of the lipogenic enzymes become virtually undetectable, as do the mRNAs of markers of mature adipocytes, such as aP_2 and TNF-α (4). The disappearance of leptin mRNA in adipocytes of rats with liver-derived hyperleptinemia indicates that ectopic leptin can suppress leptin expression in adipocytes, the cells that normally secrete it, and induce dedifferentiation (4).

In contrast to the foregoing expression profile induced in adipose tissue by liver-derived hyperleptinemia, in the endogenous adipocyte-derived hyperleptinemia of diet-induced obesity (DIO) both leptin and

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the lipogenic transcription factors and enzymes are expressed at high levels in adipose tissues, despite even higher hormone levels in the interstitium surrounding the cells. In other words the marked elevation of leptin surrounding obese adipocytes does not suppress the expression of either leptin or the lipogenic enzymes as does liver-derived leptin. This implies that, when adipocytes hypersecrete leptin, a coexpressed factor must be blocking the autosuppression of lipogenic adipocyte markers and the involution that occurs when hyperleptinemia is derived from nonadipocytes.

Flier's group has shown that SOCS-3 blocks phosphorylation of STAT-3 in CHO cells (10), making this family of cytokine signaling inhibitors a strong candidate for the role of a blocker of leptin autosuppression in adipocytes. In this study we determine if the expression levels in white adipose tissue of two members of the SOCS family, SOCS-3 and SOCS-1 are elevated in adipocyte-derived hyperleptinemia, thereby providing a possible explanation for the resistance to autosuppression. Two rat models of adipocyte-derived hyperleptinemia were employed, diet-induced obesity (DIO), and obesity induced by electrolytic lesioning of the ventromedial hypothalamus (VMH).

METHODS

Animal Groups

Rats with Hypothalamic Obesity

Ventromedial hypothalamus (VMH). VMH-lesioning was performed at the Department of Neuroscience, University of Florida (Gainesville, FL) in adult Sprague-Dawley (Harlan Sprague-Dawley, Indianapolis, IN) male rats weighing 250–300 g housed in air-conditioned rooms (22–25°C) with lights on from 0500–1900 h. Food and water were available *ad libitum*. Rats were anesthetized with 50 mg/kg of sodium pentobarbital (Abbott, North Chicago, IL) administered intraperitoneally. The VMH was destroyed bilaterally by the electrolytic lesion procedure as previously described (11). Briefly, rats were placed in the stereotaxic instrument and positioned with the nose bar set 3.3 mm below the interaural line. The electrode, consisting of an insulated stainless steel insect pin with exposed tip, was positioned 2.6 mm behind the bregma, 0.6 mm lateral to the midline and lowered to the base of the brain and then raised 0.5 mm. A direct anodal current of 2.5 mA for 15 s was passed through the electrode aimed at the VMH with a rectal electrode serving as cathode. For sham controls, the same surgical procedures were performed except that no current was passed. Rats were allowed to recover for approximately one week. They were then flown to Dallas for further studies. The foregoing procedure was approved by the University of Florida Institutional Animal Care and Use Committee, which follow the NIH Guide for the Care and use of laboratory animals.

Rats with Diet-Induced Obesity (DIO)

Sprague-Dawley rats purchased from Charles River Laboratories (Raleigh, NC) were housed in individual metabolic cages with constant temperature and 12 h of light, 12 h of darkness. Four-week-old rats were given either their usual diet of 50 g/d of standard chow (Teklad FG Rodent diet, Harlan, Teklad, Madison WI), which contains 24.8% protein, 4% fat; (3.94 Kcal/g) or a high fat diet (Teklad FG Rodent diet, Harlan Teklad, Madison WI), which contains 60% fat, 7.5% carbohydrate, 24.5% protein; 6.7 Kcal/g for 8 weeks.

Treatment with Recombinant Adenovirus Containing the Leptin cDNA (AdCMV-leptin)

Recombinant adenovirus containing the rat leptin cDNA under control of the CMV promoter (AdCMV-leptin) was prepared and administered intravenously in a dose of 10^{12} plaque-forming units as previously described (9). As a control, virus containing the bacterial-β-galactosidase gene (AdCMV-β-gal) was administered to a second group of rats (9). Control rats were diet-matched to AdCMV-leptin-treated rats.

