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ORIGINAL ARTICLE

Inhibition of SOCS-3 in adipocytes of rats with diet-induced obesity increases leptin-mediated fatty acid oxidation

Hailun Gu · Li Liu · Shuang Ma · Yali Liu · Yahao Ren · Lingling Zhai · Fei Yu · Li An · Jun Yang

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Abstract Rats with diet-induced obesity (DIO) usually experience hyperleptinemia. Thus, leptin produced by adipocytes does not deplete adipocyte fat, which implying a leptin resistance in adipocytes during overnutrition. Here, we induced hyperleptinemia in rats by feeding them a diet consisting of 45% fat. In epididymal adipose tissues, the mRNA and protein levels of a putative leptin resistant factor, suppressor of cytokine signaling 3 (SOCS-3), were increased. The mRNA levels of SOCS-3 in adipocytes differentiated from adipose-derived stromal cells (ADSCs) were higher in DIO rats than in rats on a 10% fat diet. Using SOCS-3 short hairpin RNA lentivirus interference, we found decreased expression of acetyl-CoA carboxylase mRNA (a marker of de novo lipogenesis) and increased expression of acetyl-CoA oxidase mRNA (a marker of fat oxidation) in SOCS-3-knockdown adipocytes after incubation with 50 nM leptin for 6 h. We conclude that the SOCS-3 knockdown may have increased the leptin-mediated in situ fatty acid oxidation in the DIO adipocytes, and therefore, SOCS-3 might be an excellent target for therapeutic intervention for obesity.

Keywords Suppressor of cytokine signaling 3 · Diet-induced obesity · Adipose-derived stromal cells · Leptin · Fatty acid metabolism

H. Gu
Department of Orthopaedics, Shengjing Hospital, China Medical University, Shenyang 110004, People's Republic of China

L. Liu (✉) · S. Ma · Y. Ren · F. Yu · L. An · J. Yang
Department of Nutrition and Food Hygiene, School of Public Health, China Medical University, No. 92 Beier Road, Heping District, Shenyang, Liaoning Province 110001, People's Republic of China
e-mail: liuli@mail.cmu.edu.cn

Y. Liu
College of Profession and Technology, Liaoning University of Traditional Chinese Medicine, Shenyang 110032, People's Republic of China

L. Zhai
Department of Child and Adolescent Health, School of Public Health, China Medical University, Shenyang 110001, People's Republic of China

Introduction

Obesity has become a global epidemic that is associated profoundly with coronary artery disease, hypertension, and type 2 diabetes mellitus. It is well known that the majority of obese people and rodents exhibit leptin resistance [1], which refers to the presence of hyperleptinemia and a diminished response to the signaling or weight-reducing effects of recombinant leptin [2–4]. Excessive expression of the suppressor of the postreceptor in the leptin signal transduction pathway in central and peripheral tissues is associated closely with their leptin resistance. Studies have demonstrated that the expression of suppressor of cytokine signaling 3 (SOCS-3), a negative regulator of leptin signal, can be induced by leptin, which in turn inhibits leptin signaling. RNA interference-mediated knockdown of SOCS-3 restored leptin signaling [5]. These findings suggest that SOCS-3 may play a role in regulating leptin sensitivity [6–8].

The peripheral effects of leptin, such as those found in white adipose tissues, may also help regulate body weight [9]. Leptin has direct lipolytic effects on

denervated fat pads in vivo [10], on white adipose tissues [11] and on adipocytes in vitro [12, 13]. It might be directly involved in the regulation of adipocyte metabolism [14]. Leptin functions not only in centrally mediated pathways, but also acts peripherally [12], demonstrating a potential direct paracrine or autocrine effect of leptin on adipocytes. Huan et al. [9] demonstrated that the control of body weight by leptin requires not only its actions in the hypothalamus, but also distinct autocrine/paracrine actions in the fat tissue. This finding suggests that leptin resistance at the adipocyte level might be a molecular link between obesity and type 2 diabetes. Therefore, in the obese state, adipocytes that are leptin resistant, together with the diminished response to the central signaling of leptin, promote excessive fat storage in the body.

In this study, we investigated whether leptin resistance is generated in adipocytes of rats with diet-induced obesity (DIO). We also examined whether the inhibition of SOCS-3 in adipocytes of DIO rats might lessen the blockade of lipolysis by leptin and whether the effect can be reflected in a reduction in lipogenic enzymes and an increase in fatty acid oxidation enzymes.

