

Multilevel regulation of leptin storage, turnover, and secretion by feeding and insulin in rat adipose tissue

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Abstract The mechanisms of the increased serum leptin in response to feeding are poorly understood. Therefore, we used metabolic labeling to directly assess leptin biosynthesis, secretion, and turnover in adipose tissue from 14 h-starved compared with fed 12–14 week old rats. Starvation decreased serum leptin ($-47 \pm 7\%$), adipose tissue leptin content ($-32 \pm 5\%$), and leptin secretion during 3 h of incubation ($-65 \pm 12\%$). Starvation did not affect leptin mRNA levels but decreased rates of leptin biosynthesis by tissue fragments, as determined by [³⁵S]methionine/cysteine incorporation into immunoprecipitable leptin. Insulin in vitro did not acutely increase leptin biosynthesis or rates of ¹²⁵I-leptin degradation. Pulse-chase studies showed that in adipose tissue from fed but not starved rats, insulin accelerated the secretion of [³⁵S]leptin by ~ 2 -fold after 30 and 60 min of chase. Degradation of newly synthesized leptin was slower in adipose tissue of starved than fed rats (half-lives of 50 and 150 min, respectively). Inhibitor experiments showed that both lysosomes and proteosomes contributed to leptin degradation. **In conclusion, feeding compared with starvation influences leptin production at multiple post-transcriptional levels: synthesis, tissue storage, turnover, and secretion. The insulin-stimulated release of leptin from a preformed intracellular leptin pool may contribute to increases in serum leptin levels after meals.**—Lee, M-J., and S. K. Fried. **Multilevel regulation of leptin storage, turnover, and secretion by feeding and insulin in rat adipose tissue.** *J. Lipid Res.* 2006. 47: 1984–1993.

Supplementary key words degradation • lipoprotein lipase • proteosome • lysosome • starvation • translation

Serum leptin levels and rates of secretion are proportional to body fat content (1, 2). In addition, fasting decreases and feeding and/or insulin increases serum leptin over a time course of hours, independent of body fat (3–5). Although most previous studies (6–11) have focused on regulation at the mRNA levels with starvation or insulin, we (12) and others (13–15) recently suggested that leptin production is also regulated at posttranscrip-

tional steps. Our recent study showed that in young rats (6 to 7 weeks old), short-term starvation (14 h) only minimally affected leptin mRNA levels but markedly decreased leptin release by decreasing the rate of leptin biosynthesis (16).

In addition to alterations in its synthesis, the processing of leptin in the short term (i.e., within minutes to an hour) may also be regulated (13–15, 17, 18), although reports are conflicting. Two papers report that the protein synthesis inhibitor cycloheximide (CHX) does not block the ability of insulin to stimulate leptin release from rat adipocytes (13, 14), suggesting that insulin acts on the leptin secretion step per se, whereas another study (19) concluded that insulin stimulated leptin release by increasing its synthesis.

Newly synthesized leptin may be stored, secreted, or degraded intracellularly before being secreted. Nutritional status and hormonal stimuli (i.e., insulin) could affect any of these processes to regulate leptin release. Thus, in this study, we used pulse-chase methodology to directly assess the effect of starvation/feeding and insulin on the turnover and secretion of newly synthesized leptin in adipose tissue. We also used chemical inhibitors of lysosomal and proteosomal degradation to investigate the mechanism of leptin turnover.

MATERIALS AND METHODS

Animals

Male Wistar rats were purchased from Charles River Laboratory (Wilmington, MA) and housed with free access to laboratory chow and water until they were 12–14 weeks old. The dark/light cycle was 12 h of dark (6:00 PM to 6:00 AM) and 12 h of light (6:00 AM to 6:00 PM). On the day before the experiment, rats of similar body weight were either starved for 14 h (8:00 PM to 10:00 AM) with water available or fed ad libitum. Rats were then anesthetized using CO₂ and euthanized by decapitation. Trunk blood was collected and plasma was stored at -80°C for leptin

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measurement. Epididymal and retroperitoneal fat pads were pooled and used for experiments. Both depots have similar levels of leptin mRNA and protein (our unpublished observation) and respond to starvation in a similar manner (11). Minced tissue (5–10 mg pieces) was washed with 0.9% NaCl on a 250 μ m nylon mesh, gently blot-dried, and used for experiments. Aliquots of tissue were frozen immediately in liquid nitrogen to measure initial tissue leptin content and leptin mRNA levels. The protocols were approved by Institutional Animal Care and Use Committee of the University of Maryland at Baltimore and the Baltimore Veterans Administration Medical Center.

