Regulation of the leptin content of obese human adipose tissue

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Russell, C. D., M. R. Ricci, R. E. Brolin, E. Magill, and S. K. Fried. Regulation of the leptin content of obese human adipose tissue. Am J Physiol Endocrinol Metab 280: E399–E404, 2001.—The objective of this study was to determine whether obese human adipose tissue contains preformed stores of leptin and their relationship to secreted leptin. Detergent increased detectable leptin by about twofold, suggesting that leptin is stored in a membrane-bound location. Subcutaneous tissue leptin was ~1.6-fold higher than omental, paralleling known differences in leptin secretion and expression. The amount of leptin secreted during a 3-h incubation was similar to that of extractable tissue leptin. Tissue leptin levels were maintained over the incubation. Inhibition of protein synthesis decreased tissue leptin content but did not decrease leptin secretion until after 3 h of incubation. Culture of adipose tissue for 2 days with the combination of insulin and dexamethasone, but not with either hormone alone, increased tissue leptin content about twofold in both depots. Although insulin did not affect tissue leptin content, it potentiated leptin secretion (as a % of tissue stores). These data suggest that adipose tissue leptin storage and secretion per se are regulated. Regulation of the release of preformed leptin may modulate serum leptin levels in obese humans.

secretion; insulin; dexamethasone; organ culture; cycloheximide

LEPTIN, A SMALL PEPTIDE HORMONE (16 kDa) produced primarily by adipose tissue, has broad-ranging and complex effects on neural and endocrine systems regulating body weight (30). Serum leptin levels are higher in the obese (8) but can change in both lean and obese individuals in response to hormones and nutritional state (10). Regulation of leptin output by adipose tissue has not yet been clearly defined. Because levels of leptin mRNA often correspond roughly with serum leptin and leptin accumulating in culture media (19), it has been tentatively assumed that the synthesis of leptin protein is regulated transcriptionally and that leptin secretion follows its synthesis without significant accumulation within the tissue or regulation of secretion per se.

Nonetheless, some examples of discordance between levels of leptin mRNA and leptin secretion have been reported (6, 25, 28), suggesting that posttranscriptional regulation of leptin may occur. Rates of leptin synthesis have never been directly measured in adipocytes, and studies addressing the leptin content of adipose tissue or cells are few. Attoub et al. (1) demonstrated that the leptin content of rat epididymal adipose tissue increases about twofold within 2 h of refeeding after an 18-h fast. Consistent with this finding, Barr et al. (2) showed that treatment of rat adipocytes with insulin increased tissue leptin content and secreted leptin during a 2-h incubation. Moreover, insulin increased secreted leptin within 10 min while decreasing cellular leptin, suggesting that insulin may act as a leptin secretagogue. Bradley and Cheatham (6) showed that the ability of insulin to stimulate leptin secretion persisted despite inhibition of protein synthesis. Further evidence for regulated secretion of leptin from preformed pools comes from work by Kirchgesner et al. (15), who demonstrated that cultured 3T3-L1 adipocytes secrete a substantial bolus of leptin in response to tumor necrosis factor-α (TNF-α) treatment. This effect did not depend on the synthesis of new protein, suggesting that the leptin was released from a preformed pool of leptin within the cells. These data raise the possibility that regulation of leptin storage and/or secretion could underlie the fairly rapid changes in serum leptin levels observed in vivo.

