Combined Leptin Actions on Adipose Tissue and Hypothalamus are Required to Deplete Adipocyte Fat in Lean Rats: Implications for Obesity Treatment*

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Intense hyperleptinemia completely depletes adipocyte fat of normal rats within 14 days. To determine the mechanism, epididymal fat pads from normal wild-type (+/*) and obese (fa/fa) Zucker Diabetic Fatty (ZDF) donor rats were transplanted into normal +/* and fa/fa ZDF recipients. Hyperleptinemia induced by adenovirus-leptin administration depleted all fat from native fat pads and from fat transplants from +/* donors, but not from transplants from fa/fa ZDF donors with defective leptin receptors (Lepr). In both native and transplanted +/* fat pads large numbers of mitochondria were apparent and genes involved in fatty acid oxidation were upregulated. However, +/* fat pads transplanted into fa/fa recipients did not respond to hyperleptinemia, suggesting lack of an essential leptin-stimulated co hormone(s). In +/*, but not in fa/fa rats, plasma catecholamine levels rose and both P-STAT3 and P-CREB increased in adipose tissue, suggesting that both direct and indirect (hypothalamic) Lepr-mediated actions of hyperleptinemia are involved in depletion of adipocyte fat.

In normal lean rodents the induction of hyperleptinemia by administration of recombinant adenovirus containing the leptin cDNA (AdCMV-leptin) causes all visible fat to melt away within 7 days (1). In what is probably the most striking effect of this hormone, white adipocytes are transformed into fatless cells filled with small mitochondria (2). In addition, genes such as uncoupling protein-1 (UCP-1) and peroxisome proliferator-activated receptor-γ-coactivator-1-α (PGC-1α), normally expressed in brown, but not white adipocytes, are expressed at high levels, while adipocyte markers, such as leptin and aP2, are profoundly downregulated (2). Despite their similarities to brown adipocytes, these transformed white adipocytes differ sufficiently to warrant designation as a novel derivative cell, the “post-adipocyte” (2). Although fat-depletion of adipocytes by hyperleptinemia occurs only in lean and not in obese rodents, understanding of its mechanisms may have implications for the treatment of obesity.

The fat depletion induced by experimental hyperleptinemia differs from that of naturally-occurring catabolic states, such as in starvation and insulin deficiency, in which free fatty acids (FFA) are released from the adipocytes and oxidized in the liver. The fat depletion of experimental hyperleptinemia is unaccompanied by an increase in plasma FFA or ketones (3, 4) and the molecular and morphologic changes (2) imply that the oxidation takes place within the transformed adipocytes.

The pathway by which hyperleptinemia induces transformation of adipocytes to post-adipocytes has not been clearly identified. We know that leptin-responsive centers in the hypothalamus regulate energy metabolism and feeding behavior (3-8), but there is also evidence for direct leptin action on adipocytes (9-11) and on...
other tissues (12-17). Here we report that the fat-depleting action of hyperleptinemia requires both direct leptin receptor (Lepr)-mediated action on adipocytes coordinated with Lepr-mediated stimulation of sympathetic and perhaps other centers in the hypothalamus.

**Experimental Procedures**

**Animals** - Male obese Zucker Diabetic Fatty (ZDF) homozygous (fa/fa) and wild-type (+/+) rats were bred in our laboratory and housed in individual cages with a constant temperature and 12 h of light alternating with 12 h darkness. All were fed standard chow (Teklad mouse/rat diet, Teklad, Madison, WI) and had access to chow and water ad libitum.

**Experimental Procedures** - Epididymal fat pads were isolated from six-week-old ZDF +/+ or fa/fa rats under ketamine-xylazine anesthesia. The fat pads were weighed, washed with saline and trimmed so that each transplant weighed 300 mg. Fat pads from ZDF (+/+) donors were transplanted on top of the right anterior rectus abdominis muscle of the ZDF (+/+) recipient rats and fat pads from ZDF (fa/fa) donors on the left side. The abdominal wound was then closed. In other experiments ZDF (fa/fa) rats were used as recipients. Ten days later ZDF (+/+) rats were infused with $1 \times 10^{12}$ plaque-forming units of recombinant adenovirus containing either the leptin cDNA (AdCMV-leptin) or, as an inactive control, the β-galactosidase cDNA (AdCMV-β-gal). Most animals were sacrificed 14 days after the virus injection, or in some cases at 3 or 7 days after. The abdominal wound was then closed.

