Leptin treatment markedly increased plasma adiponectin but barely decreased plasma resistin of ob/ob mice

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Leptin treatment markedly increased plasma adiponectin but barely decreased plasma resistin of ob/ob mice. Am J Physiol Endocrinol Metab 287: E446–E453, 2004. First published May 4, 2004; 10.1152/ajpendo.00488.2003.—Adipocytes, adiponectin (ApN) and leptin are two adipocytokines that control fuel homeostasis, body weight, and insulin sensitivity. Their interplay is still poorly studied. These hormones are either undetectable or decreased in obese, diabetic ob/ob mice. We examined the effects of leptin treatment on ApN gene expression, protein production, secretion, and circulating levels of ob/ob mice. We also briefly tackled the influence of this treatment on resistin, another adipocytokine involved in obesity-related insulin resistance. Leptin-treated (T) obese mice (continuous sc infusion for 6 days) were compared with untreated lean (L), untreated obese (O), and untreated pair-fed obese (PF) mice. Blood was collected throughout the study. At day 3 or day 6, fat pads were either directly analyzed (mRNA, ApN content) or cultured for up to 48 h (ApN secretion). The direct effect of leptin was also studied in 3T3-F442A adipocytes. Compared with L mice, ApN content of visceral or subcutaneous fat and ApN secretion by adipose explants were blunted in obese mice. Accordingly, plasma ApN levels of O mice were decreased by 50%. Leptin treatment of ob/ob mice increased ApN mRNAs, ApN content, and secretion from the visceral depot by 50–80%. Leptin also directly stimulated ApN mRNAs and secretion from 3T3-F442A adipocytes. After 6 days of treatment, plasma ApN of ob/ob mice increased 2.5-fold, a rise that did not occur in PF mice. Plasma resistin of T mice was barely decreased. Leptin treatment, but not mere calorie restriction, corrects plasma ApN in obese mice by restoring adipose tissue ApN concentrations and secretion, at least in part, via a direct stimulation of ApN gene expression. Such a treatment only minimally affects circulating resistin, another adipocytokine involved in obesity, diabetes; adipokines; insulin sensitivity; calorie restriction; obesity; diabetes; adipokines; insulin sensitivity; calorie restriction.

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solved in 5 mM sodium citrate (pH 4) and administered for 6 (or 3) days to T mice as a continuous subcutaneous infusion (20 μg/day) via an osmotic minipump. Although food intake was normalized at constant delivery of the lower amount of the hormone, this dosage was found to be necessary to normalize metabolic parameters, hypothermia, and uncoupling protein expression in ob/ob mice (23). The minipump (Alzet model 2001; Alza, Palo Alto, CA) was implanted in the interscapular region of mice under light anesthesia. Mice from the other groups received pumps filled with vehicle. Unlike other animals fed ad libitum, PF mice received a restricted amount of food similar to that spontaneously ingested by T mice; this amount was adjusted daily and administered in two rations (one-third at 1000 and two-thirds at 2200). At the beginning of the study, the groups of ob/ob mice were matched for body weight and fed blood glucose (see Fig. 1). They were also matched, as seen a posteriori, for fed plasma insulin and adiponectin levels sampled on day 0 (see Figs. 1 and 2).

Body weight and food intake were measured daily. On several occasions, tail vein blood was collected from fed mice (between 0800 and 0900). At the end of the experiment, the mice were killed by decapitation (between 0800 and 1000), and larger blood samples were also saved. Pairs of visceral (intrapерitoneal-retrovesical) and subcutaneous (inguinal) white fat pads and interscapular brown adipose tissue were immediately removed and either frozen in liquid nitrogen and stored at −70°C or immediately used for ex vivo studies (adipose tissue culture).

The University Animal Care Committee approved all procedures.