There is very little published information addressing tissue leptin levels in human adipose tissue. However, Zhang et al. (33) were able to detect intracellular leptin in adipocyte ceiling cultures. Furthermore, they noted that intracellular leptin levels were higher in subcutaneous than in omental adipocytes, paralleling the reported depot difference of leptin secretion and leptin mRNA (14, 21, 28). It is not known whether hormones that regulate the expression of leptin also affect the leptin content of human adipose tissue. For example, the combination of insulin plus dexamethasone increases serum leptin in humans (18) and increases leptin mRNA and leptin secretion from human adipose tissue (28) or rat adipocytes (6) in vitro, but it is not clear whether this upregulation involves increased tissue leptin tissue content as well. Glucocorticoid-in-
duced increases in serum leptin levels in humans can be explained by an increase in leptin mRNA abundance, as shown in vivo (23) and in vitro (28), but the effect of insulin is controversial. In some models, insulin increases both leptin secretion and leptin mRNA (9, 16, 20). However, we previously found that culture of adipose tissue with insulin for 1–7 days increased leptin accumulation in the media without increasing leptin mRNA (28).

In the present study, we examined the relationship between tissue leptin content and leptin secretion in adipose tissue from obese humans. We report that leptin accumulates within obese human adipose tissue and that prior culture with hormones (insulin and dexamethasone) regulates both the tissue content and secretion of leptin. Furthermore, we found that maintenance of tissue leptin levels over 3 h requires continuous protein synthesis, suggesting that pools of leptin within obese adipose tissue undergo fairly rapid turnover.

MATERIALS AND METHODS

Patient characteristics and sample preparation. Subcutaneous and/or omental adipose tissues were obtained during obesity surgery from severely obese women and men who had no metabolic or malignant disease and were not taking medications known to alter adipose metabolism [5 males, 9 females; age 40 ± 3 (SE); body mass index: 53 ± 4 kg/m²]. Omental and subcutaneous fat cell size was each 0.62 ± 0.05 μg lipid/cell. Adipose tissues were placed into Medium 199 (M199; Earle’s salts, 25 mM HEPES) at room temperature (25°C) and minced into ~5-mg fragments (28). Day 0 refers to samples analyzed on the same day of surgery. One or more aliquot(s) of tissue (~100 mg) were immediately frozen at −80°C for subsequent determination of initial tissue levels of leptin.

Tissue culture. Tissue culture was performed as previously described (12, 28, 31). Briefly, tissue fragments were cultured in serum-free M199 alone (basal) or in the presence of 7 nM insulin (Humulin, Lilly, Indianapolis, IN), 25 nM dexamethasone, or the combination of 7 nM insulin and 25 nM dexamethasone, for 24–48 h.

Leptin secretion during incubation of tissue. To measure secreted leptin, aliquots (~100 mg) of fresh (day 0) or cultured tissue were rinsed with saline and incubated in 1.5 ml M199 containing 1% BSA under an atmosphere of 95% O₂–5% CO₂ for 3 h, with shaking at 55 oscillations per minute in a 37°C water bath. Previous studies (26) have shown that the appearance of leptin in the medium is linear for 3 h. Where indicated, 10 μg/ml cycloheximide was included in the incubation for the entire period. At 3 h, incubation media and tissue pieces were frozen at −80°C for subsequent determination of leptin protein by RIA (Linco, St. Charles, MO). Data are expressed as nanograms of leptin per gram of adipose tissue.

Extraction of leptin from tissue pieces. Fresh or frozen aliquots of tissue were homogenized in a ground glass homogenizer in 3–4 volumes of cold TES (10 mM Tris, 1 mM EDTA, and 250 mM sucrose) buffer, pH 7.4, containing proteolytic inhibitors (leupeptin, 2.5 μg/ml; aprotinin, 3.5 μg/ml; and 1 mM phenethylmethylsulfonyl fluoride) and with or without 1% Triton X-100, as indicated. Homogenates were centrifuged at 14,000 rpm for 15 min at 4°C to separate the fat layer. Infranatants beneath were removed and assayed for leptin content by RIA. Preliminary studies showed that tissue homogenates made with fresh or frozen tissue were similar in leptin content (data not shown).

Leptin assay. Leptin protein within adipose tissue homogenates and incubation media was assayed using an RIA from Linco. Appropriate blanks reflected buffers and media additions (e.g., Triton X-100).

