Acute and chronic regulation of leptin synthesis, storage, and secretion by insulin and dexamethasone in human adipose tissue

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LEPTIN, the peptide hormone encoded by the obese gene, is secreted by adipose cells and plays a role in regulating food intake, energy expenditure, and adiposity, as well as immune and endocrine systems (1, 6, 23). Although deficiency of leptin (37) or the leptin receptor (25) results in obesity, most human obesity is associated with elevated leptin levels (8, 21). The mechanisms that increase plasma leptin levels in proportion to body fat are not well understood. Leptin mRNA levels and secretion during a 2-h in vitro incubation correlate with obesity (as assessed by BMI or body fat) and even more tightly with fat cell size (19, 34). The increased leptin secretion and synthesis in obese fat cells could simply be secondary to increased leptin mRNA levels. However, a number of lines of evidence (5, 29) and our studies in human and rat adipose tissue (15, 16, 28) suggest that leptin production is also regulated at posttranscriptional steps, including translation and secretion.

We (32) previously demonstrated that a substantial quantity of leptin protein resides in a detergent-sensitive compartment of adipose tissue of severely obese subjects, and we proposed that the regulated release of this preformed leptin may contribute to changes in plasma leptin that are known to take place hours after meals (14, 17, 20, 31, 33). The existence of a preformed pool could provide a mechanism that permits more rapid changes in circulating leptin than would be possible with alterations in de novo synthesis. For example, variations in the release of stored leptin may contribute to pulsatile variations (~30 min) in plasma leptin levels that are documented in both humans (18) and rats (24). To further explore this hypothesis, we examined the relationship between leptin content (reflecting the size of preformed leptin stores) and obesity (fat cell size and BMI). We also asked whether leptin content was associated with higher levels of leptin mRNA or rates of leptin biosynthesis as determined by metabolic labeling and whether insulin could acutely increase the rate of release of preformed leptin.

An additional goal of the present investigation was to understand the mechanisms that contribute to higher leptin stores in enlarged fat cells from obese subjects. Our prior studies of adipose tissue from obese subjects in organ culture showed that insulin increased media leptin without affecting leptin mRNA (as assessed by Northern analysis) and that dexamethasone increased media leptin by increasing leptin mRNA (31, 32). These studies did not directly determine whether insulin increased leptin secretion by increasing leptin biosynthesis or secretion per se. Thus, in the present work, we assessed the ability of insulin and glucocorticoid to acutely (1 h) and chronically (days) increase leptin biosynthesis and secretion in human adipose tissue.

METHODS

Subjects. All subjects gave informed written consent. Abdominal (epigastric) subcutaneous (sc) adipose tissue was sampled from nonobese (BMI <30 kg/m²) and obese (BMI >30 kg/m²) subjects undergoing elective surgery at Robert Wood Johnson University Hospital, St. Peter’s University Hospital in New Brunswick, NJ, and University of Maryland at Baltimore, MD. In addition, abdominal (hypogastric, at the level of the umbilicus) sc adipose tissue was taken by fat aspiration. For fat aspiration, the skin was anesthetized with 2% lidocaine, and 2–5 g of adipose tissue were aspirated using a 3-mm
Spirotri cannula (Unitech Instruments, Fountain Valley, CA) connected to a 50-cm³ syringe. None of the subjects had a medical history of metabolic disease such as cancer or diabetes, nor were any taking medication known to affect adipose tissue metabolism. Subjects taking beta-blockers, but not other antihypertensive drugs, were excluded. Some subjects had lost weight from prior obesity surgery but were weight stable for at least 1 mo before surgery. There were a total of 46 subjects [22 nonobese: 15 F and 7 M, BMI 25 ± 0.5 kg/m² (range 21.8–29.1 kg/m²), mean fat cell size 0.47 ± 0.15 μg lipid/cell and 24 obese individuals: 16 F and 8 M, BMI 43.5 ± 1.5 kg/m² (range 33.1–63.3 kg/m²) and mean fat cell size 0.75 ± 0.04 μg lipid/cell].

