Effects of obesity on the relationship of leptin mRNA expression and adipocyte size in anatomically distinct fat depots in mice

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LEPTIN, A HORMONE produced predominantly in adipocytes, has profound effects on food intake, energy expenditure, and metabolism (1, 7, 22, 36, 52, 54, 66). Plasma leptin concentrations are positively correlated with various indexes of fatness, such as total body fat mass, percent body fat, and body mass index, in both humans and rodents (10, 17, 44, 51), suggesting that leptin may function as a humoral signal of fat mass as part of the regulatory mechanism for energy balance (29, 53).

Leptin mRNA expression and protein secretion are regulated by many factors, including insulin, glucocorticoid, catecholamines, and TNF-α (6, 47, 48, 55, 61). Fasting acutely suppresses leptin gene expression, and feeding restores leptin mRNA expression to nonfasting levels within 4–7 h (5, 41, 48). The effects of fasting and feeding on leptin mRNA levels are in part mediated by the circulating insulin concentration and by the effects of insulin on adipocyte metabolism (38, 48). We have previously shown that leptin mRNA levels and rates of protein secretion are highly correlated with each other and with adipocyte volume in inguinal, perigonadal, and retroperitoneal fat depots of lean young mice in the postabsorptive state (10-wk-old male C57BL/6J). The slopes of the regression lines relating tissue leptin mRNA level to the average adipocyte volume are slightly higher in the perigonadal than in the inguinal and retroperitoneal fat depots, and adipocyte volume accounts for 64% of the variation of tissue leptin mRNA levels in these mice (65). These results provide a mechanistic basis for leptin’s physiological role as a humoral signal of fat mass.

Although plasma leptin concentrations are generally higher in obese than in lean humans, plasma leptin concentrations per unit of fat mass appear to be more scattered, and possibly lower, in older and obese humans compared with normal weight subjects (10, 28, 35, 45, 50, 51). The underlying mechanism for the variation of plasma leptin concentration per unit of fat mass in obese humans is not clear. Plasma leptin concentrations are also higher in obese than in lean mice (17, 31). However, the precise relationship between leptin gene expression and adipocyte size in anatomically distinct fat depots in obese mice or humans is not clear. Studies suggest that plasma leptin concentration per unit of fat mass is an important parameter for energy homeostasis in humans and rodents (4, 9, 42, 56, 62, 63). Low plasma leptin concentrations per unit of fat mass are associated with increased rates of obesity in Pima Indians and increased adiposities in Lep(ob)/ob mice (9, 42), while high plasma leptin concentrations per unit of fat mass are associated with a lean phenotype and resistance to diet-induced obesity in several mouse models (4, 56, 57, 62, 63).

In this report, we systematically examined the relationships of leptin mRNA level to adipocyte volume in anatomically distinct fat depots in four mouse models of obesity and compared the results with those of lean young mice. The specific questions we asked are the following. 1) Are leptin mRNA levels per unit of adipocyte volume similar between anatomically distinct fat depots in obese mice, and similar to those of lean young mice? 2) Does the positive correlation between leptin mRNA level and adipocyte volume extend from small to hypertrophic adipocytes in obese mice?

RESEARCH DESIGNS AND METHODS

Animals and adipose tissue sample collection. Male C57BL/6J, C57BL/6J Lep(ob)/Lep(ob), and C57BL/6J-A Perl mice were purchased from Jackson Laboratories at 8 wk of age and were maintained in a barrier facility (12:12-h light-dark cycle) with ad libitum access to regular rodent chow (Purina rodent chow 5035) until the ages indicated in the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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RESULTS. Another group of 8-wk-old male C57BL/6J mice were fed a high-fat diet (D12451, Research Diet, caloric composition: 45% fat, 20% protein, 35% carbohydrates) until they were 8 mo old (high-fat diet-fed mice, HFD). All animals were deprived of food for 5 h before death to prevent random feeding during the light cycle that might confound the results and were killed by CO₂ asphyxia between the seventh and eighth hour of the light cycle (2 PM–3 PM), as described in an earlier publication (65). Because mice feed during the dark cycle but eat very little in the light cycle when food is available in the cage, the mice at the time of death in this study were in the postabsorptive state. Ahima et al. (1) showed that plasma leptin concentrations are relatively constant and are near the median level of the diurnal variation in mice at the postabsorptive state. Inguinal, perigonadal, and retroperitoneal fat pads were dissected. One pad was immediately frozen in liquid nitrogen for RNA extraction, and the other was fixed in Bouin’s fixative for measurement of adipocyte volume in paraffin sections. Four 8-mo-old male HFD mice and two 3-mo-old female C57BL/6J Lep⁻/⁻/Lep⁻/⁻ mice were also used to isolate adipocytes from inguinal and perigonadal (epidymal in male, perimamral in female) fat depots as described below. All animal procedures have been approved by the Institutional Animal Care and Use Committee of Columbia University.