Culture of adipose explants and 3T3-F442A adipocytes. Small fragments of mouse visceral and inguinal adipose tissue (2–3 mm³; explants) were prepared and cultured in MEM with Earle’s salts, supplemented with 0.5% BSA and antibiotics as described (15). It proved necessary to culture 400-mg adipose tissue for 24 h to obtain detectable amounts of ApN secreted into medium by fat from obese mice. These experimental conditions were therefore extended to all groups of mice. ApN secretion was linear for ≈24 h in each group. Aliquots of medium were saved during the culture and stored at −20°C.

Mouse 3T3-F442A preadipocytes were grown at 37°C in 5% CO₂ in basal medium (i.e., DMEM with 1 g/l glucose containing 10% FCS, 8 mg/l biotin, and antibiotics) (38). Two days after confluence, cells were induced to differentiate in induction medium [i.e., basal medium supplemented with 17 nM insulin, 2 nM triiodothyronine (T₃), 100 nM dexamethasone, and 100 μM IBMX]. Forty-eight hours later, the induction medium was replaced by a differentiation medium (i.e., basal medium supplemented with 17 nM insulin and 2 nM T₃) that was kept until the end of the study. One-half of medium volume was renewed every day from induction onward. At the time of the experiments (day 8 after initiation of differentiation), >90% of the cells had accumulated fat droplets, as evidenced by Oil Red O staining, and leptin was added to the differentiation medium.

Northern blot analysis and real-time PCR. Total RNA from adipose tissue was subjected to Northern blot analysis (21). The cDNA probes for mouse ApN and TNF-α have been described elsewhere (15, 43). The cDNA probe for mouse resistin was obtained after RT-PCR on total RNA from mouse adipose tissue (sense primer 5’ TCCTTGTCCCTGAACTGC 3’ and antisense primer 5’ TGGAACACCGCTCATT 3’). After hybridization with the radiolabeled probes (15), optical densities (OD) of the mRNA bands on the autoradiograms and of 18S rRNA on the membranes were quantified by scanning densitometry. Levels of specific mRNA were expressed relative to those of 18S rRNA.

ApN mRNAs from 3T3-F442A adipocytes were measured by real-time PCR. Total RNA (2 μg) was reverse transcribed using oligo(dT) primers and Superscript II Reverse Transcriptase (Life Technologies, Leek, The Netherlands). Total RNA equivalents (40 ng) were amplified with iQ Syber Green Supermix (Bio-Rad Laboratories, Brussels, Belgium) containing 300 nM of each specific primer by use of the iCycler iQ Real Time PCR Detection System (Bio-Rad). The primers designed for ApN were 5’ GCAGAGATGGGACTCTGGA 3’ (sense) and 5’ CCCTTCAGCTCTGTCATTCC 3’ (antisense), and those for rat were cyclophilin 5’ AACCCACCGTGTCTCTC 3’ (sense), and those for rat were cyclophilin 5’ TGCCCTTCCTACGTTTCC 3’ (antisense). The threshold cycles (Ct) for ApN and cyclophilin were measured in separate tubes and in duplicate. The amount of ApN mRNA, normalized to cyclophilin mRNA, was expressed relative to the respective control culture condition and calculated as 2⁻ΔCt (13).

Quantification of ApN and resistin. ApN concentrations were measured in plasma, culture medium, or homogenized adipose tissue samples (15). Aliquots from plasma (0.5 μl), medium (30 μl), Western blot; 100 μl, RIA), or tissue homogenates (2.5- to 10-μg protein) were analyzed by Western blot, as described (15), or by RIA (Linco Research, St. Charles, MO). There was a close correlation between absolute ApN levels obtained by both methods (P < 0.0001). Two species of ApN were detected on Western blots (15): one that migrates like recombinant ApN as a 30-kDa band (immature form), and the other found in plasma and in culture medium that migrates as a 32-kDa band (mature, posttranslationally modified form) (see Figs. 2 and 3D). In adipose tissue homogenates, both forms of ApN could be detected (see Fig. 3C).

Resistin levels were measured in duplicate on diluted (1:5) plasma samples using an ELISA kit (Phoenix Pharmaceuticals, Belmont, CA). These results were confirmed by a mouse resistin RIA kit (Linco).

