Fat storage in adipocytes requires inactivation of leptin's paracrine activity: Implications for treatment of human obesity

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Hyperleptinemia rapidly depletes adipocyte fat in lean rats, whereas comparable hyperleptinemia produced by adipocytes in diet-induced obesity does not, implying a leptinergic blockade in adipocytes during overnutrition. Indeed, activated STAT-3 in white adipose tissue (WAT) of normal rats was less on a 60% high fat diet (HFD) than on 4% fat, despite a 10-fold higher plasma leptin. In 6 days of a HFD, mRNA of the postreceptor leptin inhibitor, suppressor of cytokine signaling-3, increased 22-fold in WAT, while leptin receptor (Lepr-b) mRNA gradually disappeared, implying leptinergic blockade at both postreceptor and receptor levels. Adipocyte-specific Lepr-b overexpression of a Lepr-b transgene completely prevented the adipocyte hypertrophy and hyperplasia and the increase in body fat induced in wild-type mice by HFD. Activated STAT-3 and AMP-activated protein kinase (AMPK), and the mRNA of lipooxidative enzymes, peroxisome proliferator-activated receptor-γ-coactivator-1α, and uncoupling protein-1 and -2 were increased in WAT. Body temperature was elevated in the transgenic mice, suggesting uncoupled fatty acid oxidation of surplus fatty acids. In conclusion, storage of surplus calories in WAT and the development of diet-induced obesity require the blockade of a latent leptin-stimulated caloric sump in white adipocytes.

autocrine/paracrine action | diet-induced obesity | hyperleptinemia | lepr-b | AMP-activated protein kinase

White adipocytes are able to perform simultaneously two seemingly antithetical functions. They can store triglyceride (TGs) while secreting leptin in concentrations that make fat completely disappear when induced experimentally in lean rodents (1, 2). The accumulation of fat in the presence of fat-depleting levels of leptin constitutes *prima facie* evidence of a blockade of its action. Teleologically, such a blockade can be viewed as essential for the primary mission of adipocytes, the extension of survival in time of famine (3) by stockpiling surplus calories. It can also be viewed as essential for the development of diet-induced obesity (DIO).

This study was designed (*i*) to obtain direct objective evidence that such a blockade exists in DIO, (*ii*) to elucidate its mechanisms, and (*iii*) to determine whether it is essential for the storage of surplus calories and the development of DIO. We provide evidence that the blockade exists, that it is caused by both postreceptor and receptor events, and, that, without it, DIO cannot occur.

Materials and Methods

Animals. Male Harlan Sprague–Dawley (SD) rats were from Charles River Laboratories (Raleigh, NC). Wild-type (+/+) Zucker diabetic fatty (ZDF) rats and C57BL/6 mice were bred locally. All were housed in individual cages in a temperature-controlled environment with 12-h light/12-h dark cycle. All rats had *ad libitum* access to water and pelleted rat chow. For the DIO study, SD rats were maintained on a standard chow diet composed of 4% fat, 24.8% protein, and 3.94 kcal/g (1 kcal = 4.18 kJ) (Teklad 4% mouse/rat diet, Madison, WI) for 1 week. At 5 weeks, half of the SD rats were placed on a pelleted high-fat diet containing 60% fat, 7.5% carbohydrate, 24.5% protein, and 6.7 kcal/g (Purina Test Diet, Richmond, IN) for 9, 13, or 19 weeks to produce DIO. The remainder were continued on the standard 4% fat diet. For DIO study involving adenovirus administration, lean ZDF (+/+) rats were used. At 5–6 weeks of age, they were fed a diet containing either 6% or 60% fat for 12 weeks. They were then infused intravenously with recombinant adenovirus containing either leptin or β-galactosidase cDNA. Transgenic studies were carried out with C57BL/6 mice (see below). For the DIO study, transgenic and control mice were fed either a 4% or 60% fat diet for 12 weeks.

Animals were killed under anesthesia with pentobarbital sodium. Nonfasting blood samples were obtained from the inferior vena cava. Fat tissues were rapidly excised, frozen in liquid nitrogen and stored at −70°C until use. Institutional guidelines for animal care and use were followed. The animal protocol was approved by the Institutional Animal Care and Research Advisory Committee of University of Texas Southwestern Medical Center at Dallas.

Transgenic Mice Production. To construct the aP2-Lepr-b transgene, a cDNA insert of pLepr-b (a gift from Cai Li, Merck Research Laboratories, Rahway, NJ) encoding mouse leptin receptor b isoform was modified by PCR to introduce a stop codon and NotI restriction site at the 3′ end of the coding region. The modified Lepr-b cDNA was subcloned into NheI-NotI sites of pSTEC-1-aP2 (from Cai Li), which was modified by a linker ligation to remove a HindIII site and generate a SmaI site at the 5′ end to generate the pSTEC-1-aP2-Lepr-b. The pSTEC-1-aP2-Lepr-b construct contained a chimeric intron, composed of a 5′ splice site from the β-globin intron and a 3′ splice site from an IgG intron, and a simian virus 40 (SV40) polyA addition site required for proper processing of the transgene mRNA *in vivo*. The DNA fragment of pSTEC-1-aP2-Lepr-b digested with SmaI was purified by using an EluTip-D column (ISC Bioexpress). Transgenic mice were produced by microinjection of purified aP2-Lepr-b DNA insert into pronuclei of fertilized mouse eggs, which were transferred into foster mothers. Transgenic founders and offspring were screened by PCR genotyping with DNA prepared from tail biopsies and specific primers.