Construction and Transfer of the SOCS-3 cDNA (AdCMV-SOCS-3) in Pancreatic Islets

To construct AdCMV-SOCS-3 total RNA was collected from the hypothalamus of lean Zucker +/+ rats. Using a TA cloning Kit, a Hind III/Xba I cut 703-bp fragment was isolated and inserted into pACCMV.pLpA vector and verified by sequence analysis. The preparation of a recombinant adenovirus containing the SOCS-3 cDNA under control of a CMV promoter (AdCMV-SOCS-3) was carried out as previously described (12).

Pancreata of 11-week-old wild-type, lean male Zucker Diabetic fatty (ZDF) +/+ rats were perfused with 1×10^{12} plaque-forming units of recombinant adenovirus containing the cDNA of SOCS-3 (AdCMV-SOCS-3) in Krebs-Ringer bicarbonate buffer with 3.5% Dextran T70, 1% BSA, 5.6 mM glucose and 5 mM each of sodium pyruvate, sodium glutamate, and sodium fumarate. Islets were then isolated and maintained in suspension culture in 60-mm petri dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, as described previously (12). The culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum, 1% of penicillin and streptomycin, and 8 mM glucose.

Measurement of Triacylglycerol Content of Perfused Pancreatic Islets

Triacylglycerol (TG) content of islets was measured with a Sigma Diagnostic Kit (Sigma, St. Louis, MO) as previously described (13).

Leptin Measurements

Blood samples were collected from the tail vein in capillary tubes coated with ethylenediamine tetraacetic acid. Plasma was stored at −20°C. Plasma leptin was assayed using the Linco leptin assay kit (Linco Research, St. Charles, MO).

Semiquantification of mRNA by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Expression of SOCS-1, SOCS-3, CPT-1, and ACO-1 in adipose tissue and perfused islets was semiquantified by RT-PCR. The linear range of PCR was found to be between 22 and 35 cycles. Samples were amplified for 28–30 cycles using previously described parameters (4). The primers are shown in Table 1.

SOCS-1 and SOCS-3 Immunoprecipitation

Using the method of Lassar *et al.* (14), 100 μg of protein was precipitated with 1:50 goat-anti-SOCS-1 and anti-SOCS-3 from Santa Cruz Biotechnology, Inc., CA. The same antibodies were used for immunoblotting at ratio of 1:1500.

TABLE 1

Sequences of PCR Primers Used in Study

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	Internal primer (5′–3′)	GenBank Accession No.
β-Actin	TTGTAACCAACTGGGACGATATGG	GATCTTGATCTTCATGGTGCTAGG	GGTCAGGATCTTCAT GAGGTAGTCTGTCAG	J00691
ACO	GCCCTCAGCTATGGTATTAC	AGGAACTGCTCTCACAATGC	GCCTGCACTTTCTTC AGCCATCTTCAACGA	J02752
CPT-1	TATGTGAGGATGCTGCTTC	CTCGGAGAGCTAAGCTTGTC	ACTCTGGTTGGAATC TGACTGGGTGGGATT	L07736
SOCS-1	CCTCGAGTAGGATGGTAGCA	CGGCAGCCGGTCAGATCTGG	CTCCTGGACGCCTGCG GCTTCTACTGGGGA	AJ243123
SOCS-3	CCATGGTCACCCACAGCAAG	CTCTGACCCTTTCTTTGCTC	ACCAGCGCCACTTCT TCACADTGAGCGTCG	AF075383

Statistical Analyses

All values shown are expressed as mean ± SEM. Statistical analysis was performed by two-tailed unpaired Student's *t* test with unequal variance.

RESULTS

Effect of liver-derived hyperleptinemia on body fat in hypothalamic and diet-induced obesity. The infusion of AdCMV-leptin causes leptin overexpression in the liver and marked hyperleptinemia. In normal rats this liver-derived leptin excess results in rapid and profound depletion of body fat (4, 9). To determine if rats with adipocyte-derived hyperleptinemia resulting from acquired obesity are less sensitive to the effects of liver-derived hyperleptinemia on body fat, we studied two models of acquired obesity, one caused by electrolytic lesions of the ventromedial nucleus of the hypothalamus (VMH) and the other with diet-induced obesity (DIO) caused by high fat feeding. We employed adenoviral gene transfer in both groups of rats to produce liver-derived hyperleptinemia.