Measurement of leptin secretion and tissue leptin content

Leptin release and tissue leptin content were measured as described previously (20). Leptin levels in tissue homogenates and incubation medium were determined with a rat leptin RIA kit (Linco, St. Charles, MO). Appropriate blanks (incubation medium and tissue lysis buffer) and an additional standard of 0.25 ng/ml were included in each assay. Leptin values are expressed as ng/ 10^7 cells or ng/g tissue over the stated time intervals.

Insulin effect on leptin secretion

Minced adipose tissue fragments from ad libitum-fed rats were incubated with or without insulin in the absence or presence of CHX (10 μ g/ml) for up to 3 h. The medium after incubation was collected and used to measure leptin.

Determination of fat cell size and fat cell number

Mean fat cell weight was determined by Coulter counting (MultiSizer III; Beckman Coulter, Fullerton, CA) of freshly isolated adipocytes or by microscopy (21). Fat cell number was determined by dividing the weight of lipid per gram of tissue, measured with Folch extraction (22), by average fat cell weight.

RNA extraction and Northern blotting

Total RNA was extracted from adipose tissue (frozen immediately: 0 min) or after incubation in the absence (basal) or presence of insulin (6 nM) for 2 h using a modified method of Chomczynski and Sacchi (23). Twelve micrograms of total RNA was separated on 1.2% agarose gels, and Northern blotting was performed as described previously (12). 18S rRNA was captured on images after electrophoresis, quantified, and used as a loading control. Data are presented as leptin mRNA/18S rRNA.

Measurement of relative rates of leptin and LPL biosynthesis

Pulse-labeling experiments were performed as described previously (17). Briefly, adipose tissue fragments from 12–14 week old rats (overnight-starved or ad libitum-fed) were preincubated in MEM (without methionine and cysteine; Sigma, St. Louis, MO) with 4% BSA, 2 mM glutamine, and 200 nM adenosine in the absence (basal) or presence of insulin (6 nM) for 1 h and pulse-labeled for 45 min with [35 S]methionine/cysteine (500 μ Ci/ml; Perkin-Elmer, Wellesley, MA) in MEM under the same hormonal conditions. After washing with saline, tissue was homogenized in lysis buffer containing proteolytic inhibitors. [35 S]leptin was immunoprecipitated with a polyclonal antibody to human leptin (Biovender, Brno, Czech Republic) from tissue homogenates and analyzed with fluorography after 4–12% SDS-PAGE. The completeness and specificity of immunoprecipitation were assessed in preliminary experiments, and the immunoprecipitated leptin migrated in the gel at the same molecular mass as 125 I-leptin standard (\sim 16 kDa). To assess rates of LPL synthesis, a second immunoprecipitation was carried out with LPL antibody (chicken anti-

bovine LPL; a gift from John Goers, California Polytechnic State University) as described previously (24). Immunoprecipitated LPL was analyzed with fluorography after 10% SDS-PAGE. Band intensities were quantified using ImageQuant software 5.0 (Molecular Dynamics, Boston, MA) after exposure to a PhosphorImager screen (Molecular Dynamics). 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.

Pulse-chase experiments

After pulse-labeling of leptin, equal weights (\sim 500 mg) of labeled tissue were divided into tubes containing chase medium (MEM with 4% BSA, 200 nM adenosine, 45 mg/l methionine, 78 mg/l cysteine, and 10 μ g/ml CHX, pH 7.4) without or with insulin (6 nM) and chased at 37°C with shaking (100 cycles/min) for up to 3 h. At each chase time point, tissue was homogenized in an equal volume of lysis buffer and medium samples were saved for the immunoprecipitation. [35 S]leptin was immunoprecipitated from incubation medium and tissue lysates at each chase time point and then analyzed by 4–12% SDS-PAGE. Dried gels were exposed to a PhosphorImager screen (Molecular Dynamics), and band intensities were quantified. [35 S]leptin was also immunoprecipitated from the initial (0 min) pulse-labeled tissue homogenates, and data are presented as percentage of the 0 min pulse in each experiment.

To test the effects of chemical inhibitors on leptin degradation, vehicle control, 5–10 μ M MG 132, or 20 μ M chloroquine were included during the preincubation (the last 15 min), pulse-labeling (1 h), and chase periods.