Results

Male Wistar rats with DIO exhibit modest leptin resistance

Male Wistar rats were fed a diet containing either 45% or 10% kcal from fat for 8 weeks. At the end of 8 weeks, one-third of the rats fed a high-fat diet (10 of 30 rats total) became obese and were identified as having DIO [15], while other 20 nonobese rats were not used in this study. In DIO rats ($n = 10$), body weight gain was 25% higher than that of the rats ($n = 5$) in control-1 group that received a 10% fat diet (Fig. 1a), consistent with the difference in the average food intake between the DIO and control-1 groups (106.7 ± 3.4 kcal/day versus 82.4 ± 1.8 kcal/day). The weights of mesenteric, retroperitoneal, and epididymal fat pads in DIO-1 group ($n = 5$) were all significantly higher than those of the control-1 group (Fig. 1b). Serum triglyceride and total cholesterol in DIO-1 group were 60% and 33% higher than those of control-1 group, respectively (Fig. 1c). The levels of serum leptin (1.30 ± 0.07 ng/ml in DIO-1 group versus 0.61 ± 0.03 ng/ml in control-1 group) were positively correlated with body weight gain

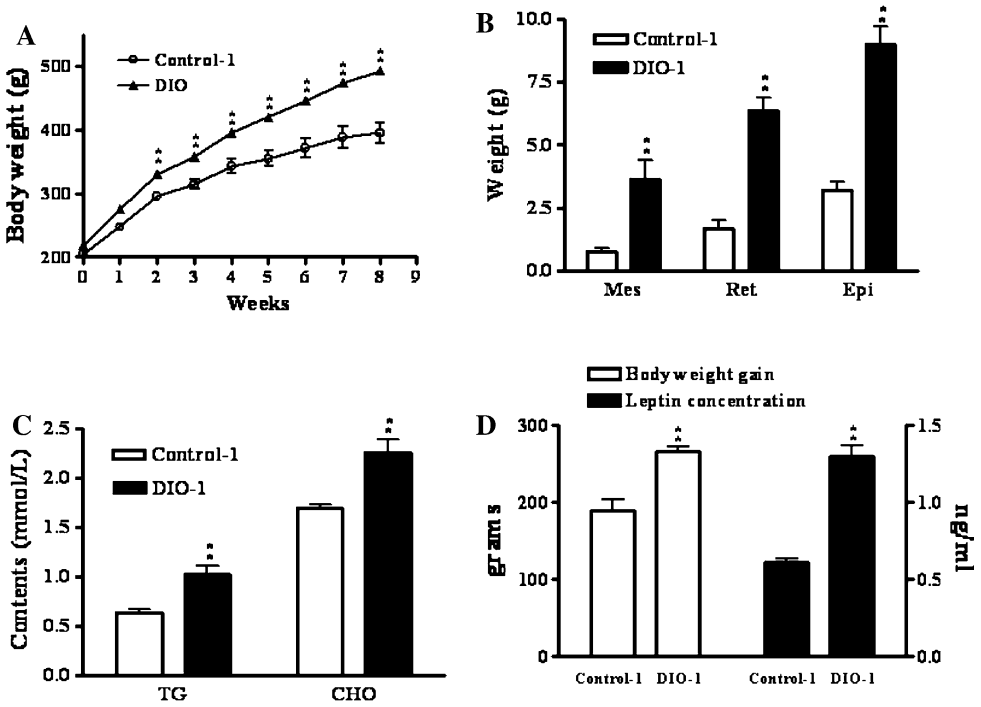

Fig. 1 Effect of high-fat feeding on body weight and serum leptin levels in male Wistar rats. **a** Body weights of control-1 rats ($n = 5$) fed a 10% fat diet and all diet-induced obesity (DIO) rats ($n = 10$) fed a 45% fat diet for the times indicated. **b** Mesenteric (Mes), retroperitoneal (Ret), and epididymal (Epi) fat pads were dissected from control-1 ($n = 5$) and DIO-1 rats ($n = 5$) at the end of 8 weeks. **c** Serum triglyceride (TG) and total cholesterol (CHO) levels of control-1 and DIO-1 rats ($n = 5$) at the end of 8 weeks. **d** Serum leptin level and body weight gain of control-1 and DIO-1 rats ($n = 5$) at the end of 8 weeks. Body weight gain was equal to the body weight difference between the end of the 8th week and the origin. All data are expressed as means ± SE; ** $P < 0.01$ versus control-1 group (Student's *t*-test)

(266.9 ± 5.1 g in DIO-1 group versus 190.2 ± 14.1 g in control-1 group) (Fig. 1d), indicating that rats with DIO exhibited modest leptin resistance.

SOCS-3 mRNA and protein are elevated in fat depots of DIO rats

To assess the potential relevance of SOCS-3 in mediating leptin resistance, we measured SOCS-3 mRNA using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and SOCS-3 protein using western blot analysis in epididymal fat depots. After 8 weeks of high-fat feeding, SOCS-3 mRNA was increased in epididymal fat pads in DIO-1 rats compared with control-1 rats (Fig. 2a). SOCS-3 protein was also elevated in DIO-1 rats compared with rats on a normal diet (Fig. 2b).

SOCS-3 mRNA levels are elevated in adipocytes differentiated from ADSCs of rats with DIO

To observe whether SOCS-3 levels were elevated in adipocytes differentiated from adipose-derived stromal cells (ADSCs) of rats with DIO, we measured SOCS-3 mRNA and protein expression using RT-PCR. As shown in Fig. 3a, the adipogenic differentiation rate of ADSCs was 60–70% as identified by oil red O staining. As shown in Fig. 3b, the SOCS-3 mRNA levels were significantly higher in adipocytes differentiated from ADSCs of rats in DIO-2 group than in control-2 group (~1.3-fold), while SOCS-3 protein levels were slightly increased but there was no significant difference between control-2 and DIO-2 groups.