Isolation and size fractionation of adipocytes. Adipocytes were isolated by collagenase digestion as previously described (43). For size fractionation, isolated adipocytes from a single fat pad (0.5–1 g) were suspended in 20 ml Krebs-Ringer bicarbonate buffer supplemented with 4% BSA and 1 g/l glucose in a 50-ml conical tube and gently mixed. Adipocytes were then allowed to float for 90 s, and cells in the bottom 10 ml of buffer were collected (small-size fraction). Ten milliliters of fresh buffer was added to the remaining adipocytes. Cells were then allowed to float again for 30–45 s, and adipocytes in the bottom 10 ml of buffer were collected (medium-size fraction). Finally, adipocytes from the top 5 ml of buffer were collected after the remaining adipocytes were mixed again in 20 ml buffer and allowed to float for 10–15 s (large-size fraction). A portion of adipocytes in each fraction was used to determine cell volume, and the remaining cells were used for RNA preparation (see below). A representative result, the size distributions of the three adipocyte size fractions from the parametral fat pad of a 3-mo-old female ob/ob mouse, is shown in Fig. 1.

Determination of the average adipocyte size of adipose tissues and the sizes of isolated adipocytes. The average adipocyte volume of adipose tissue samples was determined as previously described (3, 65). Cell volumes of isolated adipocytes were determined using a micropotograph method previously described by DiGirolamo (12) and Ashwell et al. (3). Briefly, methylene blue-stained adipocytes were photographed at ×100 magnification using a Nikon Eclipse E400 light microscope connected to a digital camera and computer. The diameters of at least 200 adipocytes (identified by their stained nuclei) from each sample were measured in the microphotographs. The average adipocyte size (expressed as μg lipid/cell) of an isolated adipocyte population (or a size fraction) was calculated using the Goldrick’s formula, volume = πd/3(3SD)² + d²)/6, where d is mean diameter of adipocytes and SD is standard deviation of adipocyte diameters, assuming lipid density = 0.915 g/ml (19).

Determination of mRNA levels using quantitative RT-PCR. Quantitative RT-PCR using β-actin as an internal control was used to determine mRNA levels of leptin, TNF-α, and glucocorticoid receptor as previously described (65). Total RNA was extracted from adipose tissue or isolated adipocytes using Trizol reagent (Invitrogen, Carlsbad, CA). About 1–5 μg of total RNA was reverse-transcribed into cDNA using random hexamers and M-MLV reverse transcriptase (Invitrogen). Complementary DNA was quantitatively amplified by PCR using specific primers for the genes of interest in combination with primers for β-actin for a limited number of cycles that were within the exponential amplification ranges for both genes. Primer sequences for leptin, TNF-α, glucocorticoid receptor, and β-actin were as previously reported (65), and mRNA levels for genes of interest were expressed as a ratio to β-actin mRNA to normalize for initial RNA input.