125 I-leptin degradation

To determine whether secreted leptin is degraded nonspecifically during incubation, adipose tissue conditioned medium was obtained by incubating tissue fragments, in the absence or presence of insulin, for 2 h at 37°C. 125 I-leptin (0.05 μ Ci/ml; Perkin-Elmer) was added to the conditioned medium and incubated for another 3 h at 37°C. Aliquots of incubation medium were taken at 0 min, 1 h, and 3 h of the incubation period and spotted on glass fiber filters (Whatman, Florham Park, NJ). After precipitating total proteins with 12% TCA, filters were washed five times with water, four times with methanol, and three times with acetone, and precipitated 125 I-leptin was determined with a γ counter (Perkin-Elmer). Precipitated 125 I-leptin was determined in the incubation medium at the beginning of each experiment, and data are presented as percentage of the initial 125 I-leptin value.

To examine tissue degradation of 125 I-leptin, adipose tissue fragments (\sim 50 mg) from overnight-starved or ad libitum-fed animals were incubated in 0.5 ml of medium (MEM + 1% BSA) containing 0.05 μ Ci/ml 125 I-leptin (Perkin-Elmer) in the absence or presence of 6 nM insulin. Aliquots of incubation medium were taken at 0 min, 1 h, and 3 h of incubation, and the amount of degraded 125 I-leptin was determined by TCA precipitation. Control incubations containing 125 I-leptin, but no adipose tissue fragments, were always run in parallel.

Statistical analyses

All data are presented as means \pm SEM. One-way or two-way ANOVA was used to determine treatment effects. When significant main effects and/or interactions were found, posthoc comparisons between treatments were made with a Bonferroni *t*-test. Significance was set at $P < 0.05$.

RESULTS

Overnight starvation decreased plasma leptin levels, tissue leptin content, and leptin secretion without affecting leptin mRNA levels

Body weight and fat cell size were not different between overnight-starved (14 h) and ad libitum-fed rats at 12–14 weeks old, respectively, but serum leptin levels ($-47 \pm 7\%$), tissue leptin content ($-32 \pm 5\%$), and leptin release during an acute 3 h incubation ($-65 \pm 12\%$) were lower in the starved group (Table 1). However, this period of starvation did not affect leptin mRNA levels (Fig. 1B).

Overnight starvation decreased relative rates of leptin biosynthesis by 50%

Overnight starvation tended to decrease [^{35}S]methionine/cysteine incorporation into adipose tissue total protein by $\sim 18\%$ [15.2 ± 3.4 (fed) vs. 12.5 ± 2.2 (starved) $\times 10^6$ cpm/ 10^6 cells; $n = 4$, NS] and significantly decreased relative rates of leptin biosynthesis by $51 \pm 13\%$ (Fig. 1A) ($P < 0.05$, $n = 4$). When these nonspecific and specific effects were combined, starvation resulted in a $70 \pm 15\%$ decrease in total leptin biosynthesis.

Resistance to the insulin effect on leptin biosynthesis

To determine whether insulin acutely stimulated leptin biosynthesis in adipose tissue from ad libitum-fed or 14 h-starved 12–14 week old rats, leptin biosynthesis was measured after a 2 h in vitro incubation. Leptin mRNA levels were maintained at initial values during the 2 h incubation, and insulin had no effect (Fig. 1B). Insulin tended to increase [^{35}S]methionine incorporation into TCA-precipitable protein by 15–25% in both the starved state [12.5 ± 2.2 (basal) vs. 14.3 ± 4.0 (insulin) $\times 10^6$ cpm/ 10^6 cells; $n = 4$, NS] and the fed state [15.2 ± 3.4 (basal) vs. 19.2 ± 5.5 (insulin) $\times 10^6$ cpm/ 10^6 cells; $n = 4$, NS]. However, insulin did not increase relative rates of leptin biosynthesis in adipose tissue from either starved or fed rats (Fig. 1A).

To test whether there was a general resistance to the insulin biosynthetic effect, the insulin effect on the relative rates of another adipose-secreted protein, LPL, was determined. In contrast to leptin, insulin increased relative rates of LPL biosynthesis in adipose tissue from both starved ($71 \pm 24\%$; $P < 0.05$, $n = 3$) and fed ($45 \pm 18\%$; $P < 0.05$, $n = 3$) rats (Fig. 1C), in agreement with pre-

vious results (24). Thus, the inability of insulin to stimulate leptin biosynthesis in these 12–14 week old rats was specific.