Before infusing the adenoviral construct containing the leptin cDNA (AdCMV-leptin), plasma leptin levels in VMH rats averaged 18.5 ± 5.3 ng/ml, and rose to 41 ± 12 ng/ml one week after the infusion of virus. In rats with sham VMH lesions plasma leptin averaged 1.2 ± 0.5 ng/ml before and 33 ± 14 ng/ml after. The lean sham VMH group lost 37 g or 13.4% of their body weight, while the obese VMH group lost 17 g, which was only 5.2% of their body weight (Table 2A). The

TABLE 2

Effects of Treatment with AdCMV-Leptin or AdCMV-β-Gal on Plasma Leptin (ng/ml) and Body Weight (g) of Rats with Acquired Obesity

A. Ventromedial hypothalamic (VMH) lesions or sham controls

		VMH + AdCMV-Leptin (n = 6)	VMH + AdCMV-β-Gal (n = 6)	Sham + AdCMV-Leptin (n = 6)	Sham + AdCMV-β-Gal (n = 6)
Body weight (g)	Before virus	330 ± 10	332 ± 11	277 ± 8	283 ± 8
	1-wk after virus	313 ± 6 (−5%)	325 ± 8 (−2%)	240 ± 11 (−13.4%)	274 ± 7 (−3%)
Plasma leptin (ng/ml)	Before virus	18.5 ± 5.3	15.7 ± 6.4	1.2 ± 0.5	1.0 ± 0.3
	1-wk after virus	40.6 ± 11.5	19.8 ± 6.9	32.5 ± 14	0.9 ± 0.2
Δ body weight (g)/Δ leptin (ng)		0.8	—	1.2	—

B. DIO rats fed a 60% fat diet or 4% fat-fed controls

		DIO + AdCMV-Leptin (n = 6)	DIO + AdCMV-β-Gal (n = 6)	4% Fat + AdCMV-Leptin (n = 6)	4% Fat + AdCMV-β-Gal (n = 6)
Body weight (g)	Before virus	472 ± 10	407 ± 14	304 ± 11	312 ± 9
	1-wk after virus	438 ± 6 (−7.2%)	398 ± 8 (−2%)	264 ± 11 (−13%)	302 ± 7 (−3%)
Plasma leptin (ng/ml)	Before virus	10.7 ± 5.1	11.4 ± 6.4	1.6 ± 0.6	1.5 ± 0.3
	1-wk after virus	42 ± 8.4	13.8 ± 4.5	37 ± 10	1.4 ± 0.3
Δ body weight (g)/Δ leptin (ng)		1.1	—	1.1	—

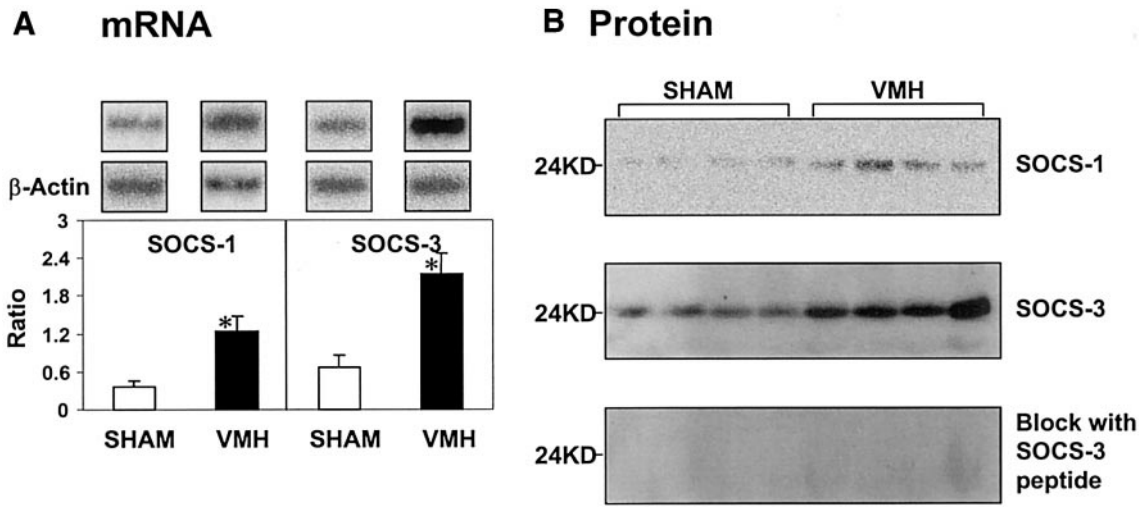

FIG. 1. SOCS-1 and SOCS-3 expression in adipocytes of rats with acquired obesity resulting from lesioning of the ventromedial nucleus of the hypothalamus (VMH). (A) SOCS-1 and SOCS-3 mRNA in epididymal white adipose tissue of obese VMH rats and sham-lesioned controls (n = 6). (B) SOCS-1 and SOCS-3 protein in white adipose tissues of obese VMH rats and sham-lesioned controls (n = 6). Representative blots are shown.