Statistical analyses. Statistica V6.0 was used for all analyses. Simple correlation analysis was used to examine the relationships between adipocyte volume and mRNA levels for leptin and glucocorticoid receptor in size fractions of adipocytes from four male HFD and two female ob/ob mice. Multiple regression analysis was used to assess effects of adipocyte size and depot of origin on leptin mRNA levels and glucocorticoid receptor mRNA levels in size fractions of adipocytes from the HFD and ob/ob mice. To further investigate depot-of-origin effects in the size fractions of adipocytes from HFD mice, the differences in intercepts and slopes of the regression lines relating leptin mRNA levels and adipocyte volume between depots were tested by regression of leptin mRNA levels on adipocyte volume, depot of origin, and interactions between depot of origin and adipocyte volume. The coefficient for the interaction term represents...
the difference in slope between the depots, and the coefficient for the depot represents the difference in intercept (65). Two-way ANOVA was used to assess differences in mRNA levels and cell size among animal models and fat depots. One-way ANOVA and post hoc comparison (Newman-Keuls test) were used to compare differences in mRNA levels and adipocyte size between fat depots in each animal model. A two-tailed $P$ value $< 0.05$ was considered to indicate statistical significance.

**RESULTS**

Decreased leptin mRNA levels relative to adipocyte volume in obese mice. mRNA levels for leptin and TNF-$\alpha$ and the average adipocyte volume were compared in inguinal, epididymal, and/or retroperitoneal adipose tissues of 11-mo-old male C57BL/6J mice (Old) that were mildly obese due to aging, 10-wk-old male C57BL/6J Lep$^{ob}$/Lep$^{ob}$ mice (ob/ob), 5-mo-old male C57BL/6J-A$^1$ mice (A$^1$), and 8-mo-old male HFD-fed mice (Fig. 1). Adipocyte volume ($P < 0.001$) (Fig. 1A) and tissue leptin mRNA levels ($P < 0.01$) (Fig. 1B), but not TNF-$\alpha$ mRNA levels ($P = 0.19$) (Fig. 1C), were significantly different among fat depots in a two-way ANOVA with animal model and fat depot as grouping variables. Within an animal model, by one-way ANOVA, adipocyte volume was significantly larger in the epididymal and/or retroperitoneal fat depots than in the inguinal fat depots in ob/ob, HFD, and Old mice (Fig. 1A), while leptin mRNA levels were significantly lower in the epididymal and/or retroperitoneal than in the inguinal fat depots in ob/ob, A$^1$, and Old mice (Fig. 1B). Thus leptin mRNA levels relative to adipocyte volume were significantly lower in the epididymal and/or retroperitoneal fat depots than in the inguinal fat depots in all the obese mouse models examined.

To assess and graphically depict the differences in tissue leptin mRNA levels relative to the average adipocyte volume in the anatomically distinct fat depots of the obese mice, and between the obese mice and lean young mice, tissue leptin mRNA levels and the average adipocyte volumes of the ob/ob, Old, A$^1$, and HFD mice were plotted (Fig. 2). Two plots are used to facilitate the distinction among models. Data from the lean young mice (65) were included in both plots, and the regression line for the pooled data set of the three fat depots of the lean young mice is shown in a dashed line. Compared with the lean young mice, tissue leptin mRNA levels relative to the average adipocyte volume were significantly lower in epididymal fat depots in all of the obese mice examined, i.e., leptin mRNA levels in the obese mice were below the regression line extrapolated from the data of the lean young mice (Fig. 2, A and B). In addition, tissue leptin mRNA levels relative to the average adipocyte volume were also lower in the inguinal fat depots in the A$^1$ and HFD mice and in the retroperitoneal fat depots of the A$^1$ and ob/ob mice (Fig. 2, A and B), compared with those of the lean young mice. These results suggest that although the obese mice differ in the etiologies of their obesity, decreased leptin mRNA level relative to adipocyte volume, especially in the epididymal fat depot, is a common feature among them.

**Relationships between leptin mRNA level and adipocyte volume within a fat pad in obese mice.** To determine the mechanism that may account for the preferential decreases of tissue leptin mRNA level relative to the average adipocyte volume in the perigonadal fat depot of the obese mice, the relationship of leptin mRNA levels and adipocyte volume within a single perigonadal and inguinal fat pad was examined. Adipocytes isolated from each perigonadal or inguinal fat pad of four HFD mice and two 3-mo-old female C57BL/6J Lep$^{ob}$/Lep$^{ob}$ mice (ob/ob) were separated into three size fractions by the flotation method described in RESEARCH DESIGNS AND METHODS. Figure 3 shows a representative result of the size fractionation, i.e., the size distributions of three adipocyte size fractions derived from the parametral fat pad of a female ob/ob mouse. The diameters (mean $\pm$ SD) for the large-, medium-, and small-size fractions were 92.6 $\pm$ 28.7, 67.0 $\pm$ 29.2, and 37.6 $\pm$ 20.4 $\mu$m, which correspond to an average adipocyte volume of 0.506, 0.225, and 0.048 $\mu$g lipid/cell, respectively, and were significantly different ($P < 0.001$).