**Microscopy** - Fat tissue was fixed with 2% glutaraldehyde in cacodylate buffer, post-fixed in osmium tetroxide, stained en bloc with uranyl acetate, dehydrated in graded ethanols and embedded in Epon 812. Semithin (1 μm-thick) and thin sections were cut with an LKB ultramicrotome. Semithin sections were photographed under phase contrast using a Zeiss photomicroscope. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips CM10 electron microscope.

**Quantitative Real-Time RT-PCR** - Total RNA was extracted by the Trizol isolation method according to the manufacturer’s protocol (Life Technologies, Gaithersburg, MD). Total RNA (2 μg) was treated with RNase-free DNase (Invitrogen, Carlsbad, CA) and first-strand cDNA was generated with the random hexamer primer in the first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA). Specific primers were designed using primer express software (Applied Biosystems) and their primer sequences were followings; ACCα, TCGAAGAGCTTATATCGCCTATGA (forward) and GGGCACGATGAAGCTAAATTC (reverse), FAS, GGAGGACGCTTCTCTGT (forward) and GCTGAATACGACCAGCACTAC (reverse), SCD-1, CGCTCCGCCACACCTTGAT and reverse GTGGTCGTGAGAAGAAGTGGAGAT, and PGC-1α, forward GCCCCAGCACAACCTCAGCATG and reverse TGGGATTGATTTTGATGGT. The sequence for the control 18S ribosomal RNA was purchased from Applied Biosystems and used as the invariant control. The real-time RT-PCR reaction contained in a final volume of 10 μl, 10 ng of reverse transcribed total RNA, 167 nM of forward and reverse primers and 2 x PCR master mix. PCR reaction was carried out in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All reactions were done in triplicate.

**Determination of TG Content of Adipose Tissue** - A portion of the fat was dissected from the native epididymal fat pad and transplants. Tissues were placed in 2 ml of homogenizing buffer containing 18 mM of tri (hydroxymethyl) aminomethane HCl (pH=7.4), 300 mM of D-mannitol, and 50 mM EGTA and 1 mM PMSF and were homogenized by using a polytron for 30 sec. Homogenates were treated with 4 ml of a 2:1 chloroform:methanol mixture for 1 h with occasional vortexing. 100 μl of homogenates was used for protein assay and lipid was further extracted. TG content was measured with TG diagnostic kit (Sigma, St. Louis, MO).

**Immunoblotting** - Total cell extracts prepared from fat tissues of rats or mice were resolved by SDS-PAGE and transferred to PVDF membrane (Amersham, Piscataway, NJ). The blotted membrane was blocked in 1 x TBS containing...
0.1% Tween and 5% nonfat dry milk (TBST-MLK) for 1 h at room temperature with gentle, constant agitation. After incubation with rabbit primary antibodies to phospho-STAT3 (Tyr705), STAT3, phospho-CREB (Ser133) and CREB (Cell Signaling Technology, Beverly, MA) 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 HRP-conjugated IgG in TBST-MLK for 1h at room temperature with agitation. The membrane was then washed three times with TBST buffer, and the proteins of interest on immunoblots were detected using an enhanced chemiluminescence detection system (Amersham). γ-tubulin was employed throughout as a loading control.

Radioimmunoassays - Plasma leptin was measured by leptin RIA kit (Linco Research Immunoassay, St. Charles, MO). Plasma catecholamines were measured with Bi-CAT RIA kit (Alpco Diagnostics, Windham, NH).

Statistics - The results in this study are presented as means ± SD and were evaluated with Student’s t test for two groups.

RESULTS

Direct Lepr-mediated action on adipocytes is necessary for depletion of fat stores by hyperleptinemia: To determine if normal leptin receptors (Leprs) on adipocytes are required for the fat-depleting action of hyperleptinemia in vivo, we transplanted into 6 week-old normal, lean ZDF (+/+) recipients a fat pad resected from a 5-6 week-old normal lean wild-type (+/+) ZDF donor rat with functioning Leprs and a fat pad from an obese ZDF (fa/fa) rat and a fat pad from an obese ZDF (fa/fa) rat with a loss-of-function mutation in the Lepr gene (18). Both fat transplants were readily visible and palpable throughout the first 10 post-operative days. If the fat-depleting effects of hyperleptinemia on adipocytes require normal Leprs on white adipocytes, fat will disappear from the 3 fat pads with functioning Leprs, i.e., from both native +/- fat pads of the recipients and from the +/+ transplant, but not from the fa/fa transplant. Alternatively, if these effects are mediated by neurotransmitted signals from leptin-responsive hypothalamic centers, only the 2 intact fat pads will lose their fat.