Statistical analysis. Two-way repeated-measures ANOVA were used to analyze hormonal effects, and post hoc t-tests were used to compare individual means. Paired t-tests were used to compare detergent and cycloheximide effects. No effect of gender on depot or hormonal effects was noted, so data were pooled before statistical analysis. Statistical significance is denoted as $P < 0.05$. Values were log-transformed to equalize the variance between conditions.

RESULTS

Detergent enhances extraction of leptin from adipose tissue fragments. As a secretory protein, leptin within adipose tissue is likely to be associated with membrane-bound compartments (endoplasmic reticulum, Golgi, secretory vesicles). To determine whether significant quantities of leptin reside in detergent-sensitive compartments, we compared extractable leptin in subcutaneous adipose tissue homogenates that had been prepared in TES in either the absence or the presence of 1% Triton X-100. Homogenates prepared with detergent consistently contained 1.5- to 2-fold higher extractable leptin than samples prepared without detergent (18.1 ± 2.9 vs. 11.0 ± 1.9, $P < 0.05$, $n = 8$; data not shown). Similar results were obtained in omental tissue and in cultured tissue regardless of hormonal additions. We therefore used a buffer consisting of 1% Triton X-100 in TES for subsequent homogenizations to maximize leptin extraction efficiency.

Omental and subcutaneous tissue leptin content and secretion. We (28) and others (14, 22) have previously found that levels of leptin mRNA and leptin secreted into the culture media are about twofold higher in subcutaneous compared with omental adipose tissue. To determine whether this depot difference was also reflected in tissue leptin content, we homogenized tissue fragments in TES + 1% Triton X-100 and found that leptin was on average 1.5-fold higher in subcutaneous than in omental tissue (17.6 ± 2.4 vs. 11.4 ± 1.2, $P < 0.005$, $n = 12$). In a subset of these subjects, we also incubated tissue from either depot in M199 containing 1% BSA and found that the amount of leptin secreted into the media over 3 h was similar to the quantity extracted from the tissue fragments (Fig. 1, $n = 7$). Analysis of the leptin remaining in the tissue fragments after the incubation showed that there was no significant decline from initial levels, indicating that leptin synthesis was sufficient to replenish tissue leptin levels despite continued secretion.

Cycloheximide decreases tissue leptin levels. To determine whether leptin secretion or the leptin content of adipose tissue depended on the synthesis of new protein, we incubated tissue fragments for 3 h in the presence of 10 μg/ml cycloheximide. Compared with control incubations, cycloheximide treatment significantly decreased leptin, within omental tissue frag-
leptin secretion dropped to control values, yet between 3 and 5 h, the increment in presence of cycloheximide was maintained at 5 h. From 0 to 3 h, the rate of leptin secretion in the tissue from a subset of subjects with cycloheximide for therefore, we treated samples of subcutaneous adipose tissue or modulates the tissue's capacity to secrete tissue from cultured adipose tissue was depleted during this latter period (CHX), suggesting that tissue leptin stores became depleted during this latter period (P < 0.05, n = 5).

Hormones regulate leptin content and secretion of cultured adipose tissue. Our previous work has shown that leptin expression is regulated by chronic culture with insulin, dexamethasone, or the combination of insulin and dexamethasone. We therefore determined whether culture for 2 days with these hormones also affects the amount of leptin present within adipose tissue or modulates the tissue's capacity to secrete leptin during a subsequent standardized incubation (in the absence of hormones). We also determined whether leptin secretion from cultured adipose tissue was dependent on protein synthesis by conducting incubations with or without cycloheximide.

Culture with insulin alone did not affect tissue leptin content in either depot (Fig. 2). Dexamethasone significantly increased tissue leptin content in omental tissue only (P < 0.05 by ANOVA, n = 5, Fig. 2). In both depots, the combination of insulin and dexamethasone increased tissue leptin to levels about twofold higher than basal (P < 0.05). In subcutaneous tissue, the effects of insulin and dexamethasone on tissue leptin content were synergistic (insulin × dexamethasone interaction P < 0.005 by repeated-measures ANOVA).