mRNA levels for leptin and glucocorticoid receptor (as a control), and the average adipocyte volumes in the size fractions of each fat pad were determined and related to one another (Fig. 4). A positive correlation between leptin mRNA level and adipocyte volume was present within each perigonadal and inguinal fat pad, but the slopes of the regression lines...
The results of the present study showed that in the obese mice in the postabsorptive state 1) leptin mRNA levels per unit data set of the size fractions from both inguinal and perigonadal fat pads of the four HFD mice, adipocyte volume ($P < 0.0001$) and depot of origin ($P < 0.05$) were significant predictors of leptin mRNA levels, accounting for 56 and 9% of the variation of leptin mRNA level, respectively. Depot of origin affected the relationship of leptin mRNA levels to adipocyte volume mainly by modifying the slopes of the regression lines in the HFD mice, which were significantly lower in the perigonadal than in the inguinal fat pads of the same mice ($P < 0.05$). The intercepts of the regression lines on the $y$-axis were not significantly different between the two depots in the HFD mice ($P = 0.57$). In contrast, adipocyte volume ($P = 0.64$) and depot of origin ($P = 0.81$) were not significant predictors of glucocorticoid receptor mRNA levels in the HFD mice (Table 1). Adipocyte volume and depot of origin were also significant predictors of leptin mRNA levels in a pooled data set of the size fractions from the two $ob/ob$ mice, accounting for 18% ($P < 0.0001$) and 16% ($P < 0.01$) of the variation, respectively. When the data from the size fractions of each animal were analyzed separately, adipocyte volume accounted for 74% ($P < 0.05$) and 50% ($P < 0.05$) of the variation of leptin mRNA levels in ob1 and ob2 mice, respectively, suggesting that individual differences in leptin gene expression between the two female $ob/ob$ mice might have weakened the predicting power of adipocyte volume on leptin mRNA levels in the pooled data set of the two $ob/ob$ mice.

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of adipocyte volume were decreased relative to the lean young mice; and 2) a positive correlation between leptin mRNA level and adipocyte volume is present from small to hypertrophic adipocytes within each perigonadal or inguinal fat pad, but the slopes of the regression lines relating leptin mRNA level to adipocyte volume were significantly lower in the perigonadal than in the inguinal fat pads.

Relative to the lean young mice, leptin mRNA levels per unit of adipocyte volume were significantly lower in the perigonadal fat depot of the four obese models examined, including ob/ob, Aδ, Ods, and HFD mice. In addition, leptin mRNA levels per unit of adipocyte volume were also decreased in Aδ and HFD mice compared with those of the lean young mice. Contrary to the finding in the lean young mice (10-wk-old C57BL/6J mice) that leptin mRNA levels per unit of adipocyte volume are slightly higher in the perigonadal than in the inguinal fat pads (65), leptin mRNA levels relative to adipocyte volume were significantly lower in the perigonadal than in the inguinal fat depots in all of the obese mice examined. Together, these results suggest that obesity per se, regardless of the etiology, has differential effects on leptin gene expression in anatomically distinct fat depots and/or decreased leptin mRNA stability. However, transcriptional regulation appears to be the primary mechanism that determines leptin mRNA levels and protein production in adipocytes (6, 13, 37, 47, 48, 58).

Data points (N = 24) consist of 3 size fractions from each inguinal and perigonadal fat pad of 4 high-fat diet-fed male C57BL/6J mice, i.e., 3 size fractions × 2 depots × 4 mice = 24 data points. β, standardized correlation coefficients; P, P value for standardized correlation coefficients; GR, glucocorticoid receptor.