At 10 days after the surgery the recipients received an intravenous injection of recombinant adenovirus containing either the leptin cDNA (AdCMV-leptin) or the β-gal cDNA (AdCMV-β-gal). The diet of the latter control rats was matched to that of the AdCMV-leptin-treated group. Plasma leptin levels rose in the AdCMV-leptin-treated group from 1.0 ± 0.2 to 81 ± 35 ng/ml during the first week, food intake declined by 43% and body weight of the +/- recipients fell within 14 d from 267 ± 33 before AdCMV-leptin treatment to 223 ± 27 g at 10 days after treatment. On external inspection and palpation, transplants from +/- donors shrank progressively in parallel with loss of body weight, while those from the fa/fa donors did not change (Figure 1A). By contrast, in AdCMV-β-gal-treated control recipients that had been diet-matched to the hyperleptinemic rats plasma leptin did not rise and no change in the size of either +/- or fa/fa transplants or in the native intact fat pads (Figure 1B) was observed (Figure 1D). On dissection of the AdCMV-leptin-treated +/- recipients, fat tissue could not be identified in either the +/+ transplant, the native fat pads or anywhere else in the body other than in the fa/fa transplant (Figure 1C). The native epididymal fat pads appeared as slender strands of vascular tissue devoid of visible fat, while the ~300 mg +/- fat transplant was reduced to a flat hypervascular red patch resembling the remnant of fat-depleted native fat pads (Figure 1C). Only 67 ± 13 mg, or 22% of the original weight of +/- transplants could be recovered from their transplantation sites (Table 1A). By contrast, in the normoleptinemic AdCMV-β-gal-treated control rats pairfed to the leptinized rats, 273 ± 16 mg or 91 % of the +/- transplants was recovered (Table 1A). The TG/protein ratio of the various transplants corresponded to the change in the weight of the transplant (Table 1A). Thus, hyperleptinemic fat depletion in the denervated +/- fat pad transplants with normal Lepr seemed to parallel that in the intact +/- native fat pads with normal Lepr and intact neurocircuitry (Figure 1C).

By contrast, the Lepr-defective fa/fa fat pads transplanted into +/- recipients did not respond to hyperleptinemia. The rounded, elevated mass that was visible and palpable through the abdominal wall immediately postoperatively was still present (Figure 1C). When excised, the weight
of fa/fa transplants averaged 224 ± 24 mg, or 75% of the original weight. Again the TG content of the transplants corresponded to their weight (Table 1A). These results indicate that depletion of TG in adipocytes requires the presence of functioning Lepr in the fat tissue.

To exclude the possibility that the unresponsiveness of the adipocytes from obese, age-matched fa/fa rats to hyperleptinemia was the nonspecific result of their much larger size, we transplanted into +/+ recipients fat pads from pre-obese 4-week old fa/fa donor rats in which adipocyte diameters approximated those of 6-week old lean rats. These fat pads also failed to respond to hyperleptinemia, evidence that receptor dysfunction, rather than adipocyte size, was the cause of the unresponsiveness (data not shown).

Comparison of morphologic transformation of adipocytes to post-adipocytes by hyperleptinemia in transplanted +/+ and fa/fa fat pads: Microscopic examination of the native and transplanted fat pads in +/+ recipient rats made hyperleptinemic by AdCMV-leptin treatment 14 days earlier confirmed the impression gained by gross examination. Phase contrast views of semithin sections of the intact native +/+ fat pads (Figure 2A) and fat pads transplanted from lean ZDF +/+ donors (Figure 2B) revealed in both the same fatless, shrunken “post-adipocytes” previously described in detail in the intact native fat pads of hyperleptinemic +/+ rats (2). At the ultrastructural level both native (Figure 2C) and transplanted cells (Figure 2D) contained the same apparent abundance of distinctive mitochondria with an electron-dense matrix (inset of Figure 2D). By contrast, phase contrast views of semithin sections of fa/fa fat transplants in hyperleptinemic +/+ recipients contained normal-appearing white adipocytes with a typical lipid droplet (Figure 2E), as did +/+ fat pads transplanted into normoleptinemic +/+ control recipients that had been treated with AdCMV-β-gal instead of AdCMV-leptin, and diet-matched to the leptinized rats (Figure 2F). In addition, the molecular changes observed in the +/+ transplants and in the native +/+ fat pads of hyperleptinemic rats (Figure 3) were not present in these normoleptinemic controls (data not shown).