In general, the secretion of leptin from cultured adipose tissue during a 3-h incubation in M199 + 1% BSA paralleled tissue leptin levels. As expected, insulin and dexamethasone were synergistic in increasing tissue leptin content in either depot (Fig. 2). Dexamethasone increased tissue leptin, or secreted leptin, in all conditions. *Effect of prior culture with hormones: Ins + Dex increased tissue leptin, or secreted leptin, over all other hormonal conditions (P < 0.05 by ANOVA). †Effect of prior culture with hormones on tissue leptin: Ins + Dex = Dex > Ins = Basal (P < 0.05 by ANOVA). ‡Insulin increased subcutaneous leptin secretion over basal (P = 0.052) in this set of subjects matched for both CHX and hormone, but P < 0.05 in a larger set of subjects matched only for hormone effect (n = 8, not shown).
lin plus dexamethasone increased leptin secretion by about twofold. Although dexamethasone increased tissue leptin content in omental tissue, it did not significantly alter leptin secretion. Prior culture with insulin did not increase tissue leptin content yet did increase leptin secretion during the acute 3-h incubation \( (P = 0.052, n = 5) \) (matched for both hormone and CHX treatment), as shown in Fig. 2. In a larger sample of subjects, the insulin effect was statistically significant \( (+37 \pm 13\%, P < 0.05, \text{with } n = 8 \text{ matched only for hormone effect}) \). As shown in Fig. 3, culture in the presence of insulin for 1–2 days increased the ratio of secreted leptin to initial levels of tissue leptin in subcutaneous tissue \( (n = 8, P < 0.05 \text{ by repeated-measures ANOVA}), \) suggesting a potentiation of leptin secretion. The effect of insulin was more variable in omental tissue, such that there was on average no clear trend toward increased leptin secretion \( (n = 8, \text{not significant, data not shown}) \).

**Cycloheximide effects in cultured tissue.** In contrast to what was found using freshly obtained adipose tissue \( (Fig. 1) \), cycloheximide decreased the amount of leptin released into the incubation medium under most conditions \( (P < 0.05, n = 5 \text{ for each depot; Fig. 2}) \). An exception was subcutaneous tissue cultured with dexamethasone alone, in which treatment with cycloheximide did not further decrease the rate of leptin release.

As in freshly obtained adipose tissue, tissue leptin content did not decline in cultured adipose tissue \( (\text{all conditions}) \) after a 3-h incubation \( (\text{Fig. 2}) \). However, treatment with cycloheximide during the incubation significantly decreased the tissue levels of leptin in cultured adipose tissue from both depots \( (P < 0.05 \text{ for CHX effect, with the exception of omental tissue cultured with dexamethasone, } P = 0.06; \text{Fig. 2}) \). We have observed that culture with dexamethasone \( (\text{alone}) \) decreases rates of total protein synthesis in adipose tissue fragments \( (\text{C. D. Russell and S. K. Fried, unpublished observation}); \) thus the inhibition of protein synthesis by cycloheximide may not impact the main-
tenance of tissue leptin levels over the time period studied here.

In tissue cultured with the combination of insulin and dexamethasone, there was a trend \( (P < 0.1, \text{Fig. 2}) \) to respond more robustly to cycloheximide compared with the other culture conditions; leptin within tissue and released into the medium was consistently decreased by \( \sim 60\% \) \( (\text{vs. control}) \) by cycloheximide in both depots. Overall, leptin secretion appeared to be more dependent on protein synthesis in cultured adipose tissue than in freshly obtained samples.