Tissue handling. Immediately after excision or aspiration, aliquots of adipose samples were frozen for RNA extraction. Additional aliquots were placed in serum-free medium 199 (M199; Invitrogen) containing 5 μg/ml gentamicin for transport to the laboratory. Upon arrival, tissue was finely minced (5–10 mg fragments) aseptically, to approximately the size of the fragments of adipose tissue from the needle aspirations, and used for experiments. Results with respect to fat cell size and leptin content and secretion, and with regard to insulin and dexamethasone effects were similar with both techniques (obtained during surgery and minced in the laboratory or through needle biopsy); thus the results were pooled and analyzed.

Determination of leptin content and release. Tissue fragments were homogenized in a ground-glass homogenizer in 300–400 μl (100 mg tissue) of cold TES (10 mM Tris, 1 mM EDTA, 250 mM sucrose) buffer, pH 7.4, containing protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) with 1% Triton X-100. Homogenates were centrifuged at 14,000 rpm (15 min, 4°C), and the internatant below the fat cake was used for measuring leptin (32). To measure leptin release, tissue fragments (~100 mg) were incubated in 1 ml of M199 + 1% BSA for 3 h at 37°C with shaking (70 cycles/min). Medium was frozen at ~80°C for subsequent determination of leptin concentration. Tissue lyses and media samples were assayed for leptin using a radioimmunoassay kit (Linco, St. Louis, MO) according to the manufacturer’s protocol. Leptin values are expressed as nanograms per gram tissue or as nanograms per 10⁶ cells over stated time periods. Appropriate blanks (incubation medium and tissue lysis buffer) and an additional standard at 0.25 ng/ml were included in each assay.

Organ culture. Aliquots of aspirated or minced surgical adipose tissue were placed in organ culture in serum-free medium (M199) in an incubator maintained at 37°C at 5% CO₂, as previously described (10). M199 contains 5 mM glucose and essential and nonessential amino acids, including glutamine. Insulin at 7 nM and/or 25 nM dexamethasone (Dex, a synthetic glucocorticoid) were added to culture medium as described. Aliquots of medium samples were saved for measuring leptin level. The results were similar between 1 or 2 days of culture; thus, data were combined. After 1 or 2 days of culture, rates of leptin biosynthesis and leptin mRNA levels were determined as described below.

Biosynthetic labeling and immunoprecipitation of leptin. To assess the effects of chronic culture on leptin biosynthetic rates, cultured adipose tissue (0.5–1 g) was incubated in 10 ml of methionine- and cysteine-free minimal essential medium (MEM; Sigma, St. Louis, MO) in a 50-cm³ polystyrene centrifuge tube containing 4% BSA, pH 7.4, and the same hormones as during culture, under an atmosphere of 95% O₂-5% CO₂ with shaking at 70 oscillations/min at 37°C. After preincubation, tissue was placed in 1 ml of the same medium with 1 μCi/ml [³⁵S]methionine/cysteine (Easy Tag Express; PerkinElmer, Boston, MA) under the same conditions and pulse-labeled for 45 min. Tissue was then homogenized in lysis buffer (1 ml/g tissue: 50 mM Tris base, 300 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, pH 7.4), containing protease inhibitors (Protease Inhibitor Cocktail Set III). Lysates were centrifuged at 2,500 rpm for 15 min at 4°C to remove the fat cake, and the internatant below the fat cake was centrifuged at 14,000 rpm for an additional 15 min at 4°C and used for immunoprecipitation as described previously (16) with 5 μl of rabbit anti-human leptin antibody (Biovendor, Brno, Czech Republic) bound to protein A-Sepharose (Sigma) for 1 h at 4°C. Pellets were washed three times with washing buffer (0.15 M NaCl, 0.2 M Tris, 0.1% Na-lauryl sarcosine, 3% Triton X-100, pH 7.4) with proteolytic inhibitors. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography (Phospholmager, Molecular Dynamics). Bands were cut out of the gel and digested in 1 ml of H₂O₂, and radioactivity was determined by liquid scintillation counting.