Plasma Measurements. Plasma leptin was measured by a Mouse/Rat Leptin ELISA kit (Crystal Chem, Downers Grove, IL). Plasma triglycerides were measured by glycerol phosphate oxidase-Trinder triglyceride kit (Sigma). Plasma free fatty acids were measured by using the Wako NEFA kit (Wako Chemical USA, Richmond, VA).

Triglyceride Content of Tissues. Mice were anesthetized with pentobarbital sodium. Tissues were rinsed with PBS (pH 7.4), dissected,

Conflict of interest statement: No conflicts declared.

Abbreviations: AMPK, AMP-activated protein kinase; DIO, diet-induced obesity; HFD, high fat diet; SD, Sprague–Dawley; SOCS, suppressor of cytokine signaling; TBST, TBS containing 0.1% Tween; TG, triglyceride; UCP, uncoupling protein; WAT, white adipose tissue; ZDF, Zucker diabetic fatty.

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and placed in liquid nitrogen immediately. Total lipids from tissues were extracted and dried under N_2 gas. TG content was assayed as described in ref. 4.

Real-Time Quantitative Polymerase Chain Reaction (RT-QPCR). Total RNA was extracted from fat tissues by TRIzol isolation method (Life Technologies, Rockville, MD). All PCR reactions were done in triplicate. mRNA was calculated by using the standard curve method. Ribosomal RNA (18S) or 36B4 RNA was used as the invariant control. Primer sequences of genes used for quantification of mRNA by RT-QPCR are shown in Table 3, which is published as supporting information on the PNAS web site.

Immunoblotting. Total cell extracts prepared from fat tissues of DIO and control rats or mice were resolved by SDS/PAGE and transferred to a poly(vinylidene difluoride) membrane (Amersham Pharmacia). The blotted membrane was blocked in 1× TBS containing 0.1% Tween (TBST) and 5% nonfat dry milk (MLK) for 1 h at room temperature with gentle, constant agitation. After incubation with primary antibodies anti-phospho-STAT-3 (Tyr-705), anti-STAT-3, anti-phospho-AMPK (Thr-172), anti-AMPK (Cell Signaling Technology, Beverly, MA), or anti-γ-tubulin (Sigma) in freshly prepared TBST-MLK at 4°C overnight with agitation, the membrane was washed twice with TBST buffer followed by incubating with goat anti-rabbit or anti-mouse HRP-conjugated IgG in TBST-MLK for 1 h at room temperature with agitation. The membrane was then washed three times with TBST buffer, and the proteins of interest on immunoblots were detected by using an enhanced chemiluminescence detection system (Amersham Pharmacia).

Quantification of Adipocyte Size. Paraffin sections of Bouins-fixed fat pads were stained with hematoxylin and eosin and analyzed by using a Zeiss Axiophot microscope equipped with an AxioCam digital camera. For each sample, five areas and 10 cells in each area were evaluated. Cell diameters were obtained by using AXIOVISION software (Zeiss) for digital imaging processing. Results are expressed as mean ± SEM.

Quantification of Body Fat. For rats, proton magnetic resonance spectroscopy was used as described in ref. 4. Proton spectra were resolved into water and fat resonances, the areas of which were quantified with the magnetic nuclear resonance spectroscopy software program NRM-1 (Tripos Associates, St. Louis). In mice, body fat was quantified by using the Bruker (The Woodlands, TX) Minispec mq7.5 NMR analyzer.

Statistical Analysis. Results obtained in this study are presented as means ± SEM and were evaluated with Student's *t* test for two groups.

Results

Evidence for Autocrine/Paracrine Blockade of Leptin During High Fat Feeding. To establish the existence of the putative blockade of leptin action on adipocytes, we fed normal SD rats a diet containing either 60% or 4% fat. After 9, 13, and 19 weeks of the 60% fat diet, the mean body weight of the rats was, respectively, 12%, 26%, and 20% more than that of the control rats receiving a 4% fat diet (Fig. 1*A*). Plasma leptin levels rose in proportion to the increase in body weight to 11.7 ± 4.3 ng/ml after 9 weeks of high fat diet versus 0.9 ± 0.02 ng/ml in the control group, 33.9 ± 10.4 ng/ml at 13 weeks versus 1.8 ± 0.5 ng/ml, and 42.3 ± 12.4 ng/ml versus 2.6 ± 1.0 ng/ml after 19 weeks (Fig. 1*B*). This level of endogenous hyperleptinemia in DIO is within a range that completely depletes adipocyte fat when induced experimentally in lean rats (2).

For direct evidence of a blockade of leptin activity in adipose tissue in DIO, we studied phosphorylation of STAT-3 in lean (+/+) ZDF rats. STAT-3 phosphorylation provides an index of signal transduction initiated by binding of leptin to its receptor, Lepr-b (5, 6). Like the SD rats, the lean (+/+) ZDF rats on 60% fat for 12 weeks developed DIO, accompanied by even higher hyperleptinemia of 63 ± 18 ng/ml. In the 6% fat-fed controls, plasma leptin levels averaged only 3 ± 0.7 ng/ml, and yet their adipose tissue P-STAT-3 was 3-fold higher than in the hyperleptinemic DIO group on a 60% fat diet (Fig. 1*C*), providing objective evidence for blockade of local leptin action during overnutrition.