Establishment of a SOCS-3-knockdown adipocyte model

To determine whether SOCS-3 knockdown can lighten the blockade of leptin action in adipocytes, we employed ADSCs as test cells to explore the effect of SOCS-3 knockdown in DIO adipocytes. The infection rate of differentiated adipocytes was 70–80% (Fig. 3c). Semi-quantitative RT-PCR and western blot analysis showed that SOCS-3 mRNA and protein were effectively inhibited by Lv-SOCS-3 by 70% and 60%, respectively, when compared with the Lv-SOCS-3-NC infection (Fig. 3d).

Effect of SOCS-3 knockdown on DIO adipocytes

To evaluate whether SOCS-3 knockdown in DIO adipocytes had an effect on fatty acid metabolism and whether leptin's anti-lipogenic action could be recovered, we measured acetyl-CoA carboxylase (ACC) and acetyl-CoA oxidase (ACO) expression in cells incubated in the absence or presence of rat recombinant leptin. Since many studies have reported that leptin can induce SOCS-3 expression [16], we first observed the effect of leptin on SOCS-3 levels in cells from rats with DIO without infection (untreated cells) or cells infected with Lv-SOCS-3 (KD) and Lv-SOCS-3-NC (NC), respectively. As shown in Fig. 4a, incubation of adipocytes with 50 nM leptin for 6 h did not modify the basal SOCS-3 mRNA levels in any of the groups. However, as shown in Fig. 4b, the expression of ACC mRNA in the KD cells with leptin incubation decreased significantly compared with that without leptin

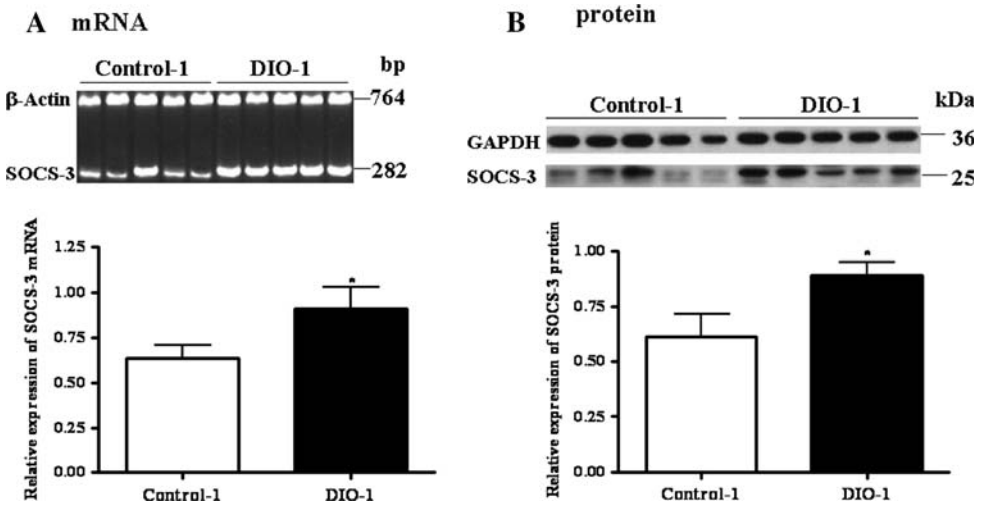

Fig. 2 Effect of 8 weeks of high-fat feeding on the expression of SOCS-3 mRNA and protein in epididymal adipose tissues. **a** The relative expression of SOCS-3 mRNA in control-1 rats on low-fat diet and DIO-1 rats on high-fat diet measured by semi-quantitative RT-PCR. **b** SOCS-3 protein blots of control-1 and DIO-1 rats measured by western blot. All data were analyzed using Scion Image 4.0 software and are expressed as means ± SE ($n = 5$), * $P < 0.05$ versus control-1 group (Student's t-test). Representative images are shown