decline in food intake in the VMH group was only half of that of the sham VMH controls (data not shown). Weight loss in g per ng/ml of incremental plasma leptin, an index of the weight-lowering activity of the hormone, was 0.8 g/ng · ml^{-1} compared to 1.2 g/ng · ml^{-1} in the sham-lesioned group (Table 2A).

In rats with DIO leptin levels averaged 11 ± 5 ng/ml before AdCMV-leptin infusion and had increased to 42 ± 8 ng/ml at 7 days after the treatment. The reduction in food intake in the obese group was only 50% of that of the lean controls (data not shown). At that time body weight had declined by 34 g or 7.2% in the obese group. In the lean controls body weight declined by 40 g or 13% (Table 2B). Weight loss in grams per ng/ml of incremental plasma leptin, an index of leptin's weight-lowering activity, was identical in the obese DIO group and lean controls (Table 2B).

Adipocyte SOCS expression in adipocyte-derived hyperleptinemia of hypothalamic obesity and diet-induced obesity. To determine if the uneven response of body fat to liver-derived hyperleptinemia in VMH and DIO rats might have been the result of SOCS-mediated postreceptor resistance of some adipocytes to the hyperleptinemia, we compared SOCS-1 and SOCS-3 mRNA and protein in the epididymal adipose tissue of VMH-lesioned with their sham-lesioned control rats. The results are shown in Fig. 1A. SOCS-1 mRNA was 3.4-fold higher and SOCS-3 mRNA 3.2-fold higher in the epididymal fat tissue of the VMH-lesioned rats than in that of sham VMH controls. Immunoblots indicated that SOCS-1 and SOCS-3 proteins were also higher (Fig. 1B). The specificity of the immunoblots was validated by the complete absence of signal after preincubating the anti-SOCS-3 antibody with a peptide containing the SOCS-3 epitope (Fig. 1B).

To determine if the relative reduction in the response to leptin in DIO might also be associated with increased SOCS expression in adipocytes, we compared SOCS-1 and SOCS-3 mRNA and protein in the epididymal fat of Sprague Dawley rats with DIO. After 8 weeks of high fat feeding, their plasma leptin averaged 10.7 ± 5.1. SOCS-1 mRNA was 3.7-fold higher and SOCS-3 3.7-fold higher in the epididymal fat of the DIO rats than in control fat tissue (Fig. 2A). SOCS-1 and SOCS-3 protein were also increased compared with nonobese littermates on a normal diet (Fig. 2B).

Effect of SOCS-3 overexpression in normal tissues on leptin-induced lipid depletion. SOCS-3 has been shown to block leptin-mediated phosphorylation of STAT-3 in CHO cells (10), but its ability to abolish a downstream biologic effect, such as its lipopenic action (2–4), has not been demonstrated. Leptin has powerful lipopenic activity in both adipocytes (4) and nonadipocytes (2). To determine if SOCS-3 can block the lipopenic action of leptin, we first attempted to overexpress it in adipocytes of normal rats by means of adenovirus gene transfer, but were unsuccessful for technical reasons. We therefore employed pancreatic islets as the test tissue, since earlier work had demonstrated that leptin in high concentrations dramatically reduces their TG content (2), as it does in adipocytes (4). β-galactosidase was overexpressed in some batches of islets to serve as a control. SOCS-3 mRNA (Fig. 3A) and protein (not shown) were increased in the AdCMV-SOCS-3-treated islets compared to the β-gal-overexpressing control islets, evidence that the maneuver had been successful (Fig. 3A).