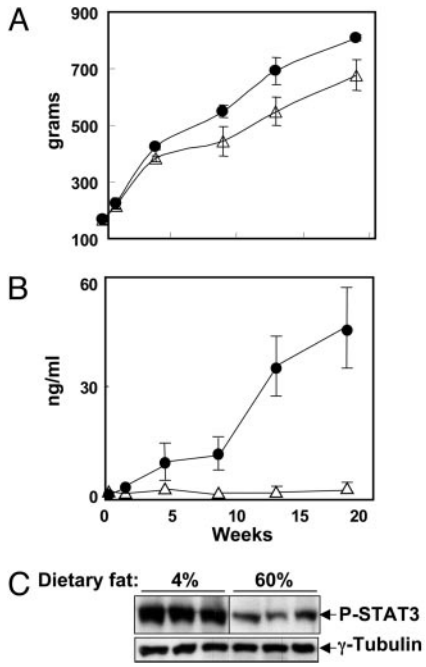

Fig. 1. Effect of high fat feeding on body weight and plasma leptin levels. (*A*) Mean (±SEM) body weight of normal 5-week-old SD rats fed a 4% (△) or 60% (●) fat diet for the times as indicated. (*B*) Mean (±SEM) plasma leptin level of 4% and 60% fat-fed rats. (*C*) Phosphorylated STAT-3 content of WAT of the rats 12 weeks after the start of the 4% or 60% fat feeding.

To determine whether the action of experimental hyperleptinemia is also blocked during high fat feeding, we induced hyperleptinemia by AdCMV-leptin administration and compared adipose tissue STAT-3 activation in normal rats on either a 6% or a 60% fat diet. In lean rats on the 6% fat diet for 12 weeks, the AdCMV-leptin treatment produced hyperleptinemia of 68 ± 5 ng/ml (Fig. 2*A*), associated with a 100 ± 10 g loss of body weight and disappearance of body fat, as measured by magnetic nuclear resonance spectroscopy (Fig. 2*B*). P-STAT-3 content of the fat pad remnant, an index of direct leptinergic action, rose 3.2-fold (Fig. 2*C*). In the DIO rats on the 60% fat diet for 12 weeks, AdCMV-leptin treatment, which added exogenous hyperleptinemia to the preexisting endogenous hyperleptinemia of DIO, raised the plasma leptin levels to >128 ng/ml (Fig. 2*A*), twice that of the 6% fat-fed group. Despite the extreme hyperleptinemia, no fat loss could be detected by magnetic nuclear resonance spectroscopy (Fig. 2*B*), and the P-STAT-3 content of their fat rose by only 1.8-fold (Fig. 2*C*). These results indicate that leptin signaling and fat depletion by intense hyperleptinemia, whether adipose-derived tissue or experimentally induced, are greatly reduced by high fat feeding.

Mechanisms of the Blockade of Leptin. The blockade of leptin action on adipocytes could be the result of changes at the receptor or postreceptor levels. Down-regulation of the leptin receptor has been reported in the hypothalamus of high fat fed rodents (7). To determine whether Lepr-b is also reduced in adipocytes during high fat feeding, we compared adipocyte Lepr-b mRNA in the two groups and its relationship to leptin expression. Plasma leptin levels

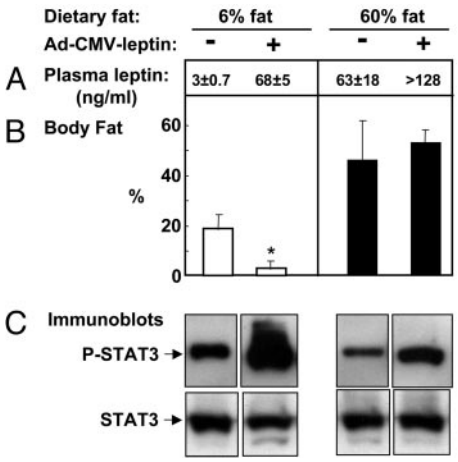

Fig. 2. Comparison of the effect of overnutrition on the response of adipose tissue to exogenous hyperleptinemia induced by i.v. administration of AdCMV-leptin (+) or AdCMV-β-galactosidase as a control (−). (*A*) Mean (±SEM) plasma leptin levels. (*B*) Mean (±SEM) body fat, measured by magnetic nuclear resonance spectroscopy. (*C*) Phosphorylated and total STAT-3 content, measured by immunoblotting, of WAT in normal (+/+) ZDF rats fed either 6% or 60% fat.

had increased by the sixth day of the 60% fat diet, and leptin mRNA in white adipose tissue (WAT) had risen 2-fold by the fourth week (Fig. 3*A*), an increase that persisted throughout the period of observation. Lepr-b mRNA began a slow decline on the fourth day of the 60% fat diet but required 19 weeks to reach unmeasurable levels (Fig. 3*B*); expression of adipocyte Lepr-b seemed to be inversely related to the progressively rising levels of leptin mRNA ($R = 0.8343$) and to the plasma levels of leptin ($R = 0.8724$, Fig. 1*B*).