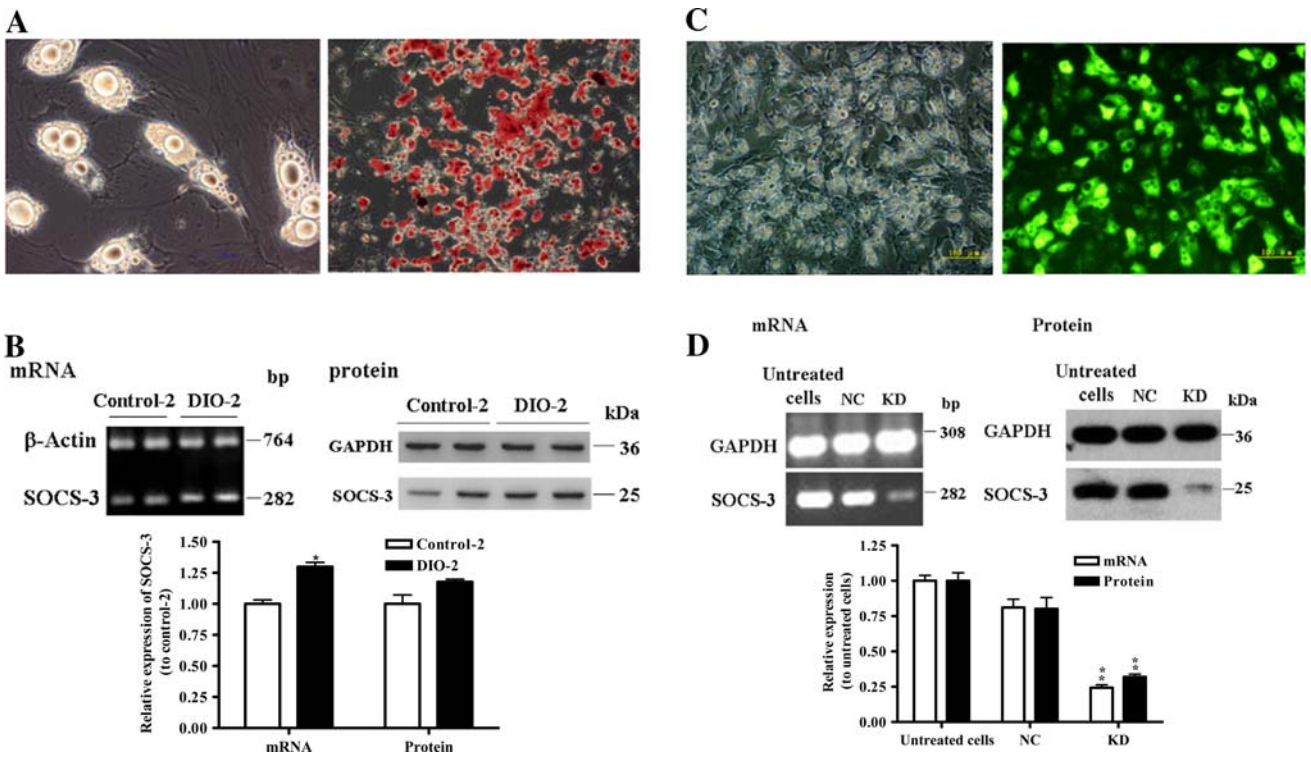

Fig. 3 Establishment of a SOCS-3-knockdown adipocyte model. **a** Differentiated adipocytes from ADSCs (microscopic images 400×) with oil red O staining (microscopic images 100×). ADSCs were cultured in the differentiation medium for 10 days. On day 10, the lipid accumulation in the cells was evaluated by oil red O staining. ADSCs were generated and differentiated as described under "Materials and methods" section. **b** SOCS-3 mRNA was elevated in adipocytes differentiated from ADSCs of rats with DIO. At day 10 of differentiation, adipocytes from control-2 rats on a 10% fat diet ($n = 3$) and DIO-2 rats ($n = 5$) were washed twice with PBS (pH 7.4), and SOCS-3 mRNA and protein levels were determined by RT-PCR and western blot, respectively. * $P < 0.05$ versus control-2 group. **c** Lv-SOCS-3 infected differentiated adipocytes. At day 10 of differentiation, adipocytes were infected with Lv-SOCS-3 (MOI = 50); 24 h after infection, the medium was replaced with fresh DMEM/F12 complete medium, and the incubation was continued for another 72 h before the infection rate was observed by fluorescence microscopy. (Fluorescence field and light field, respectively, microscopic images 200×). **d** SOCS-3 mRNA and protein levels were decreased in SOCS-3-knockdown adipocytes (Lv-SOCS-3 infection, KD). All data are expressed as means ± SE ($n = 5$); ** $P < 0.01$ versus untreated cells (no lentivirus infection) and cells with negative control shRNA lentivirus infection (Lv-SOCS-3-NC infection, NC) (one-way analysis of variance). The image is representative of three similar experiments

incubation. Additionally, after leptin incubation, the ACC mRNA levels in the KD cells were much lower than that in the untreated cells. On the other hand, ACO mRNA was increased significantly with leptin incubation in the KD cells compared with the NC and untreated cells. Although the expression of ACO mRNA in the KD group did not show marked increases compared with that without leptin incubation, the leptin-stimulated ACO mRNA expression in the KD group by approximately 1.7-fold (0.57 ± 0.07 and 0.97 ± 0.06 arbitrary units for cells without or with leptin, respectively; $n = 5$, $P = 0.08$).