Table 1. Multiple regression analysis of effects of adipocyte size and depot of origin on mRNA levels of leptin and glucocorticoid receptor in adipocyte size fractions from inguinal and perigonadal fat pads of four 8-mo-old high-fat diet-fed male C57BL/6J mice

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>N</th>
<th>R²</th>
<th>β</th>
<th>P</th>
<th>Cell size</th>
<th>Depot origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin mRNA level</td>
<td>24</td>
<td>0.66</td>
<td>0.82</td>
<td>&lt;0.0001</td>
<td>0.31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GR mRNA level</td>
<td>24</td>
<td>0.17</td>
<td>−0.11</td>
<td>0.64</td>
<td>0.05</td>
<td>0.81</td>
</tr>
</tbody>
</table>

The decreased leptin mRNA level per unit of adipocyte volume suggests that leptin mRNA expression per gram of fat mass is decreased in the obese mice, even when it is assumed that total RNA yield per gram of fat mass is the same, but not decreased, in the obese mice, relative to that of the lean young mice. It should be noted, however, that leptin expression per cell is not necessarily lower in large adipocytes from obese mice than in small adipocytes from lean young mice. On the contrary, leptin mRNA expression per cell is likely to be higher in large adipocytes than in small adipocytes because the absolute leptin mRNA levels in the obese mice were higher than or comparable to those of the lean young mice (Fig. 1), and total RNA yield per cell is also likely to be higher in large adipocytes than in small adipocytes. It is apparent that leptin mRNA levels per gram of fat mass were lower in the obese mice than in the lean young mice because the increase in adipocyte size, or the amount of fat mass per cell (μg lipid per cell), was not paralleled by the increase of leptin mRNA expression per cell in the obese mice. The decrease of leptin mRNA levels per gram of fat mass may be due to decreased rates of transcription and/or decreased leptin mRNA stability. However, transcriptional regulation appears to be the primary mechanism that determines leptin mRNA levels and protein production in adipocytes (6, 13, 37, 47, 48, 58).

Increased rates of transcription and protein synthesis of leptin are required for the increase of plasma leptin concentration induced by feeding and for the long-term stimulatory effects of glucocorticoid and insulin on leptin secretion (6, 37, 47, 58). Our previous study also showed that rates of leptin protein secretion are positively correlated with tissue leptin mRNA levels, suggesting that leptin is constitutively secreted, and that the quantity of the preformed pool of intracellular leptin is likely to be small (65). Thus decreased leptin mRNA levels per gram fat mass will likely lead to decreased levels of leptin protein production per gram of fat mass in the obese mice. In summary, the results of the present study suggest that obesity in mice is apparently associated with a relative deficiency of leptin gene expression, i.e., lower leptin production per gram of fat mass, relative to that of lean young mice, which may contribute to further weight gain and the development of obesity-related metabolic disorders.

Russell et al. (47) reported that in morbidly obese men and women, leptin mRNA levels are lower in omental than in abdominal subcutaneous adipocytes even though the sizes of adipocytes are larger in omental than in subcutaneous fat depots. These results suggest that decreases of leptin mRNA expression relative to adipocyte volume may also occur in the omental fat depot in obese humans and that individuals with excessive visceral adiposity may have lower plasma leptin concentrations per unit of fat mass compared with those with excessive subcutaneous adiposity. The results on the relationship between body fat distribution and plasma leptin concentration per unit of fat mass in the literature are conflicting. While some studies found that plasma leptin concentrations per unit of fat mass are negatively correlated with waist-to-hip ratios in both obese men and women (30, 34), others found no significant effect of body fat distribution on circulating leptin concentrations per unit of fat mass in either obese men or women (25, 39, 46). Variations in the physiological characteristics of the research subjects of each study, such as adiposity, age, and gender, may partly account for the discrepancy. A systematic study on the relationship between adipocyte size and leptin gene expression in anatomically distinct fat depots in both lean and obese individuals may be necessary to resolve the discrepancy.