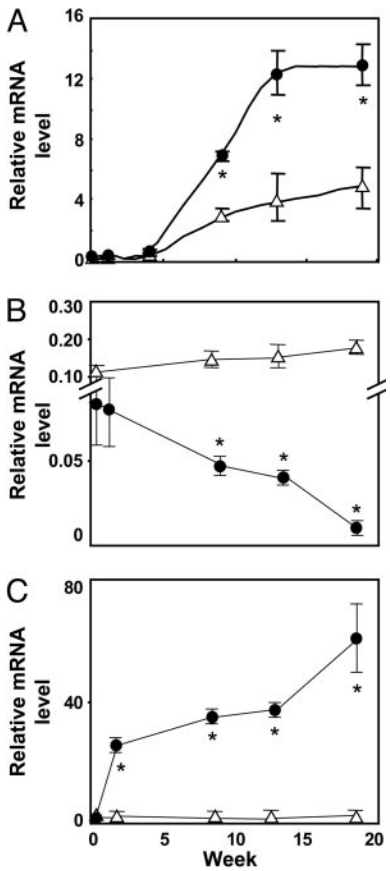

Fig. 3. Effect of 20 weeks of overnutrition on the expression profiles of leptin, the leptin inhibitor, SOCS-3, and the leptin receptor, Lepr-b, in adipose tissue of normal rats. (*A*) The mRNA of leptin. (*B*) Lepr-b. (*C*) SOCS-3 in WAT of normal lean SD rats fed either a 4% (△) or 60% (●) fat diet (*, $P < 0.05$).

Evidence of leptin resistance in the high fat-fed rats preceded the biologically significant reduction in Lepr-b mRNA by several weeks, raising the possibility of an early postreceptor level blockade of leptin action on adipocytes. Suppressor of cytokine signaling-3 (SOCS-3) has been identified as a leptin resistance factor in the hypothalamus (8) and was reported to be increased in the WAT of DIO rats after 8 weeks of high fat feeding (9). To determine the chronology of a rise in SOCS-3 relative to the rise in plasma leptin and the decline in Lepr-b, we measured its mRNA in the adipose tissue at multiple time points in rats on the 60% and 4% fat diets. SOCS-3 mRNA expression did not change during the first 2 days of high fat feeding, but at 6 days, when plasma leptin had risen 2.4-fold, it was 22-fold higher (Fig. 3*C*). After 9, 13 and 19 weeks of 60% fat feeding, with plasma leptin ranging from 13- to 19-fold higher than in 4% fat-fed controls, SOCS-3 mRNA was 40- to 60-fold greater than in the controls. Thus, the robust up-regulation of SOCS-3 after 1 week of high fat feeding suggested that a postreceptor mechanism initially prevents leptinergic paracrine activity as the local levels of leptin begin to rise and before Lepr-b mRNA down-regulation becomes substantial. However, by the time leptin mRNA and plasma leptin have approached peak levels, Lepr-b expression has been drastically reduced.

Essentiality of Lepr-b Down-Regulation in Adipocyte Fat Storage and DIO. To determine whether down-regulation of Lepr-b plays an essential role in the ability of fat cells to store excess calories and undergo hypertrophy and hyperplasia, we transgenically overexpressed Lepr-b by using the aP2 promoter, which is relatively specific for adipocytes (Fig. 4*A*). Adipocyte Lepr-b mRNA was markedly increased in the white and brown fat of transgenic mice. Immunoblotting with an anti-Lepr-b antibody that does not detect the receptor in any tissue of wild-type mice easily detected Lepr-b protein in both white (Fig. 4*B*) and brown (data not shown) fat tissues but not in the hypothalamus or other tissues. Thus, the overexpression of Lepr-b in the transgenic mice was confined to white and brown adipocytes.

To determine the physiologic impact of constitutive adipocyte-specific overexpression of Lepr-b on body weight and energy balance during overnutrition, we measured food intake and body weight of Lepr-b-transgenic and wild-type mice placed on a 60% fat diet for 12 weeks beginning at 5 weeks of age. The wild-type mice consumed an average of 2.9 ± 0.3 g per day, whereas the transgenic mice consumed 3 ± 0.1 g per day (not significant). Body weight (Fig. 4*C*) and body fat measured by the Bruker NMR analyzer (Table 1) were significantly lower in all transgenic mice than in the wild-type controls, all of which developed massive obesity within 12 weeks of 60% fat feeding. Every transgenic mouse remained as slender on a high fat diet (HFD) as on a 4% fat diet (Fig. 4*D*).

Morphometric analysis of the fat tissue of transgenic mice obtained after 12 weeks of HFD revealed a mean adipocyte diameter of 44 ± 1 μm compared to 113 ± 3 μm in wild-type controls on the same diet (Fig. 4*E*). Interestingly, on the 4% fat diet, transgenic mice exhibited no obvious phenotypic differences nor statistically significant differences in body weight or body fat (Table 1), nor were there significant differences in the adipocyte diameters of wild-type (59 ± 2 μm) and Lepr-b-transgenic (62 ± 2 μm) mice. The results indicate that HFD-induced adipocyte hypertrophy and hyperplasia cannot occur unless leptinergic action of adipocytes is blocked.