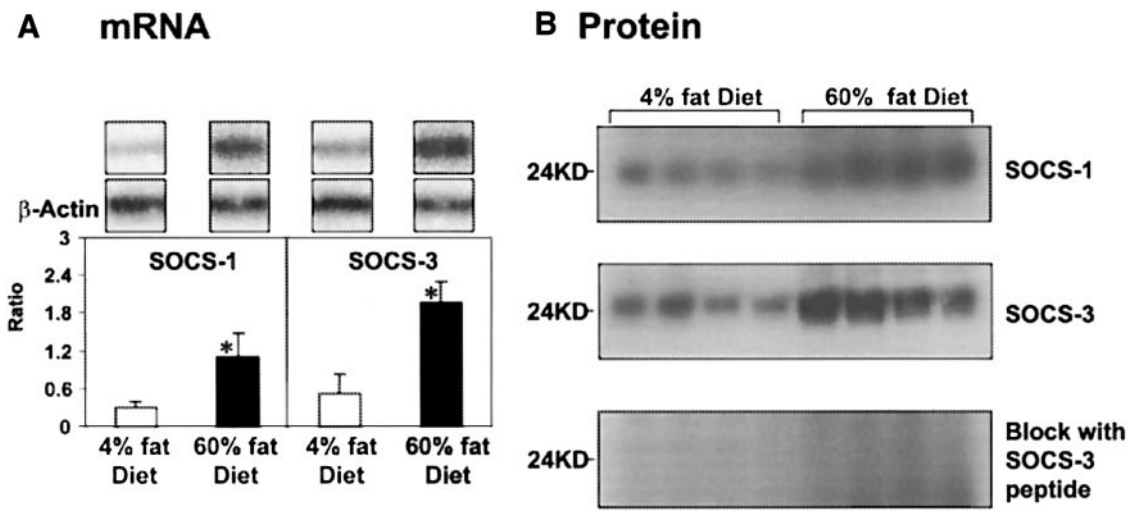

FIG. 2. (A) SOCS-1 and SOCS-3 mRNA in epididymal white adipose tissues of rats with diet-induced obesity (60% fat diet) or of lean controls (4% fat diet) ($n = 6$). (B) SOCS-1 and SOCS-3 protein in epididymal white adipose tissue of normal rats on a diet containing 4% fat and in rats with diet-induced obesity resulting from a diet containing 60% fat ($n = 6$).

The incubation of both groups of islets in 20 ng/ml of recombinant leptin caused an 85% reduction in TG content in the β-gal-overexpressing control islets ($P < 0.01$), but only a 22% decline ($P > 0.05$) in SOCS-3-overexpressing islets (Fig. 3B).

Since the lipopenic effect of leptin has been ascribed to an increased expression of the enzymes of fatty acid oxidation (4), for further validation of these results we measured the mRNA of acyl CoA oxidase (ACO) (Fig. 3C) and carnitine palmitoyl transferase-1 (CPT-1) in the two groups of islets cultured in 20 ng/ml of leptin (Fig. 3D). A highly significant ($P < 0.01$) increase in both ACO and CPT-1 mRNA was observed in leptin-cultured islets overexpressing β-gal, while neither was increased by leptin in the SOCS-3 overexpressing islets ($P > 0.05$).

DISCUSSION

In this study we have attempted to determine the mechanism by which some white adipocytes of rats with nongenetic obesity appear to resist both the lipopenic and involutional effects of their own adipocyte-derived hyperleptinemia, in contrast to the response of lean rats to liver-derived hyperleptinemia which causes marked loss of TG and reversion to a phenotype resembling preadipocytes (4, 9).

It makes teleological sense to suggest that in order for endocrine cells to meet an increased physiologic demand for a high rate of secretion, they must somehow block autosuppression by their own secretory products. For example, even though the concentration of insulin secreted into the pericapillary space surrounding the pancreatic β-cells is at least 1000-fold higher than that in the systemic circulation (15), β-cells maintain a very high level of β-cell-derived insulin secretion; and yet, comparable hyperinsulinemia from an exogenous insulin source profoundly suppresses β-cell secretion (16). The findings with respect to leptin hypersecretion are similar to insulin secretion. In obesity the interstitial concentration of leptin surrounding adipocytes must be extremely high and yet the level of leptin expression is increased. The implication is that endocrine cells must have a means of preventing autosuppression by their secretory products in order to maintain a high output.

One of several mechanisms by which autosuppression of an endocrine cell could be blocked is by coexpression of a factor that interferes with the autosuppressive signal transduced by that secretory product. SOCS-3 has been shown by Bjorbaek *et al.* (10) to block the action of leptin on STAT-3 phosphorylation, a principal pathway of leptin signal transduction, and here we demonstrate in pancreatic islets that a major biologic action of leptin, TG-lowering, is largely prevented by overexpression of this suppressor of cytokine signaling. The demonstration that SOCS proteins in adipocytes are increased in two models of acquired obesity with adipocyte-derived hyperleptinemia is consistent with a role in their resistance to autosuppression by leptin.