The foregoing findings demonstrate that the action of hyperleptinemia on the fat metabolism of white adipocytes in lean rats requires the presence of wild-type (+/+) Lepr on adipocytes but does not require neural connections to the hypothalamus.

Molecular responses to hyperleptinemia in intact versus transplanted fat: We previously reported that the transformation of white adipocytes induced by hyperleptinemia was accompanied by equally striking array of molecular changes (2). For example, PGC-1α, a gene that regulates mitochondrial biosynthesis in brown adipocytes (19), but is not normally expressed in white adipocytes, is greatly upregulated by hyperleptinemia, while the normally high expression level of lipogenic enzymes, such as acetyl CoA carboxylase-2 (ACC2), fatty acid synthase (FAS) and stearoyl CoA desaturase-1 (SCD-1), is profoundly suppressed in adipose tissue after hyperleptinemia (2). To determine if the hyperleptinemia-induced expression profile in the fat pads transplanted from normal rats matched that of intact fat pads, we compared the mRNA of ACC2, FAS, SCD-1 and PGC-1α by means of real-time RT-PCR (Figure 3). PGC-1α was increased and ACC2, FAS and SCD-1 were reduced in both the intact native fat pads and in the wild-type fat transplants from hyperleptinemic wild-type rats, but not in fa/fa fat pads from Lepr-defective obese ZDF fa/fa rats. However, the magnitude of the PGC-1α response in the +/+ transplant fat was less than in the native +/+ fat pad, possibly reflecting the loss of normal innervation and circulation.

Phospho-CREB and phospho-STAT3 are both increased in fat tissue during fat depletion by hyperleptinemia: Lepr-mediated leptin signaling is transduced by signal transduction and activation of transcription (STAT)-3 (20), while catecholamine signaling is transduced by cAMP response element-binding protein (CREB) (21). To determine at the tissue level if both direct leptin-mediated and indirect catecholamine-mediated actions on adipocytes could be involved in hyperleptinemic fat depletion, we compared the activation of the respective transcription factors during fat depletion of intact native adipose tissue of normal mice and rats. We observed a major increase in P-STAT3 to a peak at 3 days after induction of hyperleptinemia, followed by a decline at 7 days (Figure 4A), consistent with direct leptin action. P-CREB remained at baseline levels at 3 days but was markedly increased at day...
7 (Figure 4A), consistent with indirect leptin action via hypothalamic sympathetic centers. At day 7 no adipocyte fat was detected microscopically in the fat pads (Figure 5B).

STAT3 and CREB activation was also observed in fat transplants from normal rats with hyperleptinemia (Figure 4A, center panels), but not in fa/fa rats (Figure 4A, right panels). However, the increases in P-STAT3 and P-CREB/CREB ratio were less than in the intact fat pads, perhaps reflecting the surgical disruption of the normal circulation. This combination of upregulation of the adipose tissue oxidative machinery, in concert with adrenergic enhancement of lipolysis, could explain how fat stores are depleted by hyperleptinemia without the increase in plasma free fatty acid levels that characterizes other forms of fat depletion (11, 13).

**Fat pads from +/+ donors do not respond to hyperleptinemia when transplanted into obese fa/fa recipients:** The foregoing results indicate that direct Lepr-mediated action of leptin on adipose tissue is necessary for fat depletion, but they do not indicate whether or not direct action is sufficient for the effect. To determine if leptin action on the regulatory centers of the hypothalamus might also be required, we repeated the foregoing transplantation experiments using leptin-unresponsive, obese ZDF fa/fa rats as recipients. When +/+ fat pads were transplanted into fa/fa recipient rats, equivalent hyperleptinemia had no fat-depleting action; in fact, they accumulated more TG than the fa/fa transplants (Table 1B and Figure 5A). Their unresponsiveness could signify either lack of a required co-hormone normally stimulated by leptin (22-25) via hypothalamic sympathetic centers, such as catecholamines, and/or a blockade of the direct action of leptin on the +/+ fat pad transplant by a circulating factor present in the plasma of the fa/fa recipients. The greater accumulation of fat in +/+ fat pads could reflect the fact that normal adipocytes are able to undergo hypertrophy when transplanted into a hyperlipidemic environment, whereas the obese adipocytes from hyperlipidemic fa/fa donors had already undergone maximum hypertrophy before transplantation.