**DISCUSSION**

We present here the first report of measures of leptin contained within human adipose tissue fragments. Our results suggest that leptin accumulates within adipose tissue of obese individuals rather than being rapidly secreted upon synthesis, as has been suggested by studies of adipose tissue from nonobese rats \( (2) \). We found that the amount of leptin within adipose tissue differs between omental and subcutaneous depots and can be increased by culture with hormones \( (\text{insulin + dexamethasone}) \). Human adipose tissue secretes an amount of leptin equal to its tissue content within 3 h, and this occurs without a decline in tissue levels. Furthermore, studies with cycloheximide suggest that the maintenance of leptin levels in adipose tissue over 3 h depends on new protein synthesis.

The twofold increase in leptin detected when we homogenized with detergent strongly suggests that at least one-half of the leptin within human adipose tissue is membrane bound. This value is probably a minimal estimate, because some leakage of leptin from membrane compartments during homogenization cannot be ruled out. Leptin detected in the absence of detergent may represent leptin that is present in extracellular space or capillaries or is receptor bound at the cell surface \( (4) \). It will be important to know whether the subcellular and tissue distribution of leptin is different in human adipocytes from lean and obese individuals and whether it can be altered by effectors such as insulin.

Several previous studies have examined the effects of hormones on leptin mRNA and/or leptin secretion in human adipose tissue \( (13, 14, 16, 21–23, 28) \). None has directly analyzed changes in tissue leptin content. Thus it was not known whether hormones could change leptin accumulation within the tissue or whether they simply increased the rate of synthesis or secretion. We found that culture of adipose tissue with the combination of insulin and dexamethasone increased tissue leptin stores over basal and single hormone levels. Dexamethasone alone has been shown to increase leptin mRNA and secretion in vivo and in vitro \( (13, 18, 23) \). Two-day culture with dexamethasone similarly increased tissue leptin content over basal levels.

Barr et al. \( (2) \) investigated the subcellular location of leptin within nonobese rat adipocytes and found that the staining pattern was consistent with endoplasmic reticulum residence, rather than Golgi or secretory

\[ \text{Fig. 3. Hormonal regulation of secretion capacity. Data are shown as } \text{leptin secreted over 3 h divided by initial tissue leptin content } \times 100 \text{ to illustrate the ability of insulin to potentiate leptin secretion in subcutaneous adipose tissue. B, basal; I, insulin; D, dexamethasone; and ID, insulin + dexamethasone. Values are means } \pm \text{ SE; } n = 8. \]

\[ *P < 0.05 \text{ by ANOVA.} \]
vesicles. Barr et al. hypothesized that leptin was rapidly secreted without intracellular retention. However, Roh et al. (27) recently characterized a leptin-containing compartment in rat adipose cells. In addition, Kirchgessner (15) found that TNF-α increased leptin 5.5-fold from 3T3-L1 adipocytes within 6 h. This increase in secretion was ablated by brefeldin A (an inhibitor of post-Golgi transport) but not inhibited by cycloheximide, indicating that the pool of leptin secreted was preformed and stored within the cells, probably in a Golgi or pre-Golgi compartment. Treatment with cycloheximide for 9 h (without TNF-α present) had little effect on secreted leptin, but the accumulation of cellular leptin was not determined in these experiments. When we incubated human adipose tissue with cycloheximide for 3 h, tissue leptin levels were consistently decreased by ≥30%.