Discussion

Rats with DIO usually exhibit hyperleptinemia, indicating that leptin produced by adipocytes does not deplete adipocyte fat and that with overnutrition, there is leptin resistance in adipocytes. In this study, we used high-fat feeding (45% kcal from fat) to induce rat obesity. We observed that serum leptin, triglyceride, and total cholesterol levels were all elevated in DIO rats, indicating a relative reduction in the response to leptin in DIO rats. Indeed, leptin treatment can deplete adipocyte fat in lean rodents [17]. However, in DIO rats, adipocytes enlarge with fat and secrete greater amounts of leptin [18, 19], and the interstitial concentrations of leptin surrounding adipocytes are within the fat-depleting ranges [20], but they still lost the ability to deplete fat. The reasons may be (*i*) adipocyte-derived hyperleptinemia associated with obesity is needed to maintain the vital function of fuel storage and conservation, so the adipocytes mount a powerful defense against their own leptin to prevent wasteful loss of their fat stores [17], or (*ii*) excessive expression of SOCS-3 might be likely mechanisms of autocrine/paracrine action blockade of the increasing leptin levels secreted by adipocytes. Our data show that a high-fat diet brought about

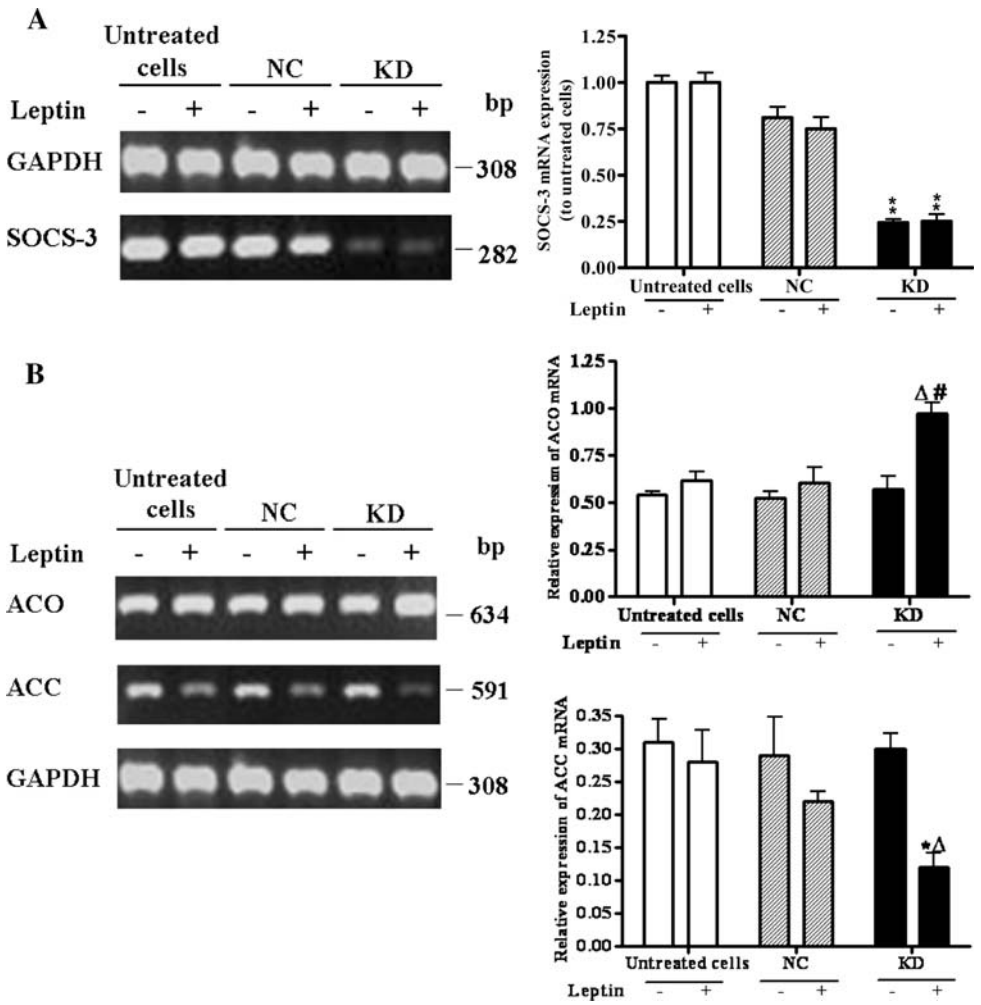

Fig. 4 SOCS-3 knockdown enhances the effect of leptin on fatty acid metabolism in DIO adipocytes. **a** The effect of leptin on SOCS-3 mRNA expression in DIO adipocytes. At day 10 of differentiation, adipocytes were infected with Lv-SOCS-3 (KD) or Lv-SOCS-3-NC (NC) (MOI = 50); 96 h after infection, cells were washed twice with PBS at pH 7.4 and incubated in the absence or presence of rat recombinant leptin in serum-free culture medium for 6 h at 37°C, 5% CO_2. The cells were subsequently harvested, and the relative expression of mRNA was determined by semi-quantitative RT-PCR. Scion Image4.0 software was used to analyze optical density values of the PCR products. All experiments were conducted in triplicate, and data are expressed as means ± SE, ** $P < 0.01$ versus NC and KD. **b** The effect of leptin on ACC and ACO mRNA expression in DIO adipocytes. The experimental procedure was the same as **a**. * $P < 0.05$ versus KD without leptin incubation; $^{\Delta}$ $P < 0.01$ versus untreated cells with leptin incubation; $^{\#}$ $P < 0.01$ versus NC with leptin incubation