In both wild-type and Lepr-b-transgenic mice, the 60% fat diet was associated with a ≈10-fold higher plasma insulin than on the 4% fat diet. Because there was no difference in plasma glucose

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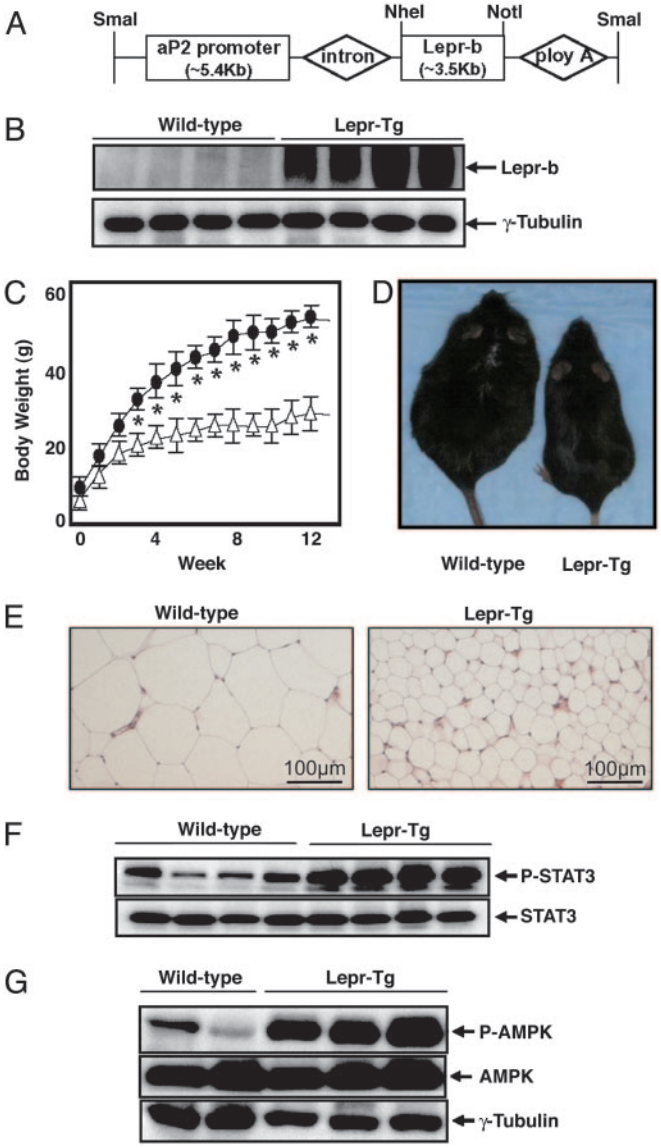

Fig. 4. The adipocyte-specific Lepr-b transgene and its obesity-preventing phenotype. (*A*) Map of the aP2-Lepr-b transgene construct. (*B*) Immunoblot for Lepr-b in WAT from Lepr-b-transgenic mice and wild-type controls. (*C*) Mean (±SEM) body weight of Lepr-b-transgenic mice (△) and wild-type controls (●) on a 60% fat diet. *, $P < 0.001$. (*D*) Gross appearance of a wild-type (*Left*) and a Lepr-b-transgenic (*Right*) mouse after 12 weeks on a 60% fat diet. (*E*) Comparison of WAT of a wild-type and a Lepr-b-transgenic mouse after 12 weeks on a 60% fat diet. (*F*) Activated STAT-3 (P-STAT-3) in WAT of wild-type and Lepr-b-transgenic mice on a 60% fat diet. (*G*) Activated AMP-activated protein kinase (P-AMPK) in WAT of the wild-type and Lepr-b-transgenic mice on a 60% fat diet.

(Table 1), this finding suggests that the prevention of obesity by Lepr-b overexpression in adipocytes failed to prevent insulin resistance. Ectopic fat content of liver, heart, and skeletal muscle was increased to the same degree by high fat feeding in the 2 groups (Table 1). This fact implies that the caloric surplus ingested by the transgenic mice exceeded the calories oxidized through the unblocked paracrine action of leptin on adipocytes.

Pathway of the DIO Resistance in aP2-Lepr-b-Transgenic Mice. To determine whether the differences between adipose tissue of Lepr-b-transgenic and wild-type mice on the high fat diet reflect unblocked paracrine action of endogenous leptin on the adipocytes, we again used phosphorylated STAT-3 as a marker of leptinergic action via Lepr-b. STAT-3 phosphorylation was significantly increased in the fat tissue of the transgenic mice (Fig. 4*F*), consistent with a direct autocrine/paracrine effect of endogenous leptin on adipocytes.

We have observed that depletion of adipocyte fat by exogenous leptin is associated with profound autosuppression of leptin mRNA (10). Therefore, we compared leptin mRNA in the two groups. In Lepr-b-transgenic mice on the 60% fat intake, the increase in leptin mRNA in their WAT was significantly less than in wild-type controls (Table 2), despite a 40-fold increase in plasma leptin levels in the controls (Table 1). This finding provides strong evidence of autosuppression of leptin expression by endogenous leptin acting via the transgenic Lepr-b.