Other analytical procedures. Blood glucose was measured using a glucometer (Elite, Bayer, Brussels, Belgium). Plasma insulin, corticosterone, total cholesterol, and triglyceride levels were determined as described (5, 44). Protein concentrations in tissue homogenates were measured by the Bradford method.

Statistical analysis. Results are given as means ± SE for the indicated number of mice. Multiple comparisons were carried out by ANOVA or by repeated-measures ANOVA followed by the Newman-Keuls test, and comparisons between two conditions were by unpaired or paired Student’s t-test or by a nonparametric test when appropriate. Differences were considered statistically significant at P < 0.05.

RESULTS

Initial body weights and daily food consumption of ob/ob mice were about twofold higher than those of L mice (Fig. 1A). Except for a decrease in food consumption that occurred immediately after surgery in all groups of mice, these parameters remained rather stable in L and O mice throughout the
study. In contrast, leptin-treated mice presented a progressive decrease in body weight (about −15% at day 6) and a persistent and marked reduction in food intake. PF mice behaved similarly: their body weight and food intake paralleled those of T mice.

Weights of brown adipose tissue from the interscapular region and white fat pads from two depots were heavier in O than in L mice (on the average ~4-fold for brown fat and ~13-fold for white fat; Table 1). As described (23), leptin treatment caused a marked decrease in brown adipose tissue weight, likely as a result of enhanced sympathetic tone (45). Both leptin and calorie restriction reduced inguinal (34), but not retrovesical, fat mass of obese mice (Table 1).

Obese mice were hyperglycemic and hyperinsulinemic compared with lean mice (Fig. 1B). Leptin treatment resulted in a normalization of plasma glucose and insulin levels, which decreased to values similar to those of L mice from the 6th or the 3rd day onward. Mere calorie restriction in PF mice also decreased to values similar to those of L mice from the 6th or the 3rd day onward. Likewise, leptin, but not pair-feeding, normalized the hypercholesterolemia that otherwise occurred in O mice. Treatment with the adipokine also attenuated plasma triglycerides (Table 1).

ApN circulates as a 32-kDa isoform in plasma of the four groups of mice (Fig. 2). Initial plasma ApN concentrations were 50% lower in ob/ob mice than in lean mice. These levels remained fairly stable in O and L mice during the experiment. Leptin treatment for 6 days induced a 2.5-fold rise in circulating ApN that actually exceeded L values, whereas no changes occurred in PF mice. ApN mRNA levels were measured in several adipose tissue sites. In brown adipose tissue, the ApN gene was not affected

### Table 1. Effects of leptin treatment on weight of several fat depots and on plasma lipid levels in ob/ob mice

<table>
<thead>
<tr>
<th>Adipose tissue region</th>
<th>Lean Mice (L)</th>
<th>ob/ob Mice</th>
<th>Pair-fed (PF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brown</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>0.14±0.02</td>
<td>0.50±0.10*</td>
<td>0.21±0.05†‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.53±0.04*</td>
<td></td>
</tr>
<tr>
<td><strong>White</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.40±0.07</td>
<td>5.78±0.50*</td>
<td>4.36±0.20†‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.05±0.27†‡</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.30±0.06</td>
<td>3.37±0.22*</td>
<td>3.22±0.11*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.42±0.15*</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma lipid levels, mmol/l</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>2.59±0.15</td>
<td>5.42±0.28*</td>
<td>2.91±0.16†‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.71±0.28†‡</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.35±0.18</td>
<td>1.85±0.41</td>
<td>0.81±0.05†‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.05±0.02</td>
<td></td>
</tr>
</tbody>
</table>