We next examined the P-STAT3 and P-CREB in the unresponsive +/+ fat pads transplanted into the fa/fa recipients, treated with AdCMV-leptin. As expected, neither STAT3 nor CREB in native intact fat pads or in fa/fa transplants were activated by hyperleptinemia (Figure 5B). The lack of an increase in P-STAT3 in the +/+ fat transplants was unexpected and could signify a circulating blocker of direct leptin action on adipocytes in the plasma of the fa/fa recipients. The lack of an increase in P-CREB was expected and is attributed to leptin-unresponsiveness of hypothalamic sympathetic nuclei in fa/fa recipients.

**Catecholamines as co-hormones for hyperleptinemic fat depletion:** The hyperleptinemia-induced increase in P-CREB in +/+ fat tissue suggested that catecholamines could play a co-hormonal role in the fat-depletion observed in normal rats by stimulating lipolysis to provide substrate for the leptin-upregulated oxidative machinery of the white adipocytes (2). If so, leptin-stimulated catecholamines levels in plasma of lean +/+ rats should be higher than in leptin-unresponsive obese fa/fa rats. In fact, we observed that hyperleptinemia stimulated an increase in plasma levels of both norepinephrine and epinephrine in +/+ rats, but not in fa/fa rats (Table 2), confirming earlier work by others (22-25). Nevertheless, basal norepinephrine levels in the fa/fa rats were higher than leptin-stimulated levels in the +/+ rats. It is not clear why this was not accompanied by a higher basal level of P-CREB.

**Circulating blockers of hyperleptinemia:** The lack of an increase in P-STAT3 in response to hyperleptinemia in +/+ fat pads transplanted into fa/fa rats could be due to a circulating blocker of leptin action. At least 3 potential circulating blockers of leptin action have been reported. Two of these, the soluble leptin receptor and C-reactive protein (CRP), have already been shown to be increased in the circulation of fa/fa ZDF rats (26, 27) and to impair the action of leptin (27, 28).

Another potential candidate blocker is hyperlipidemia, which is believed to impede leptin transport across the blood-brain barrier (29). It seemed possible that the marked hyperlipidemia of obese fa/fa ZDF rats might also block direct action of hyperleptinemia on peripheral tissues. To test this possibility, we administered gemfibrozol in order to lower plasma TG of the fa/fa recipients beginning 7 days prior to inducing the hyperleptinemia. Although the plasma TG levels...
of the gemfibrozol-treated ZDF fa/fa recipients declined from 530 ± 174 to 106± 32 mg per dl, hyperleptinemia still failed to reduce either the weight or TG content of +/+ fat transplants in fa/fa recipients (data not shown). Nor was there an increase in PGC-1α or UCP-1 or -2 expression in +/+ fat pads transplanted into fa/fa recipients. These negative findings exclude hyperlipidemia as the cause of a block of hyperleptinemic action on the hypothalamus, and point instead to the previously established elevations of soluble Lepr and CRP as a possible cause of the leptin unresponsiveness.

**DISCUSSION**

These findings indicate that fat depletion of adipocytes by hyperleptinemia requires a combination of direct leptinergic and indirect hypothalamic actions of leptin. The direct action, which is mediated, at least in part, through activation of STAT3, upregulates the oxidative machinery of white adipocytes and enhances oxidation of the fatty acyl CoA (2). The indirect leptin action is presumed to stimulate hypothalamic sympathetic centers to release catecholamines (22) and activate CREB in the fat tissue. The fact that hyperleptinemia stimulated plasma epinephrine and norepinephrine leptin-responsive levels in +/+ rats, but not in the leptin-unresponsive fa/fa rats is consistent with a co-hormonal role for catecholamines in the fat depletion. The lipolytic action of catecholamines on adipocytes would increase the availability of fatty acyl CoA for mitochondrial oxidation, while reducing stored TG (Figure 6). The uncoupling proteins upregulated by hyperleptinemia (2) would dissipate the energy generated as heat. The glycerol thus generated enters the circulation unaccompanied by free fatty acids (4). The lipolysis that results from this supraphysiologic hyperleptinemia (Figure 6) differs from both physiologic and pathophysiologic adipocyte lipolysis of starvation and insulin deficiency, in which both FAs and glycerol are released into the circulation to provide fuel for other tissues. Even though hyperleptinemia causes profound suppression of insulin levels, the resulting weight loss differs strikingly from that of insulin deficiency, in which there is a marked increase in free fatty acids and glucagon-stimulated ketosis with loss of lean body mass as well as fat. In weight loss induced by hyperleptinemia, by contrast, glucagon is suppressed, there is no increase in fatty acids or ketones, and weight loss is confined to fat tissue with no loss of lean body mass. This is probably the result of marked insulin sensitivity despite hypoinsulinemia.