Mechanism of the DIO Resistance of Lepr-b-Transgenic Mice. Reduced TG accumulation in the adipocytes without reduced food intake could be due to steatorrhea, increased release of free fatty acids from adipocytes, or increased uncoupled intracellular fatty acid oxidation. Because neither the stools nor the plasma free fatty acid levels of transgenic mice differed from the wild-type mice (Table 1), we attributed the reduced accumulation of fat in the adipocytes of the 60% fat-fed Lepr-b-transgenic mice to increased leptin-stimulated uncoupled FA oxidation in adipocytes, i.e., a "futile cycle" in which much of the ingested fat was oxidized soon after its delivery to the fat cells with dissipation of the energy as heat. To test this possibility, we measured in fat tissue the phosphorylation state of AMPK, a key regulator of fatty acid oxidation (11). P-AMPK was clearly increased in the fat tissue of the transgenic mice (Fig. 4*G*), consistent with increased oxidation of fatty acids. A significant reduction in the mRNAs of acetyl CoA carboxylase (ACC)-β, coupled with an increase in carnitine palmitoyl transferase-1 (CPT-1) and acyl CoA oxidase (ACO), was also consistent with increased fatty acid oxidation (Table 2). Moreover, peroxisome proliferator-activated receptor-γ-coactivator-1α (PGC-1α), which stimulates mitochondrial biogenesis (12), was increased 9-fold in transgenic fat. The mRNAs of uncoupling protein (UCP)-1 and -2 were increased 9- and 5-fold, respectively (Table 2), which would suggest that the energy was dissipated as heat. Wild-type mice also exhibited significant increments in UCP-1 and -2. Finally, body temperature was modestly elevated in the Lepr-b transgenics (37.8 ± 0.2°C vs. 37.3 ± 0.19°C in wild-type; $P = 0.008$) (Table 1).

A reduction in TG synthesis is suggested by the down-regulation of diacylglycerol acyl transferase-1 and -2 (DGAT-1 and -2) and of glycerol-3-phosphate acyl transferase (GPAT) in the transgenic mice of the high fat diet (Table 2).

Despite the 60% fat diet, the adipocytes of transgenic mice did not undergo hypertrophy or hyperplasia, as indicated, respectively, by measurement of their diameters and their body fat mass. Expression of insig-1, which reportedly limits the degree of adipocyte hyperplasia during overnutrition (13), was significantly increased in the fat-fed Lepr-b-transgenic mice to a greater degree than in the wild-type mice (Table 2). Expression of pref-1, a preadipocyte marker that inhibits maturation of preadipocytes into new adipocytes (14), was increased in transgenic fat, perhaps accounting for the lack of hyperplasia. FOXO1, which also negatively regulates adipogenesis (15), was markedly up-regulated (Table 2).

Effect of Brown Fat Resection on the Phenotype of Lepr-b-Transgenic Mice. Because the Lepr-b is overexpressed in brown and white fat, it seemed possible that increased uncoupled oxidation of fatty acids could have been localized in brown adipocytes. To assess its contribution relative to that of white adipocytes, all visible brown fat was resected from six Lepr-b-transgenic mice and six intact transgenic mice on a 60% fat diet. After 5 weeks of high

Table 1. Metabolic profiles of wild-type and transgenic Lepr-b mice fed 4% or 60% fat diets for 12 weeks

		4%			60%		
Measurement	*n*	Wild-type	Lepr-b-Tg	*P* value	Wild-type	Lepr-b-Tg	*P* value
Food intake, g	8	3.65 ± 0.32	3.91 ± 0.29	NS	2.97 ± 0.27	3.04 ± 0.11	NS
Body weight, g	8	30.28 ± 1.80	30.88 ± 2.19	NS	45.35 ± 2.84	34.29 ± 2.93	0.00002
Body fat, g	8	6.62 ± 1.65	5.02 ± 1.67	NS	18.80 ± 1.02	9.05 ± 2.68	0.000004
Body fat/body weight, %	8	21.70 ± 4.05	16.16 ± 4.56	NS	41.40 ± 1.66	26.45 ± 7.81	0.0008
Body temperature, °C	8	37.37 ± 0.30	36.92 ± 0.44	NS	37.35 ± 0.19	37.82 ± 0.18	0.0077
Glucose, mg/dl	8	207.61 ± 44.72	190.80 ± 42.13	NS	210.33 ± 27.94	198.18 ± 43.63	NS
Insulin, ng/ml	4	0.495 ± 0.105	0.469 ± 0.087	NS	46.46 ± 14.96	49.36 ± 9.93	NS
Leptin, ng/ml	8	1.53 ± 1.27	1.451 ± 0.958	NS	61.43 ± 4.87	11.31 ± 2.26	0.00003
Resistin, ng/ml	4	1.36 ± 0.22	1.18 ± 0.31	NS	6.76 ± 1.08	1.56 ± 0.55	0.00062
TG, mg/dl	8	61.86 ± 18.39	61.64 ± 20.67	NS	65.73 ± 15.48	46.09 ± 10.86	0.031
FFA, mM	8	0.810 ± 0.300	0.895 ± 0.182	NS	0.738 ± 0.164	0.680 ± 0.187	NS
Liver TG, mg/g	5	22.13 ± 12.22	22.02 ± 15.05	NS	133.99 ± 54.12	166.20 ± 11.49	NS
Muscle TG, mg/g	5	2.09 ± 0.72	1.87 ± 0.92	NS	28.30 ± 4.17	26.97 ± 6.59	NS
Heart TG, mg/g	5	2.67 ± 1.21	3.32 ± 1.77	NS	6.01 ± 2.21	4.60 ± 1.23	NS

FFA, free fatty acids; NS, not significant; Tg, transgenic.

fat feeding, the intact transgenic mice weighed 38.5 ± 0.4 g and the transgenic mice without brown fat weighed 37.4 ± 0.4 g. Thus, with Lepr-b overexpressed in white adipocytes, the weight gain caused by loss of brown fat did not occur, suggesting that white adipocytes had usurped their role.