an increase in SOCS-3 expression in epididymal adipose tissue that agrees well with many reported studies [21–23]. This increase in SOCS-3 is likely to be a mechanism to block autosuppression of adipose tissue by leptin, allowing adipocytes to meet the elevated physiological demand of this factor, as has been previously reported [23] for other models of nongenetic obesity. The serum leptin level of rats with DIO seemed lower than some published data [20, 23, 24], which perhaps is a result of strain differences between Wistar and Sprague–Dawley rats, short-time feeding, different dietary fat proportions or the type of fat used in the diet. The development of obesity in rats on a high-fat diet might partly be associated with increased SOCS-3 expression in adipocytes and their resistance to autosuppression by leptin [23].

Next, we focused our attention on the effects of SOCS-3 inhibition in adipocytes. Unfortunately, we could not infect mature adipocytes with lentivirus successfully because their lifespans were too short for infection and subsequent experiments. Therefore, we used ADSCs to induce adipogenic differentiation, because primary cultures of ADSCs more closely reflect the in vivo characteristics of the tissue from which they are derived [25], as they have not been passed continuously in culture. Stromal-vascular cells from

preobese fetuses (late term) may be inherently more sensitive to adipogenic agents than stromal-vascular cells from lean fetuses [26]. Compared to lean mice, tissue factors was elevated in both mature adipocytes and stromal-vascular cells in obese mice [27]. Primary cultures of ADSCs derived from rats subjected to nutritional manipulations [28] represent unique model systems for delineating the intrinsic influences on preadipocyte replication, recruitment, and differentiation. In this study, SOCS-3 mRNA expression in adipocytes differentiated from the ADSCs of rats with DIO was significantly higher than that in rats on a 10% fat diet. This result is consistent with the SOCS-3 expression in epididymal adipose tissues of control and DIO rats, suggesting that adipocytes from ADSCs are useful to study the effects of SOCS-3 inhibition.

In rats exhibiting hyperleptinemia as a result of adenovirus-mediated leptin gene transfer or in lepr-b-transgenic mice overexpressing lepr-b in white and brown fat tissues, free fatty acids are oxidized inside the adipocytes, which are known as "in situ" oxidation [17, 20]. The increased expression of fatty acid oxidation enzymes, such as carnitine palmitoyltransferase I (CPT-1) and ACO [29], and the down-regulation of genes encoding lipogenic enzymes, such as ACC alpha, might contribute to the increased fatty acid oxidation inside adipocytes [17, 29]. We believe that the decline in "in situ" oxidation ability of leptin in adipocytes of rats with DIO may contribute to obesity formation, and high-level SOCS-3 might be one of the culprits. Therefore, to evaluate whether SOCS-3 knockdown in DIO adipocytes could rescue the anti-lipogenic action of leptin, we incubated SOCS-3-knockdown adipocytes with rat recombinant leptin and measured the expression of ACC and ACO mRNA. The treatment of adipocytes with 50 nM leptin for 6 h increased the cellular level of SOCS-3 by 50% [16], which indicates that a dose of 50 nM leptin could have an effect in adipocytes in vitro [30]. The data reported herein showed that the basal SOCS-3 mRNA levels were lack of response to leptin in adipocytes in any of the groups. The most possible reason was that the adipocytes derived from DIO tissues might generate leptin resistance to a certain extent and leptin signaling was inhibited due to excessive SOCS-3. So exogenous leptin cannot further induce increases in SOCS-3. In addition, leptin did not modify the ACC and ACO mRNA levels in adipocytes in the untreated and NC groups, suggesting that the anti-lipogenic action of leptin was inhibited in those cells. On the contrary, SOCS-3 knockdown in adipocytes from DIO rats partly restored leptin activity, as shown by the lower expression of ACC mRNA and higher expression of ACO mRNA in the KD group with leptin incubation. As leptin may decrease ACC and increase ACO by inhibiting lipogenesis and stimulating lipolysis [12, 31], the inhibition of SOCS-3, a potent negative regulator of leptin signaling, in DIO adipocytes at least partly enhanced leptin-stimulated fat metabolism.

Leptin can activate PI3-kinase, MAPK, and STAT3 in a variety of tissues, including WAT in vivo [32–34], and stimulate AMP-activated protein kinase (AMPK) activity [17] and fatty acid oxidation in lean myotubes, but not in obese subjects [35]. Based on the results of this study, the possibility that excessive SOCS-3 expression in DIO adipocytes inhibits one or several leptin signal transduction pathways, such as Jak-STAT, AMPK, might be considered. SOCS-3 knockdown, which reduced SOCS-3 expression to a great extent, attenuated the inhibition of the leptin signal and restored the lipopenic action of leptin.