Sampling and measurements were made at end of study (day 6) in lean (L), untreated ob/ob (O), leptin-treated (T) ob/ob, and pair-fed (PF) ob/ob mice. Values are means ± SE of 14, 4, 6, and 7 mice of the second series for weight, and for 9, 8, 8, and 7 mice chosen at random in both series for lipids. *P ≤ 0.05 vs. L; †P ≤ 0.05 vs. O; ‡P ≤ 0.05 vs. PF [ANOVA or t-test (triglycerides)].
ApN concentrations were expressed as OD per milligram of tissue (not shown). ApN secreted in medium by adipose explants from mice of the four groups was measured after 24 h of culture (Fig. 3D). Only the 32-kDa species (mature isoform) was secreted by either lean or ob/ob explants. The pattern of ApN secretion paralleled that of overall tissue ApN. Depot-specific differences in secretion were observed in L mice ($P < 0.05$ between regions). In addition, ApN secretion was markedly suppressed in both depots of O mice (by 70 and 50% in visceral and inguinal fat, respectively; $P < 0.05$ or less). Eventually, leptin treatment partially restored ApN secretion in visceral fat (65% increase compared with O values), whereas calorie restriction was without effect.

Because TNF-$\alpha$ is involved in obesity-related insulin resistance and is known to downregulate ApN secretion (17, 36), we examined whether the correction of circulating ApN levels brought about by leptin was explained by changes in TNF-$\alpha$. TNF-$\alpha$ mRNA levels were measured in visceral and inguinal fat of the four groups of mice (Table 2). In lean mice, TNF-$\alpha$ mRNA levels were expressed more in visceral than in inguinal fat. Compared with L mice, the TNF-$\alpha$ gene was overexpressed in inguinal fat of O mice (4.5-fold). However, leptin treatment or calorie restriction did not modify TNF-$\alpha$ mRNA abundance (whether expressed per 18S rRNA or mg tissue) in any depots at any time (data not shown (day 3) and Table 2 (day 6)).

To assess whether leptin directly affected ApN gene expression, this adipocytokine was added to the culture medium of fully differentiated 3T3-F442A adipocytes for 6 days. After 24 h of culture, leptin increased ApN gene expression and secretion by 200 and 50%, respectively (Fig. 4).

Besides ApN, we examined whether leptin treatment of obese mice could also influence resistin, another adipocytokine, which is believed to play a role in the pathogenesis of the obese insulin-resistant syndrome (Table 2). In lean mice, resistin mRNA levels exhibited a depot-specific pattern of expression similar to that of ApN and TNF-$\alpha$. As reported (32, 39, 51), resistin mRNA levels were decreased in visceral fat of O mice. Leptin treatment for 6 days markedly suppressed resistin gene expression in both depots of ob/ob mice (60–70% compared with O values), whereas mere calorie restriction induced a smaller decrease (~25%) in the visceral depot only (Table 2). Differences between all groups were amplified further when data were expressed per milligram of adipose tissue (not shown). The effect of leptin on resistin mRNA levels was not detected at day 3 (not shown). As expected for a cytokine inducing insulin resistance, plasma resistin levels were ~25% higher in O mice than in L mice. Leptin treatment for 6 days tended to slightly decrease plasma resistin levels (11% compared with O values; $P = 0.099$), whereas pair-feeding was ineffective.

**DISCUSSION**

Leptin treatment, but not mere calorie restriction, reverses low circulating ApN levels in ob/ob mice, at least in part by restoring ApN concentrations and secretion in the visceral depot.

Plasma ApN levels were reduced in leptin-deficient ob/ob mice, as described in leptin-resistant db/db mutants (36, 52). Concomitantly, ApN mRNA levels were downregulated in
circulating ApN levels. By contrast, chronic dietary restriction
ob/ob
strikingly increased plasma ApN levels of (2) and obese humans (53).

centrations and secretion that occurred in a depot-specific
manner, as reported for thiazolidinediones (10). This restoration
was preceded by a rise in ApN mRNA levels. Although
the increases in adipose tissue ApN mRNA, content, and
secretion were substantial (~50–80% of untreated O or PF
values), these changes (per g tissue) may appear relatively
small compared with values of L mice. However, they could be
involved in the correction of plasma ApN, as obese T mice still
preserved a large excess of ApN-producing white fat mass (see
Table 1). The rise in ApN mRNA triggered by leptin was
transient: detected at day 3, but no longer at day 6 of treatment.
One may raise the possibility that the (over)correction of
plasma ApN observed in T mice had exerted a negative
feedback on its own mRNA levels at day 6. Alternatively,
pulsatile, rather than continuous, administration of leptin (7)
could be required to produce a sustained rise in the mRNA
levels. As in humans (19), in our mice, acute leptin treatment
(48 h) did not affect plasma ApN levels. Yet, changes in
ApN mRNA levels may already have occurred, as shown in
our study.