Control experiments showed that the immunoprecipitated leptin migrated in the gel at the same molecular mass as [³⁵S]-leptin standard (~16 kDa). Control immunoprecipitations with nonimmune rabbit serum did not show any band at the position of leptin. Furthermore, competition with excess unlabeled leptin during the immunoprecipitation abolished the radlolabeled leptin band. Labeling of leptin was linear with time for 60 min, and no [³⁵S]methionine-labeled leptin appeared in the medium during the 45-min pulse period. The insulin effect on leptin biosynthetic rates was similar between day 1 and day 2; thus, data were combined for analysis.

Incorporation of the label into TCA-precipitable protein was determined, and rates of leptin biosynthesis under different conditions were normalized to the differences in TCA-insoluble protein to calculate relative rates of leptin biosynthesis.

Acute effects of insulin and Dex on leptin biosynthesis. To test the effects of insulin and/or Dex on leptin biosynthesis, freshly obtained adipose tissue fragments were preincubated with no hormones (basal), insulin (7 nM), Dex (25 nM), or insulin + Dex for 1 h and pulse-labeled with [³⁵S]methionine/cysteine for 45 min under the same conditions as the preincubation.

Pulse-chase experiments. After pulse-labeling of leptin under basal conditions, equal weights (~500 μg) of labeled tissue were either homogenized in lysis buffer or chased in medium containing excess unlabeled methionine and cysteine (MEM with 4% BSA, 200 nM adenosine, 45 mg/l methionine, 78 mg/l cysteine, 10 μg/ml cycloheximide, pH 7.4) at 37°C with shaking (100 cycles/min), in the conditions of basal (without hormones), insulin (7 nM), Dex (25 nM) or Ins + Dex. After a 1-h chase, tissue was homogenized in lysis buffer and medium samples were saved for the immunoprecipitation. [³⁵S]leptin was immunoprecipitated from incubation medium and tissue lyses and then analyzed on ~12% SDS-PAGE. [³⁵S]leptin was also immunoprecipitated from the initial (0') pulse-labeled tissue homogenates, and data were presented as percent 0' pulse in each experiment.

RNA extraction. Total RNA was extracted from adipose tissue (frozen immediately at time 0' and after 2-day organ culture in vitro) using a modified method of Chomczynski and Sacchi (7). Total RNA was treated with DNase (RNase-Free DNase Set, Qiagen), and the quality of RNA was checked with electrophoresis.

Quantitative real-time PCR. Reverse transcription was carried out in a reaction containing 1 μg of total RNA, using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). The leptin mRNA expression level was determined relative to cyclophilin A mRNA level by real-time PCR using commercially available assay-on-demand Taqman primers and probe on an ABI Prism 7900HT system (Applied Biosystems, Foster City, CA). Data were obtained as threshold cycle (Ct) values. Relative gene expression levels of leptin were calculated using the formula \(2^{\Delta \Delta Ct} = 2^{\Delta \Delta Ct}
\), where \(\Delta Ct\) = Ct leptin - Ct cyclophilin A
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Determination of fat cell size and number. Mean fat cell weight was determined by Coulter counting of osmium-fixed fragments of adipose tissue, as described by Hirsch and Gallian (11) or by microscopy (22). Fat cell number was determined by dividing the weight of lipid in tissue [measured with Folch extraction (9)] by mean fat cell weight. Both methods gave comparable results.

Statistical analyses. All data are means ± SE. Prior to statistical analysis, data were log transformed to normalize variance. One-way or two-way analysis of variance was used to determine treatment effects vs. the appropriate control group at a specific time point. When significant main effects and/or interactions were found, post hoc comparisons between treatments were made with a Bonferroni t-test. Significance was set at the P < 0.05 level.