However, since there is no current evidence to prove that catecholamines are required for hyperleptinemic depletion of adipocyte fat, other hypothalamic factors could also be involved. Cocaine- and amphetamine-regulated transcript (CART) (30), previously shown to be upregulated by intense hyperleptinemia in normal but not in fa/fa rats (31), and to increase P-CREB in certain neurons (32), is also a prime candidate for a co-hormonal role. CART tends to increase lipid oxidation (33), and may therefore contribute to the fat-depleting action of intense hyperleptinemia.

The resistance of +/+ fat pads to hyperleptinemia when transplanted into obese fa/fa ZDF recipient rats could result from a circulating blocker of direct hyperleptinemic action on the fat transplant, or from lack of one or more leptin-stimulated co-factors essential for hyperleptinemic fat depletion, or both. Hypertriglyceridemia, one of the circulating factors proposed to block the hypothalamic action of leptin (29), was excluded as the cause of the unresponsiveness of the +/+ fat pads to hyperleptinemia when they had been transplanted into fa/fa recipients. However, soluble Lepr remains a highly likely candidate as a circulating blocker in fa/fa rats since is it increased in the ZDF fa/fa rats (26), and is known to impair leptin action (28). More recently, elevated C-reactive protein levels of obesity have been reported to attenuate leptin action (27). Either or both of these factors could explain the unresponsiveness of +/+ fat pads to leptin.

Interestingly, in fa/fa recipients the accumulation of fat was greater in the +/+ transplants than in the fa/fa transplants (Figure 4A). The mechanism of the difference is not known but could well reflect the fact that adipocytes transplanted from fa/fa rats had already undergone maximal hypertrophy in their obese hosts, while the adipocytes from lean rats had an untapped capacity to undergo hypertrophy.

The bi- or multi-hormonal requirements for fat depletion in white adipocytes may help
explain why diet-induced obesity fails to respond to therapy with recombinant leptin (34). Not only does Lepr-b disappear from the fat during overnutrition (35), thereby eliminating the direct leptinergic response to the hormone, but the adrenergic response to leptin may be attenuated by leptin resistance in the hypothalamus (36). The reversal of leptin resistance at both these sites will be necessary if the fat-depleting activity of leptin is to be exploited therapeutically for the treatment of obesity.

The results of this study, taken together with the report of de Luca et al. (36), suggest that sharp differences exist between reversal of established obesity and prevention of its development. It is clear from their study that Lepr-b expression in the central nervous system of db/db mice can prevent the overaccumulation of fat in adipocytes by regulating energy balance in the absence of peripheral expression of Lepr-b. However, the findings of this study indicate here that the rapid depletion of adipocyte fat by hyperleptinemia, in lean rats, as distinct from preventing obesity, requires not only the hypothalamic actions of leptin, but also the presence of functioning Lepr on adipocytes. The failure of leptin treatment to reverse human obesity (34) can perhaps be explained by the underexpression of Lepr-b on adipocytes of diet-induced obesity, as recently reported in overfed rodents (35).

In summary, it appears that in normal, lean rodents the rapid disappearance of body fat caused by intense hyperleptinemia involves both direct action of leptin on the adipocytes, and indirect action of leptin on leptin-responsive hypothalamic centers. Reversal of obesity with leptin therapy may require recognition of this dual requirement for leptin’s lipid-depleting action on adipocytes.