In conclusion, our results indicate that the SOCS-3 knockdown in adipocytes differentiated from ADSCs of rats with DIO increased in situ fatty acid oxidation and rescued the lipopenic activity of leptin. Inhibiting SOCS-3 might be a therapeutic intervention for obesity. However, further details of the regulatory mechanism and of how to avoid its interference with other cytokines, such as insulin, interleukin and growth hormone, must be determined.

Materials and methods

Experimental animals

Male Wistar rats weighing 150–170 g purchased from the Animal Center of China Medical University were housed individually with a control temperature of 20 ± 2°C, a relative humidity of 50 ± 10%, and a 12 h light and dark cycle. All animal procedures were conducted under an animal protocol approved by the Institutional Animal Care and Use Committee of China Medical University.

All animals were maintained on a standard chow diet containing 10% kcal from fat for 1 week before the experimental phase. Three rats were picked randomly named as control-2 group to feed a 10% fat diet for later SOCS-3 determination of adipocytes. Other rats were then given either their usual diet of standard chow, as the control-1 group ($n = 5$), or a home-made high-fat diet, which contained 45% kcal from fat, as the high-fat diet group ($n = 30$), for 8 weeks. The high-fat diet was made up of 73% standard chow diet plus 20% lard, 7% casein (Aoboxing Biotech Company Ltd, Beijing, China), and trace amounts of multiple vitamins. Food and water were available ad libitum. After 8 weeks of feeding, rats in the high-fat diet group whose body weight gains were more than those of the average body weight gain plus 1 SD in the control-1 group were regarded as rats with DIO. Half of the rats with DIO were selected randomly as DIO-1 group and the remaining DIO rats were grouped as DIO-2. Rats in control-1 and DIO-1 groups were anesthetized with 50 mg

of sodium pentobarbital (Sigma, St. Louis, MO) per kilogram body weight administered intraperitoneally after fasting 12 h. Serum, mesenteric, retroperitoneal, and epididymal fat pads were obtained and stored at −80°C. Rats in DIO-2 group were used to obtain ADSCs for adipogenic differentiation and lentivirus infection.

SOCS-3 shRNA lentiviral vector construction and generation

The details of SOCS-3 short hairpin RNA (shRNA) lentiviral vector construction have been described in our previous study [36]. Briefly, four siRNA sequences were designed against the rat SOCS-3 sequence using on-line siRNA software (http://ihome.ust.hk/~bokcmho/siRNA/siRNA.html). A negative control sequence cited by many other papers was chosen [37, 38]. Double-stranded DNA oligos containing a specific targeting sequence were designed on the basis of the siRNA sequences described above and synthesized by Genechem Co. Ltd (Shanghai, China). The synthesized DNA oligos consisted of 19 nucleotide (nt) sequences from the target transcript, separated by a short spacer from the reverse complement of the same sequences and five thymidines (T5) as the termination signal and two sticky ends for ligating into vectors. The shRNA expression vectors were generated by inserting annealed oligo sequences into the digested pGCSIL-GFP vectors (Genechem Co. Ltd) between *Age*I and *ECo*RI sites. The resulting lentiviral plasmid, which was sequenced and confirmed to have no cloning-introduced mutations, together with other two package accessory plasmids (Genechem Co. Ltd) co-transfected 293 T cells (ATCC, Denmark) to generate lentivirus. The lentivirus were amplified and concentrated. The virus titer was determined with end point dilution by counting the numbers of infected green cells under fluorescence microscopy 96 h after infection [39]. Rat intestinal epithelial cells (IEC6) (ATCC, Denmark) were used to infect the generated lentivirus to obtain the most effective siRNA target sequence, which was used to infect adipocytes differentiated from ADSCs.

Isolation and differentiation of primary ADSCs

For ADSCs isolation, epididymal fat depots from control-2 ($n = 3$) and DIO-2 groups ($n = 5$) were resected under aseptic conditions. The fat pads were minced into pieces in DMEM/F12 medium (Hyclone, Thermo scientific, Utah). Adipose tissue fragments were digested in the presence of type II collagenase (200 U/ml, 3 ml/g tissue, Sigma) at 37°C in a shaking water bath at 110 rpm/min for 60–90 min. After incubation, the tissue remnants were removed by filtration through a 250-μm nylon mesh, and samples were centrifuged at 200×*g* for 10 min to pellet the ADSCs. The supernatant with the mature adipocytes was discarded, and the pellet was resuspended in erythrocyte lysis buffer incubated for not more than 10 min and centrifuged at 200×*g* for 5 min. After washing two times, DMEM/F12 was added to the pellet, and the pellet was filtered through a nylon screen with a pore size of 25 μm. The cells passing the filter were pelleted by centrifugation and then resuspended in DMEM/F12 containing 10% fetal bovine serum (FBS) (Clark bioscience, Seabrook, MD).