References for this article have been excluded for brevity. For a complete list of references, please refer to the full-text version of the article.
RESULTS

**Intracellular leptin content was highly correlated with mean fat cell weight and BMI.** Tissue leptin content was highly correlated with BMI (Fig. 1A: in females, $r = 0.85$, $P = 0.0004$, $n = 12$; in males, $r = 0.85$, $P = 0.014$, $n = 7$) and mean fat cell weight (Fig. 1B: in females, $r = 0.70$, $P = 0.011$, $n = 12$; in males, $r = 0.88$, $P = 0.010$, $n = 7$). Tissue leptin content tended to be greater in females than in males, as expected (13). For 10 females, we measured both leptin release during an acute 3-h in vitro incubation and tissue leptin content

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Fig. 1. Leptin content and release are correlated with BMI and fat cell size. A: relationship between BMI and tissue leptin content. B: relationship between fat cell size and tissue leptin content. C: relationship between tissue leptin content and leptin release during 3-h acute incubation. D: relationship between fat cell size and leptin release during 3-h acute incubation. Leptin content and release from fragments of adipose tissue and fat cell size were measured as described in METHODS. Relationship between variables was calculated by linear regression analysis. Pearson $r$ and $P$ values are shown.

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Fig. 2. Rates of leptin synthesis are tightly correlated with fat cell size, BMI, and tissue leptin content. A: relationship between fat cell size and rates of leptin biosynthesis. B: relationship between BMI and rates of leptin biosynthesis. C: relationship between tissue leptin content and leptin biosynthesis. D: relationship between mRNA levels and leptin biosynthesis. Relationship between variables was calculated by linear regression analysis. Pearson $r$ and $P$ values are shown.
and found that leptin release was highly correlated with tissue leptin content (Fig. 1C: $r = 0.82, P = 0.003$) and fat cell size (Fig. 1D: $r = 0.68, P = 0.03$). Also, higher leptin content was associated with higher leptin mRNA ($r = 0.86, P = 0.014, n = 7$ F; data not shown).

**Biosynthetic rates of leptin correlated with tissue leptin content and adiposity (fat cell size or BMI) but not with leptin mRNA levels.** Rates of leptin biosynthesis correlated well with fat cell size ($r = 0.89, P < 0.01, n = 8$; Fig. 2A), BMI ($r = 0.85, P = 0.0004$; Fig. 2B), and tissue leptin content ($r = 0.85, P = 0.007$; Fig. 2C). On average, relative rates of leptin biosynthesis were 2.5-fold higher in obese compared with nonobese subjects [nonobese (BMI <30): 0.037 ± 0.004% vs. obese (BMI >30): 0.084 ± 0.003% of TCA-precipitable protein, $P = 0.003$]. Biosynthetic rates of leptin did not significantly correlate with leptin mRNA levels (Fig. 2D), probably due to small sample size, but this weak association further suggested the potential importance of a posttranscriptional mechanism(s) regulating leptin production.

**Chronic treatment with insulin or Dex increases leptin biosynthesis: additive effects of insulin and Dex on leptin.** To study the mechanism that maintains high leptin contents in obese adipose tissue, we tested the effects of chronic insulin, Dex, or insulin + Dex on leptin mRNA, biosynthetic rates, and media leptin accumulation by use of adipose organ culture. Although Dex increased the abundance of leptin mRNA levels, treatment with insulin did not affect leptin mRNA levels either in the absence or presence of Dex, in agreement with our previous report (31) (Fig. 3A). Insulin tended to increase, but Dex suppressed, total protein synthesis (incorporation of [35S]methionine/cysteine into TCA-precipitable protein) (basal, 80 ± 11.9; insulin, 92 ± 14.8; Dex, 59 ± 8.2; insulin + Dex, 88 ± 14.3 × 10^6 cpm/g tissue). Thus, relative rates of leptin biosynthesis were presented as percent total protein synthesis. Relative rates of leptin synthesis in adipose tissue cultured with insulin were 2.5-fold higher than basal control (Fig. 3B). Dex alone increased relative rates of leptin biosynthesis 2.9-fold compared with the basal condition. When insulin was added in