Discussion

As adipocytes enlarge with fat, they secrete greater amounts of leptin (4, 16), a hormone that depletes adipocyte fat in lean rodents (2, 3). In DIO, the concentrations of leptin in the interstitial fluid surrounding adipocytes in DIO are within the

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Table 2. Quantitative PCR analysis of mRNAs in the adipose tissue of wild-type and transgenic Lepr-b mice by using 18 s as the invariant control

	4% fat			60% fat		
Genes	Wild-type (*n* = 5)	Lepr-b-Tg (*n* = 5)	*P* value	Wild-type (*n* = 5)	Lepr-b-Tg (*n* = 5)	*P* value
AMPKα1	4.25 ± 0.31	5.67 ± 0.66	NS	5.45 ± 0.75	11.97 ± 2.90	0.05
AMPKα2	0.62 ± 0.49	0.68 ± 0.44	NS	0.28 ± 0.11	0.68 ± 0.22	0.05
PPARα	0.09 ± 0.003	0.07 ± 0.01	NS	0.047 ± 0.001	0.50 ± 0.13	0.050
PPARγ	7.57 ± 1.18	11.21 ± 3.18	NS	8.41 ± 1.77	44.84 ± 8.69	0.036
PPARδ	2.90 ± 0.68	4.17 ± 0.49	NS	2.17 ± 0.74	10.14 ± 2.91	0.009
PGC1α	4.68 ± 0.64	8.80 ± 0.66	0.005	2.70 ± 0.79	23.88 ± 12.50	0.05
LXRα	1.57 ± 0.07	1.99 ± 0.09	NS	1.45 ± 0.48	3.86 ± 0.73	0.032
RXRα	0.63 ± 0.09	0.42 ± 0.01	NS	0.62 ± 0.18	0.003 ± 0.001	0.017
SREBP-1c	0.78 ± 0.08	1.43 ± 0.17	NS	0.70 ± 0.21	4.89 ± 2.07	NS
C/EBPα	5.05 ± 1.05	5.73 ± 0.10	NS	3.41 ± 1.01	10.49 ± 2.55	NS
C/EBPβ	1.86 ± 0.35	2.95 ± 0.37	NS	2.68 ± 0.64	10.96 ± 3.26	0.023
C/EBPδ	16.49 ± 3.44	16.78 ± 2.93	NS	16.15 ± 4.28	108.9 ± 11.0	5E-04
ChREBP	6.36 ± 0.80	9.06 ± 0.06	NS	4.08 ± 1.13	12.25 ± 3.47	NS
Pref-1	177.4 ± 20.3	191.5 ± 33.4	NS	16.18 ± 7.07	288.8 ± 68.3	0.012
Insig-1	1.43 ± 0.31	2.86 ± 0.26	0.011	2.62 ± 0.91	9.36 ± 2.29	0.050
FOXO1	6.91 ± 2.09	10.44 ± 1.03	NS	5.96 ± 1.52	35.40 ± 7.85	0.033
FOXC2	1.98 ± 0.54	1.58 ± 0.42	NS	0.93 ± 0.18	2.66 ± 0.20	8E-04
IR	1.97 ± 0.20	2.24 ± 0.33	NS	0.34 ± 0.11	6.22 ± 2.99	0.048
IRS1	4.99 ± 0.79	5.19 ± 2.01	NS	2.30 ± 1.14	5.86 ± 2.26	NS
IRS2	4.81 ± 0.63	3.44 ± 1.41	NS	4.95 ± 0.90	0.47 ± 0.36	0.019
ACO	0.16 ± 0.01	0.22 ± 0.06	NS	0.26 ± 0.07	0.76 ± 0.16	0.016
CPT1	0.22 ± 0.05	0.24 ± 0.08	NS	0.45 ± 0.08	1.56 ± 0.20	0.010
ACCα	3.02 ± 0.72	4.02 ± 0.85	NS	0.67 ± 0.16	0.41 ± 0.15	NS
ACCβ	2.51 ± 1.05	1.09 ± 0.06	NS	0.51 ± 0.13	0.14 ± 0.13	0.025
FAS	1.02 ± 0.10	2.02 ± 1.01	NS	2.03 ± 0.22	1.17 ± 0.57	NS
GPAT	0.28 ± 0.05	0.24 ± 0.02	NS	0.20 ± 0.04	0.087 ± 0.003	0.023
DGAT1	1.16 ± 0.13	1.10 ± 0.18	NS	0.94 ± 0.26	0.25 ± 0.12	0.05
DGAT2	0.63 ± 0.15	0.44 ± 0.20	NS	1.07 ± 0.29	0.08 ± 0.05	0.013
MCD	0.32 ± 0.04	0.25 ± 0.01	NS	0.22 ± 0.07	0.32 ± 0.15	NS
SCD	2.97 ± 0.01	2.91 ± 0.56	NS	1.41 ± 0.26	2.17 ± 0.50	NS
Leptin	1.21 ± 0.40	3.49 ± 2.19	NS	8.29 ± 3.06	2.73 ± 1.26	0.05
Resistin	4.98 ± 1.57	6.71 ± 2.38	NS	5.46 ± 1.62	1.56 ± 0.95	0.018
Lepr-b	0.03 ± 0.01	0.67 ± 0.28	0.05	0.001 ± 0.002	1.89 ± 1.34	0.05
SOCS3	0.12 ± 0.01	0.43 ± 0.32	NS	0.60 ± 0.18	0.94 ± 0.59	NS
UCP1	8.88 ± 2.37	8.81 ± 1.33	NS	56.15 ± 8.81	81.15 ± 2.46	0.05
UCP2	2.63 ± 0.86	3.33 ± 0.70	NS	6.34 ± 0.53	16.35 ± 0.35	0.005
UCP3	23.35 ± 1.85	22.28 ± 3.48	NS	30.67 ± 7.77	52.89 ± 7.70	0.027

Values represent the mean ± SEM of triplicate determinations in five different animals. NS, not significant; Tg, transgenic.

fat-depleting range. The ability of adipocytes to undergo hypertrophy and hyperplasia despite hyperleptinemia implies that a powerful leptinergic blockade protects their vital fat-storing function from the antilipogenic action of leptin. It also means that this leptinergic blockade is essential for obesity.