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**FOOTNOTES**

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FIGURE LEGENDS

Fig 1. Gross appearance of transplanted and intact native epididymal fat pads in hyperleptinemic and normoleptinemic wild-type (+/+ ZDF recipient rats and in obese (fa/fa) ZDF recipients. A. External view of a fat pad from a +/+ donor (left) and an fa/fa donor (right) transplanted into a hyperleptinemic +/+ recipient treated with AdCMV-leptin. B. External view of a +/+ transplant (left) and an fa/fa transplant (right) in a normoleptinemic +/+ control recipient. The control recipients had been treated with AdCMV-β-gal and were diet-matched to hyperleptinemic rats. C. View of the exposed native +/+ fat pads on the gauze pad and the +/+ (left) and fa/fa (right) transplants in the hyperleptinemic +/+ recipient of Panel A. D. View of the +/+ and fa/fa transplants and native fat pads of the AdCMV-β-gal treated +/+ recipient of Panel B.

Fig 2. A. and B. Phase contrast views of semithin sections of native epididymal fat pads of +/+ rats (A) and of fat transplants they received from lean wild-type +/+ donor rats (B) obtained at 14 days after induction of hyperleptinemia by AdCMV-leptin administration. C. and D. Electron microscopic appearance of native +/+ epididymal fat pads of wild-type recipients (C) and fat pads transplanted from lean +/+ donors (D). The adipocytes of both native +/+ and transplanted +/+ fat pads have been transformed into shrunken, wrinkled "post-adipocytes" devoid of fat droplets but with a profusion of mitochondria with an electron dense matrix. This appearance is characteristic of hyperleptinemic fat depletion of white adipocytes (see inset). E. Phase contrast views of semithin sections of fa/fa fat pads transplanted from obese ZDF donors into +/+ recipients indicate unresponsiveness to hyperleptinemia, with normal-appearing white adipocytes containing a large lipid droplet. F. Phase contrast views of semithin sections of a fat pad transplant from a normal wild-type +/+ donor into a normal wild-type +/+ control without hyperleptinemia. These normoleptinemic rats had been treated with AdCMV-β-gal instead of AdCMV-leptin and were diet-matched to the hyperleptinemic rats. Both native and transplanted adipocytes retain the appearance of normal white adipocytes.

Fig 3. The effect of hyperleptinemia on the mRNA of relevant genes in native +/+ fat pads of +/+ recipients and in fat transplants from +/+ and fa/fa donors. Hyperleptinemia was induced by AdCMV-leptin administration (■). AdCMV-β-gal was administered to control animals (□), which were then diet-matched to the hyperleptinemic group. mRNA levels are normalized for 18S mRNA. ACC-2: acetyl CoA carboxylase-2; FAS: fatty acid synthase; SCD-1: stearoyl desaturase-1; PGC1α: peroxisome proliferator-activated receptor-γ-coactivator 1α. (* p<0.05; ** p<0.01; N=4 or 5).

Fig 4. A. Representative immunoblots for P-STAT3, STAT3, P-CREB and CREB from intact fat of lean +/+ rats (far left) and transplanted fat pads from +/+ (center) and fa/fa (right) donors 3 and 7 days after induction of hyperleptinemia. B. Ratio of mean densitometric readings of P-STAT/STAT3 and P-CREB/CREB ratios. γ-tubulin was employed throughout as a loading control. C. Representative sections of intact fat pads of lean +/+ rats before (0) and at 3 and 7 days after induction of hyperleptinemia (left) and transplanted fat pads from +/+ (center) and fa/fa (right) donors. The unleptinized group is marked “control” rather than “0” because they received AdCMV-b-gal treatment 3 days before their fat was obtained. *p<0.01; **p<0.05; #p<0.03.