**Fig. 3. Effects of chronic insulin and/or dexamethasone (Dex) treatment on leptin mRNA levels (A), relative rates of leptin biosynthesis (B), and leptin release into culture medium from adipose tissue fragments (C).** sc Adipose tissue from obese individuals (BMI, 38 ± 0.87) was cultured for 1 ($n = 1$) or 2 ($n = 7$) days in serum-free medium containing no hormones (Basal, B), 7 nM insulin (I), 25 nM Dex (D), or insulin + Dex (ID). Leptin mRNA expression levels after culture, relative to cyclophilin A mRNA levels, were determined by quantitative real-time PCR. Relative rates of leptin biosynthesis after culture with insulin and/or Dex were determined as described in METHODS. Equal amounts of TCA-precipitable counts were loaded in each lane for the representative fluorograph shown above in B. Leptin levels in culture medium were determined by RIA. Bars with different letters are significantly different from each other (at least, $P < 0.05$).
the presence of Dex, insulin further increased leptin biosynthetic rates, such that the combination of insulin and Dex increased relative rates of leptin more than 5.2-fold. Insulin and Dex effects on leptin biosynthesis paralleled leptin accumulation in culture medium (Fig. 3C). These data suggest that chronic exposure to insulin and Dex regulates leptin biosynthesis through different mechanisms: Dex regulates leptin by acting at the mRNA level, but insulin may regulate leptin by acting at the posttranscriptional level.

Insulin acutely increased secretion of newly synthesized leptin in human adipose tissue. A 1-h and 45-min treatment (preincubation + pulse labeling period) with insulin and/or Dex did not acutely affect total protein synthesis (data not shown) or relative rates of leptin biosynthesis in human adipose tissue (Fig. 4A). To directly test whether insulin and/or Dex affect the rates of leptin secretion from human adipose tissue, we conducted pulse-chase experiments. Adipose tissue was preincubated and pulse-labeled in the absence of any hormones and then chased in the conditions of basal (no hormones), insulin, Dex, or insulin plus Dex for 1 h. In the absence of insulin, ~20% of newly synthesized leptin was secreted into incubation medium (Fig. 4B). Dex alone did not increase the secretion of newly synthesized leptin. Insulin, however, significantly increased the rate of leptin secretion in the absence or presence of Dex (+83%, basal vs. insulin alone: 19 ± 4.2 vs. 33 ± 5.6% of 0 pulse, n = 3, P < 0.01; +68%, Dex vs. insulin + Dex: 19 ± 5.5 vs. 31 ± 6.9% of 0 pulse label, n = 3, P < 0.01).

[35S]leptin remaining inside tissue and released into medium were summed to determine whether newly synthesized leptin was disappearing (being degraded) from the system and whether insulin and/or Dex affected this process (Fig. 4B). In the absence of insulin (basal or Dex alone), ~30% of newly synthesized leptin disappeared from the system, whereas the insulin stimulation of secretion was associated with decreased degradation (~10%). Dex, added in the absence or presence of insulin, did not affect the degradation of newly synthesized leptin during the 1-h chase.

DISCUSSION

Our results demonstrate that the tissue content of leptin is increased in proportion to increased fat cell size in obesity and is maintained by higher rates of leptin biosynthesis. Furthermore, studies in organ culture suggest that chronic effects of insulin and glucocorticoid upregulate leptin biosynthesis through post- and pretranscriptional mechanisms, respectively. Thus, the higher leptin content per fat cell of obese adipose tissue may result from chronic exposure to hyperinsulinemia and higher local cortisol levels in vivo. Furthermore, we show that, in addition to the maintaining “basal” or spontaneous release of leptin over the long term, insulin can acutely stimulate the rate of release of preformed (biosynthetically labeled) leptin, as we have previously observed in rat adipose tissue (16). Thus, our data suggest that the insulin stimulation of leptin secretion from a stored intracellular pool may contribute to meal-induced variations in serum leptin levels that are documented in humans as well as rats (3, 18, 35, 36).