In this report, we have identified two likely mechanisms capable of blocking the paracrine action of the increasing leptin levels secreted by adipocytes as they store TG. A large increase in expression of SOCS-3, a postreceptor inhibitor of leptin (8), appears in adipocytes by the sixth day of high fat feeding, at which point leptin levels are slightly elevated. Later, as the hyperleptinemia becomes more intense, the decline in Lepr-b mRNA becomes substantial, and by 19 weeks of high fat feeding, Lepr-b mRNA reaches undetectable levels. Thus, a combination of postreceptor- and receptor-level leptin blockades appears to minimize potential leptinergic interference with fat storage.

The importance of Lepr-b down-regulation in the pathogenesis of DIO was confirmed in a transgenic mouse model that constitutively expresses Lepr-b on fat cells, even when fed a high fat diet. This model remains slender on the same 60% high fat intake that causes massive obesity in the wild-type controls. The adipocyte diameter of these mice on the 60% fat diet is no larger than on a 4% fat diet. In the WAT of Lepr-b-transgenic mice, STAT-3 phosphorylation is increased, whereas leptin mRNA is suppressed, suggesting that adipocyte-derived leptin is able to act via the transgenic Lepr-b to autosuppress leptin expression and deplete adipocyte fat. The increases in the transgenic WAT of P-AMPK and the mRNAs carnitine palmitoyl transferase-1 and peroxisome proliferator-activated receptor-γ-coactivator-1α are consistent with increased mitochondrial fatty acid oxidation, whereas the decrease in acetyl CoA carboxylase-β, DGAT-1 and -2, and GPAT mRNA suggests decreased lipogenesis. The increase in UCP-1 and -2 mRNA and in body temperature points to uncoupled oxidation of surplus calories and dissipation of the energy as heat. The findings fit well with an earlier study (17) in which adipocyte-selective reduction of leptin receptors by antisense RNA caused obesity and increased lipogenesis.

Other potentially important differences in the expression profile of WAT in Lepr-b-transgenic mice include insig-1, reported to "brake" the distention of adipocytes with fat (13), and pref-1, a secreted preadipocyte marker that inhibits maturation of preadipocytes into adipocytes (14). Insig-1 up-regulation, coupled with the net reduction in fatty acid accumulation, would explain the prevention of adipocyte hypertrophy, whereas the increased pref-1 expression and the striking rise in Foxo1 mRNA may account for the lack of adipocyte hyperplasia (14, 15). The lack of expansion of the adipocyte mass of the transgenic mice during overnutrition suggests a *forme fruste* of generalized lipodystrophy, in which adipocytes, while present, are incapable of augmenting their storage capacity to accommodate the level of overnutrition.

Indeed, lipid accumulation in the liver, skeletal muscle, and heart of HFD-fed transgenic mice was no less than in HFD-fed wild-type mice, despite increased fatty acid oxidation in adipocytes. The similar hyperinsulinemia suggests comparable degree of insulin resistance. This finding implies that any restriction in the ability of the adipocytes to enlarge their storage capacity to accommodate caloric surplus, whether due to absence of adipocytes, as in generalized lipodystrophy, or inability of adipocytes to expand, as in Lepr-b-transgenic mice, will predispose to ectopic lipid deposition of unoxidized surplus calories.

Lepr-b down-regulation could be an insulin-mediated or, at least, insulin-requiring phenomenon, because it seems tightly coupled to the increase in adipocyte TG and the up-regulation of leptin expression, which, like lipogenesis, is insulin-requiring (18). Indeed, adipocyte-specific interruption of insulin signaling (19) prevents both DIO and its complications, i.e., ectopic lipid overaccumulation and insulin resistance.

The study reveals in white adipocytes the existence of a previously unrecognized, Lepr-b-mediated energy-regulating system. This "short-loop" leptinergic system is independent of the far more complex hypothalamic "long loop" energy regulation. Both are rapidly turned off during overnutrition, thereby permitting the conservation of surplus calories and facilitating DIO. Teleologically, one wonders why such a potential caloric sump would evolve, only to be inactivated during overnutrition. One possibility is that the leptinergic blockade in adipocytes during overnutrition may be variable and that variations in the adipocyte blockade may determine proneness to obesity. Interestingly, preliminary studies of WAT from morbidly obese patients undergoing bariatric surgery reveal virtually undetectable levels of Lepr-b mRNA, in contrast to young, lean volunteers, in whom it varies over a broad range from very low to very high (N. Abate, W. Cook, and E. Livingston, personal communication).

Finally, down-regulation of the leptin receptor provides a rational explanation for the failure of obesity treatment with leptin. A period of starvation prior to leptin therapy might enhance its efficacy.

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