Fig 5. A. Gross appearance of intact native fat pads of an fa/fa obese ZDF rat 14 days after induction of hyperleptinemia (left) and of transplanted fat pads from a +/+ donor and a fa/fa donor into a fa/fa obese ZDF recipient made hyperleptinemic. B. Representative immunoblots for P-STAT3, STAT3, P-CREB and CREB in intact fat pads (far left) and a +/+ fat transplant (center) and an fa/fa transplant (right). C. Ratio of mean densitometric readings of P-STAT/STAT3 and P-CREB/CREB ratios. γ-tubulin was employed throughout as a loading control.
Fig 6. A proposed mechanism for fat depletion by AdCMV-leptin-induced hyperleptinemia in normal rodents. The virus infects the liver, which produces intense hyperleptinemia. STAT3 (P-STAT3) is activated in adipose tissue via leptin receptors. This causes upregulation of mitochondrial biogenesis in adipocytes. Meanwhile leptin, acting via leptin receptors in the hypothalamus, stimulates sympathetic nuclei to increase catecholamines, which activate CREB (CREB-P) in adipose tissue. Lipolysis of triacylglycerol (TG) increases, but the fatty acyl CoA (FA) is oxidized to CO₂ and H₂O, and the glycerol is released.
Table 1. Effects of hyperleptinemia on epididymal fat transplants. Plasma leptin and body weight change of hyperleptinemic and normoleptinemic wild-type (+/+)(A) and obese (fa/fa) ZDF recipients (B) are compared 9 days after treatment with either AdCMV-β-gal or AdCMV-leptin, together with the weight and TG/protein ratio of the transplants. All fat pads weighed 300 mg at the time of transplantation. AdCMV-β-gal-treated rats were diet-matched to the AdCMV-leptin-treated rats.

| Recipients | Plasma leptin (ng/ml) | Body Wt Change (g) | Transplant +/+ donors | Transplant fa/fa donors |
|------------|----------------------|--------------------|-----------------------|------------------------|
|            |                      |                    | Wt (mg) | Tg/protein | Wt (mg) | Tg/protein |
| A. ZDF (+/+) |                      |                    |            |            |            |            |
| AdCMV-leptin | 81 ± 35             | ↓44 ± 11           | 67 ± 13↑†  | 20 ± 4↑†   | 224 ± 24 | 404 ± 178 |
|             | (↓78%) (n=15)       |                    | (n=6)     |            | (↓25%) (n=15) | (n=6)     |
| AdCMV-β-gal | 1.1 ± 0.2           | ↑5 ± 5             | 273 ± 16  | 438 ± 384 | 253 ± 33 | 329 ± 57  |
|             | (↓9%) (n=8)         |                    | (n=6)     |            | (↓16%) (n=8) | (n=6)     |
| B. ZDF (fa/fa) |                    |                    |            |            |            |            |
| AdCMV-leptin | 122 ± 12            | ↑88 ± 2            | 570 ± 56↑# | 585 ± 169 | 283 ± 38 | 329 ± 57  |
|             | (↑190%) (n=3)       |                    | (n=3)     |            | (↓6%) (n=3) | (n=3)     |
| AdCMV-β-gal | 24 ± 6              | ↑115 ± 12          | 725 ± 104↑# | 577± 155 | 290 ± 48 | 319 ±117  |
|             | (↑242%) (n=4)       |                    | (n=4)     |            | (↓3%) (n=4) | (n=4)     |

*p<0.01 vs ZDF(fa/fa) and AdCMV-leptin
†p<0.01 vs ZDF(fa/fa) and AdCMV-β-gal
#p<0.01 vs ZDF(fa/fa)



Table 2. Effect of adenovirus-induced (AdCMV-leptin) hyperleptinemia on plasma epinephrine and norepinephrine levels (ng/ml) in +/+ and fa/fa ZDF rats (n=4).

|       | AdCMV-βgal | AdCMV-leptin | p value |
|-------|------------|--------------|---------|
| +/+   | Epinephrine| 10±4.6       | 21.2±7.9| 0.05   |
| +/+   | Norepinephrine| 13.3±2.1 | 25.4±9.6| 0.05   |
| fa/fa | Epinephrine| 6.8±4.6      | 5.5±1.4 | 0.61   |
| fa/fa | Norepinephrine| 31.2±11.4  | 26.7±6.5| 0.52   |
Figure 3
**Figure 4**

**INTACT FAT**

Lean (+/+)

- β-gal
- Leptin

- P-STAT3
- STAT3
- P-CREB
- CREB

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**TRANSPLANTED FAT**

Lean (+/+)

- β-gal
- Leptin

Fatty (fa/fa)

- β-gal
- Leptin

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**B**

- P-STAT3/STAT3 ratio
- P-CREB/CREB ratio

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**C**

Days after AdCMV-leptin

0 3 7

Control 3 7

Control 3 7

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Figure 5
Figure 6
Combined leptin actions on adipose tissue and hypothalamus are required to deplete adipocyte fat in lean rats: Implications for obesity treatment
Byung-Hyun Park, May-Yun Wang, Young Lee, Xinxin Yu, Mariella Ravazzola, Lelio Orsi and Roger H. Unger

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