Studies in rats support the hypothesis that the size of the cellular leptin pool is likely to have physiological significance. Walker et al. (35) found that administration of glucose caused a prompt increase in serum leptin, independent of alterations in leptin mRNA, in fed rats with higher adipose tissue leptin content but not in fasted rats with low adipose tissue leptin content. In addition, the magnitude of ultradian leptin pulses is greater in obese than in lean humans (36), and this could be related to their larger stores of preformed leptin.

The higher rates of leptin biosynthesis in obese adipose tissue were not highly correlated with levels of leptin mRNA but were tightly correlated with leptin content. These results
suggest that posttranscriptional mechanisms contribute to leptin storage such that a greater proportion of the newly synthesized leptin is retained within the tissue. Consistent with our prior observations (31), chronic culture with insulin increased leptin release without altering leptin mRNA levels, here determined by quantitative RT-PCR. Moreover, we show directly that chronic culture with insulin increases relative rates of leptin biosynthesis. In addition, culture with Dex upregulated leptin mRNA levels. Thus, glucocorticoids and insulin have complementary effects to upregulate leptin synthesis. We therefore speculate that the chronic hyperinsulinemia and local hypercortisolemia [generated by higher 11β-hydroxysteroid dehydrogenase activity in obese adipose tissue (27)] contributes to the higher rates of leptin biosynthesis that we documented in adipose tissue of obese subjects.

Although chronic treatment with insulin and/or Dex increased relative rates of leptin biosynthesis, acute treatment (for almost 2 h) did not. The mechanism whereby insulin increases leptin translational initiation and/or elongation via mTOR-dependent mechanisms merits further investigation (26).

Insulin, with or without Dex, stimulated the release of pulse-labeled leptin into the medium. We therefore conclude that, similarly to rat adipose tissue and adipocytes (2, 16, 30), the insulin stimulation of leptin secretion in human adipose tissue contributes to higher rates of leptin release in the fed compared with the fasted state. The subcellular localization of the “stored” leptin within human adipose tissue is not known. Using confocal microscopy, Barr et al. (2) suggested that insulin could increase transport of leptin from the endoplasmic reticulum (ER) toward the plasma membrane. Using subcellular fractionation methods, however, Roh et al. (30) found that most leptin in rat adipocytes was localized in low-density microsomes (Golgi complex, trans-Golgi network, and endosomes) rather than in the ER. Examining adipose tissue from nonobese subjects by electron microscopy, Bornstein et al. (4) found leptin within small vesicles near the plasma membrane of human adipocytes. Future studies are needed to examine the effects of obesity and fat cell size on the subcellular localization of putative storage pools, as well as on the mechanisms regulating the translocation of leptin in response to hormonal and nutritional stimuli.

In summary, our results clearly demonstrate that, for the example of leptin, the endocrine function of the adipocyte is regulated at multiple pre- and posttranslational steps. Chronically, high local glucocorticoids upregulate leptin mRNA levels, and chronic exposure to insulin further enhances leptin biosynthesis at the translational level and leads to the higher “basal” secretion of leptin from hyper trophyed fat cells in obesity. The high rates of leptin biosynthesis apparently lead to an accumulation of leptin stores within adipose tissue, and the release of this preformed leptin is stimulated by insulin, providing a mechanism for short-term changes in serum leptin in response to hormonal or nutritional changes. Furthermore, the enlarged fat cells of obese individuals are clearly highly responsive to the chronic and the acute stimulatory effects of insulin on leptin synthesis and secretion, respectively. Thus, although obese fat cells may become insulin resistant with respect to glucose transport or lipolysis (12), with regard to the action of insulin on leptin the obese fat cell is not insulin resistant. We speculate that the insulin regulation of leptin production and secretion may help compensate for leptin resistance and contribute to the functioning of the leptin system in obesity.

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