There was a difference in the effect of cycloheximide on secreted leptin between freshly excised and 2-day-cultured adipose tissue. Cycloheximide treatment for 3 h was sufficient to decrease leptin secretion ~40% in cultured tissue, but a 5-h treatment was required to obtain a similar effect in fresh tissue. Possibly, some residual in vivo factor modulated the dependence of leptin secretion on protein synthesis in the freshly obtained tissue. These data suggest that changes in leptin synthesis may not immediately affect rates of secretion, because preformed leptin is sufficient to maintain output. However, these results must be cautiously interpreted, because cycloheximide is a general inhibitor of protein synthesis and may be affecting other proteins involved in the processing, secretion, or degradation of leptin. Additionally, a caveat in studies of adipose fragments is that, upon secretion from the adipocyte, leptin may be detained by travel through extracellular space and capillaries before reaching the medium. Studies using biosynthetic labeling of leptin will be needed to address the time course of secretion of newly synthesized leptin. To address the question of how effective cycloheximide is at inhibiting protein synthesis in tissue fragments, we incubated fresh subcutaneous adipose tissue in the presence of 35S-labeled methionine, with or without cycloheximide (10 μg/ml). These preliminary data show that, in the presence of cycloheximide, incorporation of 35S was 61% lower at 1 h and 87% lower at 3 h, relative to control. Therefore, it appears that cycloheximide inhibition of protein synthesis is potent and rapid in adipose tissue fragments.

We studied adipose tissue from obese humans. Preliminary studies indicate that the leptin content of adipose tissue from lean individuals is lower than that from the obese (unpublished observation). Recent data from Bortoluzzi et al. (5) showed that the subcellular distribution of leptin varies between obese and nonobese rodents. In normal mice and rats, leptin was mainly localized to the low-density microsomal fraction of the adipocytes. However, in fat cells from obese mice, the leptin was found to be widely distributed throughout the low- and high-density microsomal fraction, as well as in the plasma membrane fractions. Similar results were found when leptin was overexpressed in transently transfected rat adipocytes. The authors suggest that the secretion machinery is saturated at high levels of leptin expression. Our detection of considerable leptin levels within human adipose tissue suggests the relevance of this model, and a systematic comparison of leptin synthesis and secretion between lean and obese subjects will further clarify this issue.

The literature is controversial as to whether insulin affects leptin mRNA. Insulin increases leptin mRNA levels in some cell lines and in rat adipose, but it tends not to increase leptin mRNA in human adipose tissue (7, 13, 28). Despite this, insulin has been found to increase leptin secretion in subcutaneous tissue (16, 28). We have reported that insulin significantly increases leptin accumulation in the culture media (~20–30%) without changing leptin mRNA levels (28). As an anabolic hormone, insulin can act nonspecifically to increase overall protein synthesis, potentially leading to an increase in the amount of leptin secreted. In this study, we compared the level of extractable tissue leptin with the amount of leptin secreted over a 3-h incubation. In tissue cultured in the presence compared with the absence of insulin, there was a greater ratio of leptin secreted to tissue leptin. Although these results do not rule out an insulin effect on translation of leptin mRNA, they do suggest that prior culture with insulin potentiates leptin secretion from obese human adipose tissue. There are no previous reports describing hormonal effects on tissue leptin content in humans; however, Barr et al. (2) similarly found that insulin increased leptin secretion while maintaining levels of tissue leptin. The secretion of adipocyte complement-related protein of 30 kDa (3, 29) and lipoprotein lipase (24) is specifically increased by insulin, even in the presence of cycloheximide. Insulin-sensitive vesicle sorting has been suggested as a potential mechanism underlying this form of regulated secretion (3).

In summary, we show that culture with insulin and dexamethasone regulates the leptin content of obese human adipose tissue. Adipose tissue secretes an amount of leptin approximately equal to tissue leptin levels in 3 h of time, indicating a fairly rapid turnover of leptin stores. Although adipose tissue of obese humans contains substantial tissue leptin, the maintenance of tissue leptin levels and rates of leptin secretion is dependent on new protein synthesis. Importantly, the time (4–7 h) required for alterations in serum leptin in response to feeding (17) or hormones (10, 16, 23, 32) observed in vivo is similar to the time required for inhibition of protein synthesis to affect tissue leptin content and leptin secretion (3–5 h) in vitro. The presence of leptin stores within obese human adipose tissue suggests that changes in leptin output in response to nutritional and hormonal stimuli may be mediated by regulation of leptin secretion as well as leptin synthesis.

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