For the preparation of primary cultures and differentiation of ADSCs, isolated cells from epididymal fat pads were cultured and differentiated according to Hausman's method [40], with little modification. Briefly, ADSCs at a density of 1.5×10^4 cells/cm^2 were plated in 12-well plates in DMEM/F12 containing 10% FBS for 24 h; then the medium was changed to remove nonattached cells and the culture was continued until a confluent cell monolayer was obtained. Cells were then incubated in a differentiated medium (DMEM/F12 containing 10% FBS supplemented with 50 μg/ml gentamicin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 100 nM dexamethasone, 700 nM insulin,17 μM pantothenate, 33 μM biotin, 10 μg/ml transferring, 1 nM triiodo-L-thyronine and 1 μg/ml rosiglitazone). IBMX and rosiglitazone were withdrawn after 3 days of exposure. After 10 days differentiation, oil red O (Sigma) staining was used to evaluate the differentiation rate. Adipocytes were washed twice with PBS (pH 7.4), collected and stored at −80°C for SOCS-3 mRNA and protein determination.

Adipocyte infection

At day 10 of differentiation, the adipocytes from DIO-2 rats were infected with a SOCS-3 shRNA lentivirus (Lv-SOCS-3, KD) and a negative control shRNA lentivirus (Lv-SOCS-3-NC, NC) at a multiplicity of infection of 50 (MOI = 50). Twenty-four hours after infection, the medium was replaced with fresh DMEM/F12 complete medium, and the incubation was continued for another 72 h before the infection rate was counted by fluorescence microscope. Five days after infection, the cells were washed twice with PBS (pH 7.4) and incubated in the absence or presence of rat recombinant leptin (Sigma), at doses of 50 nM in serum free culture medium for 6 h.

Biochemical measurements

Blood samples were collected from the abdominal aorta. Serum leptin was measured using a murine Leptin ELISA kit (Diagnostic systems laboratories, Inc, Webster, TX). The average intraassay and interassay coefficients of variation were 6.1% and 5.1%, respectively. The detection limit was 0.04 ng/ml. Serum triglycerides and total

Table 1 Sequence(s) of PCR primer(s)

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	GenBank Accession no.
β-Actin	TTGTAACCAACTGGGACGATATGG	GATCTTGATCTTACTGGTCCTAGG	J00691
GAPDH	AGATCCACAACGGATACATT	TCCCTCAAGATTGTCAGCAA	BC059110
SOCS-3	TCACCCACAGCAAGTTTCC	GGATGCGTAGGTTCTTGGTC	AF075383
ACC	GCAGTCTCCCAACTCCTA	CGTTCTACCCAGTCCTTTA	J03808
ACO	GCCCTCAGCTATGGTATTAC	AGGAACTGCTCTCACAATGC	J02752

cholesterol were determined by using commercial kits (Sigma). The concentrations of protein in the tissue and cultured cells lysate were measured by using a BCA protein assay (Pierce Biotechnology, Rockford, IL). The samples in all procedures were measured in duplicate.

RNA extraction and RT-PCR

The total RNA in adipose tissues and cultured cells was extracted using the TRIzol (Invitrogen, Carlsbad, CA) isolation method. The expression of SOCS-3, ACC, ACO, β-Actin, and GAPDH mRNA was semi-quantified using an RT-PCR kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Briefly, the cDNA template was denatured for 5 min at 94°C followed by 30–35 cycles with the following steps: denaturation, 1 min at 94°C annealing, 30 s at 50–57°C and elongation, 45 s to 1 min at 72°C. Reactions were finished with a final extension of 10 min at 72°C. PCR products were loaded on 2% agarose gel electrophoresis and followed by an analysis of optical density value by using Scion Image 4.0 software. All PCR reactions were done in triplicate. The primers are shown in Table 1.

Protein extraction and immunoblot analysis

Epididymal adipose tissues and cultured cells were harvested and homogenized in lysis buffer (50 mM Tris–HCl, pH 7.5, 0.1 mM Na_3VO_4, 1% Nonidet P-40, 25 mM NaF, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, and 1% protease inhibitor mixture (Sigma)). Cells were sonicated on ice (setting: 5 s each for four times, 200 W), and insoluble portions were removed by centrifugation at 15,000×g at 4°C for 1 h. Protein samples were boiled for 5 min in 1× SDS sample buffer (50 mM Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 0.02% bromophenol blue) containing 2%-mercaptoethanol. The proteins on the gels that were separated by SDS-PAGE were transferred onto a polyvinylidene difluoride membrane for 3 h at 4°C. The membrane was blocked with 5% fat-free milk for 1 h at room temperature, washed three times with TTBS, and incubated with goat anti-rat SOCS-3 (H103) polyclonal antibodies (1:300 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution, Pierce, Rockford, IL) for 45 min at room temperature. Enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK) was used to obtain signals. The blots were quantified by using Scion Image 4.0 software.

Statistical analysis

All data are expressed as means ± SE. Statistical analyses were performed by two-tailed unpaired Student's t-test with unequal variance or one-way analysis of variance. $P < 0.05$ was considered as significant.

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