Insulin stimulated leptin secretion independent of its effects on leptin synthesis

Although insulin did not increase leptin translation in adipose tissue of 12–14 week old rats, it could increase leptin release and hence serum leptin levels by stimulating the process of leptin secretion per se. To address whether insulin increases leptin secretion independent of protein synthesis, adipose tissue fragments were incubated for up to 3 h with or without insulin and/or CHX. Leptin release from adipose tissue fragments was linear for up to 3 h in the absence or presence of insulin (Fig. 2A), suggesting that adipose tissue in vitro actively synthesizes and secretes leptin. At each time point, insulin significantly increased leptin secretion: $36 \pm 9\%$ ($P < 0.01$, $n = 9$) at 1 h, $23 \pm 4\%$ ($P < 0.05$, $n = 9$) at 2 h, and $27 \pm 8\%$ ($P < 0.05$, $n = 9$) at 3 h. When protein synthesis was prevented with CHX ($10 \mu\text{g/ml}$), leptin concentration in the medium reached a plateau at 1 h in both the presence and absence of insulin. Even when leptin synthesis was blocked with CHX, insulin produced a small but consistent increase in leptin release at 1 h, by $15 \pm 4\%$ ($P < 0.05$, $n = 9$) (Fig. 2B).

In the presence of insulin and CHX, medium leptin concentration increased from 1 to 2 h of incubation [14.7 ± 5.7 (1 h) vs. 19.9 ± 3.6 (2 h) ng leptin/g tissue; $P = 0.012$], but it tended to decrease after 2 h (16.4 ± 2.4 ng leptin/g tissue at 3 h; $P = 0.06$ vs. 2 h value). Thus, at 3 h, medium leptin did not differ between basal and insulin treatment in the presence of CHX.

Insulin increased the secretion of newly synthesized leptin from fed rat adipose tissue

To directly address whether insulin increases leptin secretion from rat adipose tissue, we carried out pulse-chase experiments with CHX present during the chase. This experimental design also allowed us to assess the turnover of newly synthesized leptin (i.e., whether any leptin was degraded over the 3 h chase period and whether this process was affected by insulin or starvation). As shown in Fig. 3, [^{35}S]leptin declined at a similar rate under basal and insulin conditions, to $29 \pm 6\%$ (basal) and $30 \pm 3\%$ (insulin) of initial values ($n = 4$, NS), but the fate of the labeled leptin differed. Insulin stimulated the secretion of

TABLE 1. Effects of overnight starvation on characteristics of rats, and leptin expression levels

Condition	Body Weight	Fat Cell Size	mRNA	Serum Leptin	Tissue Content	Leptin Release
	<i>g</i>	$\mu\text{g lipid/cell}$	<i>arbitrary units</i>	<i>ng/ml</i>	$\text{ng}/10^7 \text{ cells}$	$\text{ng}/10^7 \text{ cells}/3 \text{ h}$
Fed	342.5 ± 13.3	0.171 ± 0.013	1.04 ± 0.18	7.6 ± 0.4	109.6 ± 10.1	130.7 ± 19.8
Starved	351.3 ± 22.0	0.162 ± 0.005	1.01 ± 0.17	4.0 ± 0.8^a	77.4 ± 17.0^a	45.0 ± 5.86^a

Male Wistar rats (12–14 weeks old) were used for the experiments. One day before the experiment, rats of similar body weight were either starved for 14 h with water available or fed ad libitum. Body weight was measured individually, and trunk blood was collected to measure plasma leptin levels. Cell size, leptin mRNA expression levels, and tissue leptin content were measured as described in Materials and Methods. Data (means \pm SEM of at least three independent experiments) were analyzed with two-way ANOVA followed by Bonferroni post paired *t*-test.

^a $P < 0.01$, starved versus fed.