The size-fractionation experiments were necessary to eliminate potential confounding factors, such as differences related to the physiological conditions of individual animals and/or fat depots, and allowed us to examine the relationship of leptin mRNA level and adipocyte volume in adipocytes within a single fat pad and to compare the relationships between anatomically distinct fat pads in the same individual mice. These experiments revealed that, as in lean young mice, a close positive correlation between leptin mRNA level and adipocyte volume is present within each inguinal or perigonadal fat pad in the HFD and ob/ob mice, providing a further support for leptin’s physiological role as humoral signal of fat mass. Contrary to the finding in the lean young mice (65), the slopes of the regression lines relating leptin mRNA level to adipocyte volume were significantly lower in perigonadal adipocytes than
in inguinal adipocytes in both HFD and ob/ob mice. Together, these results suggest that the positive correlation between leptin mRNA level and adipocyte volume is an intrinsic property of adipocytes that is not disrupted by obesity or adipocyte hypertrophy and that changes in the systemic physiological conditions and local environment of a fat depot may modify the slopes of the regression lines in anatomically distinct fat depots independently. The results further suggest that leptin mRNA levels per unit of adipocyte volume are decreased in all of the adipocytes across the size range, not preferentially in the hypertrophic adipocytes, in the perigonadal fat pad of the obese mice. Paracrine actions are likely responsible for the decreases of leptin mRNA levels in the perigonadal fat depot although the nature of the paracrine factor(s) is not clear. Adipocytes and other nonadipocyte cells in adipose tissues, such as preadipocytes, fibroblasts, macrophages, and endothelial cells, secrete a growing number of bioactive molecules (adipokines) besides leptin, such as TNF-α, IL-1β, IL-6, adiponectin, resistin, plasminogen activator inhibitor-1, and angiotensinogen, which may function as potential paracrine factors (2, 8, 11, 33, 40). Adipocyte hypertrophy is associated with increased macrophage infiltration into adipose tissues, especially in the intra-abdominal fat depots, and increased expression levels of TNF-α, IL-6, and IL-1β (18, 24, 60). These cytokines, and possibly other secreted proteins from macrophages, have complex effects on leptin gene expression, adipocyte metabolism, and insulin sensitivity (15, 20, 21, 26, 49, 61, 64) and may play a role in the decrease of leptin mRNA expression per unit of adipocyte volume in the perigonadal fat depot of the obese mice.

The underlying mechanism for the close correlation between leptin mRNA level and adipocyte volume is not clear. Similar to the correlation between leptin mRNA level and adipocyte volume in mice, uptake rates of glucose and palmitate are positively correlated with adipocyte size in 14 wk-old lean and obese (fa/fa) Zucker rats in both basal and insulin-stimulated states (23). Foley et al. (16) also showed that the uptake rate of glucose or nonmetabolizable 3-O-methylglucose by adipocytes is a function of cell size in the presence or absence of insulin, although the stimulation of insulin on glucose uptake varies from 11-fold in small adipocytes to 3.5-fold in large adipocytes. In both studies, the slopes of the regression lines relating the rate of glucose or methylglucose uptake to adipocyte size are lower in the basal state than in the insulin-stimulated state, suggesting that the rate of glucose uptake is a function of adipocyte size regardless of the status of insulin and that insulin stimulation may affect the slope of the regression line. Rates of de novo fatty acid/acylglycerol synthesis are also positively correlated with adipocyte size within a fat pad in 3-mo-old pigs (14). Given that leptin gene expression is sensitive to the feeding status of animals and the metabolic status of adipocytes (1, 5, 27, 38, 41, 48, 59), it is possible that the rate of anabolic flux in adipocytes is a mechanistic basis for the positive correlation between adipocyte volume and leptin mRNA expression. Because the obese and lean young mice examined in this and the previous studies were in the postabsorptive state, i.e., non-insulin-stimulated, nonfasted state, it would be of great interest to examine how feeding, insulin stimulation, and fasting affect the relationship between leptin mRNA levels and adipocyte volume in lean, insulin-sensitive mice and in obese, insulin-resistant mice. Further elucidation of the mechanisms that regulate leptin mRNA expression in relation to adipocyte size in anatomically distinct fat depots will facilitate our understanding of the physiology of body weight regulation and the mechanisms for the relationship between fat distribution and metabolic consequences of obesity.

GRANTS

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