A surprising feature of the response of diet-induced obesity to the liver-derived hyperleptinemia was the fact that the absolute loss of body weight in grams was substantial in obese rats, and yet was small in terms of total weight. In an earlier study of DIO rats receiving 60% fat, the loss of body fat was quantified by magnetic resonance spectroscopy and did not differ in absolute terms from age-matched lean controls on a 4% fat diet (11). Thus, only when weight loss is calculated as per-

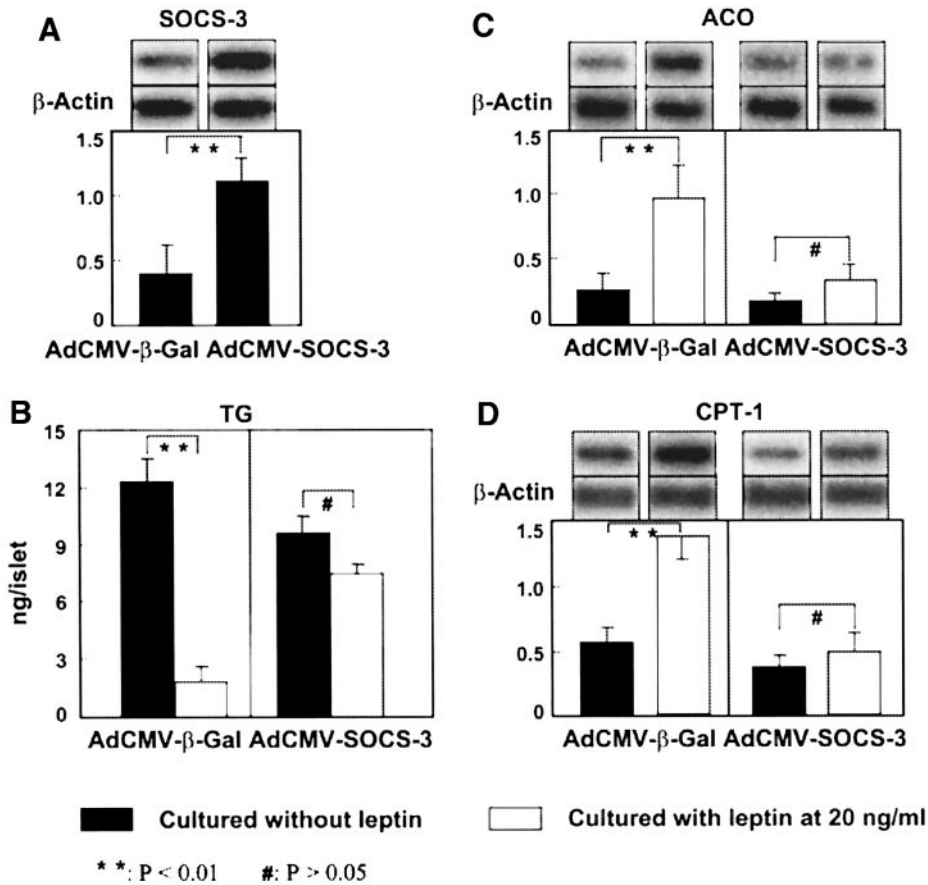

FIG. 3. Effect of adenovirus-induced overexpression of SOCS-3 or β-galactosidase in pancreatic islets of normal rats on their response to the lipopenic action of recombinant leptin. (A) The level of SOCS-3 mRNA in islets treated with AdCMV-β-gal or with AdCMV-SOCS-3. (B) Triacylglycerol (TG) content of AdCMV-β-gal or AdCMV-SOCS-3-treated islets after incubation with 20 ng/ml of recombinant leptin. (C) Acyl CoA oxidase (ACO) mRNA in the foregoing islets after incubation with leptin. (D) Carnitine palmitoyl transferase-1 (CPT-1) mRNA in these islets after incubation with leptin. $n = 6$.

cent of body weight, are obese rats clearly less sensitive than lean normoleptinemic rats to the fat-depleting action of the two- to fourfold elevation in liver-derived hyperleptinemia. One possible explanation for this observation is that in acquired obesity some adipocytes, perhaps those present prior to the onset of obesity, remain normally sensitive to leptin, while most adipocytes are not. The possibility of adipocyte heterogeneity will require further study.

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