Several mechanisms could theoretically contribute to leptin-
induced stimulation of ApN mRNA levels. First, reduced fat
A stimulatory effect of leptin on ApN gene expression and secretion from 3T3-F442A adipocytes. Fully differentiated cells were cultured without (□, control medium) or with 10 nM leptin (▪) for 6 days. Left: ApN gene expression was measured by real-time PCR. ApN mRNA levels normalized to cyclophilin mRNA levels were expressed as % of respective control conditions. Right: ApN secreted in medium after 24 h of culture was measured by RIA (control medium) or with 10 nM leptin (leptin). Results are means ± SE for 3 (left) or 6 (right) independent experiments. *P < 0.05, †P = 0.06 for effect of leptin.
circulating resistin levels. Despite halved mRNA levels in one fat depot, plasma levels of resistin were elevated in ob/ob mice. This may result from enlarged resistin-producing adipose tissue mass (49) and additional resistin production by nonadipose tissue (mainly white blood and mononuclear cells, splenocytes, and pituitary gland) (35, 39, 40). Resistin is indeed involved in insulin resistance and in other inflammatory processes (35). Unlike acute fasting (29, 48), calorie restriction for 6 days barely affected resistin parameters. In contrast, leptin treatment for the same period blunted resistin mRNA levels in both depots of obese mice. Accordingly, plasma resistin levels tended to be slightly decreased in leptin-treated mice. In humans, acute administration of leptin did not alter plasma resistin (33). In our study, the decrease of circulating resistin, if any, was tiny compared with the drop in mRNA levels. This could be explained by the contribution of nonadipose tissues to systemic resistin levels and/or by compensatory mechanisms acting at a step distal to the mRNA in adipose tissue of obese T mice. Both processes could ultimately limit the profound negative effect of leptin on resistin mRNA in fat tissues.

The decrease in plasma adiponectin associated with obesity suggests that an obesity-related factor or process participates in the downregulation of ApN synthesis. Our study proposes that leptin deficiency or resistance to its action (as seen in the common forms of human obesity) may contribute. On the other hand, our work underscores the potential confounding role of ApN during leptin treatment. Because leptin and ApN share several metabolic properties (enhanced insulin sensitivity, fatty acid oxidation, thermogenesis, and weight loss), some of the effects classically attributed to leptin may be indirectly mediated or amplified by a rise in ApN levels. The slight decrease in resistin levels could enhance insulin sensitivity further.

**Conclusions.** Leptin treatment, but not mere calorie restriction, corrects plasma ApN in obese mice by restoring adipose tissue ApN concentrations and secretion, at least in part via a direct stimulation of ApN gene expression. Such a treatment only minimally affects circulating resistin. ApN restoration could, in concert with leptin, contribute to the metabolic effects classically observed during leptin administration.

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**REFERENCES**


AJP-Endocrinol Metab • VOL 287 • SEPTEMBER 2004 • www.ajpendo.org
RISE IN ADIPONECTINEMIA AFTER LEPTIN TREATMENT


29. Kahn SE, Olefsky JM, Buchanan TA, and Scherer PE. Adipose tissue resistin expression is severely suppressed in obesity and associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration: cross-sectional and interventional studies in normal, insulin-resistant, and diabetic subjects. J Clin Endocrinol Metab 88: 4848–4856, 2003.


33. Makimura H, Mizuno TM, Bergen H, and Mobbs CV. Adiponectin is stimulated by adrenomedullary in ob/ob mice and is highly correlated with resistin mRNA. Am J Physiol Endocrinol Metab 283: E1266–E1271, 2002.