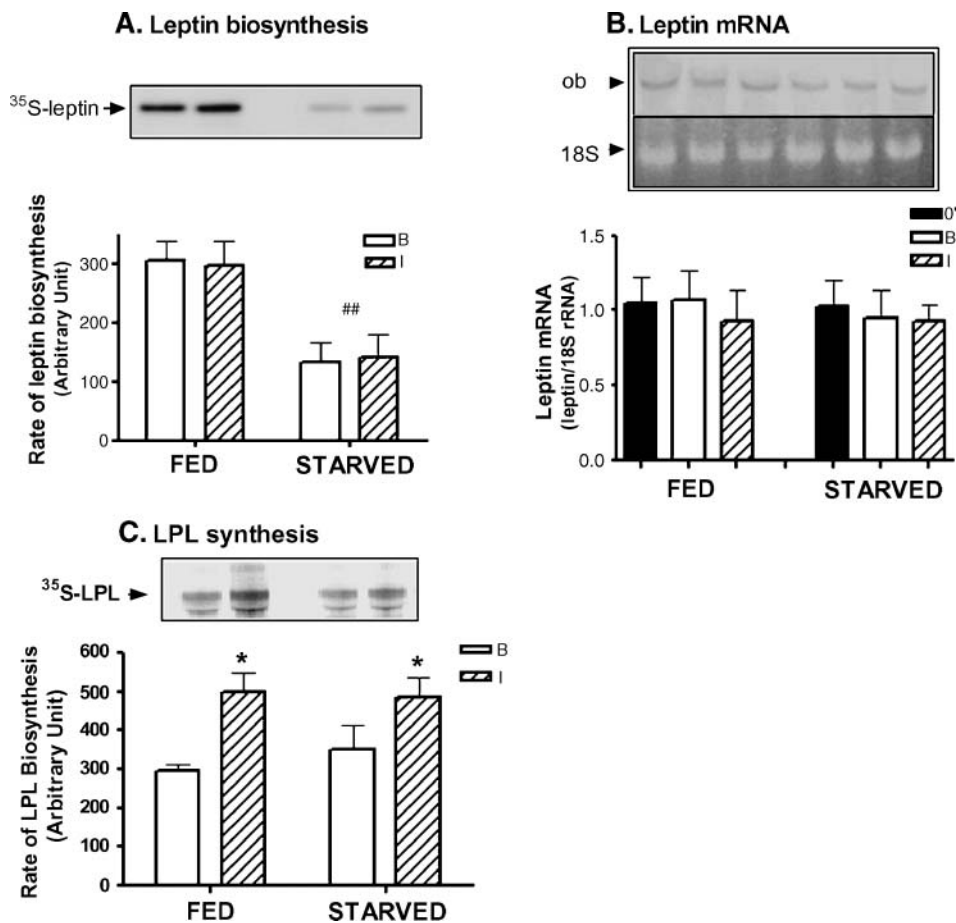


Fig. 1. Effects of overnight starvation and acute insulin treatment (6 nM, 2 h) on the relative rates of leptin biosynthesis (A), leptin mRNA levels (B), and LPL biosynthesis (C) in adipose tissue from 12–14 week old rats. A: Minced adipose tissue fragments from overnight-starved or ad libitum-fed rats were metabolically labeled with [³⁵S]methionine/cysteine in the absence (B, basal; white bars) or presence of 6 nM insulin (I; gray bars) as described in Materials and Methods. In each experiment, adipose tissue from one group of starved and fed rats was used for comparison. Data are presented in arbitrary units (means \pm SEM, $n \geq 3$ individual experiments) after correcting for the differences in TCA-precipitable protein. Data were analyzed by two-way ANOVA followed by Bonferroni post *t*-test for the insulin effect. A representative fluorograph of three or more independent experiments is shown. $^{##} P < 0.01$, starved versus fed. B: For Northern blotting, 12 μ g of total RNA extracted from tissue frozen at 0 min (0'; black bars) and after treatment with (gray bars) or without (white bars) insulin for 2 h was loaded and probed using [³²P]dCTP-labeled human leptin insert as described in Materials and Methods. Data (means \pm SEM of leptin mRNA/18S rRNA, $n \geq 3$ individual experiments) were analyzed by two-way ANOVA followed by Bonferroni post *t*-test for the insulin effect. A representative blot is shown. C: LPL was immunoprecipitated with chicken anti-human LPL antibody and protein A-Sepharose-bound rabbit anti-chicken IgG from tissue lysates after leptin immunoprecipitation as described in Materials and Methods. The same amounts of TCA-precipitable protein were loaded in each lane for these experiments. Data (means \pm SEM, arbitrary units, $n = 3$ individual experiments) were analyzed by two-way ANOVA followed by Bonferroni post paired *t*-test for the insulin effect. A representative fluorograph is shown. $^{*} P < 0.05$, by Bonferroni post *t*-test for the insulin effect.

newly synthesized leptin from adipose tissue of ad libitum-fed rats by 1.8-fold at 30 min [$14 \pm 5\%$ (without insulin) vs. $25 \pm 5\%$ (with insulin); $P < 0.05$, $n = 3$] and by 2.4-fold after 60 min of chase [$20 \pm 4\%$ (without insulin) vs. $48 \pm 7\%$ (with insulin); $P < 0.01$, $n = 4$]. In the presence of insulin, the disappearance of leptin from the tissue from 30 to 60 min of chase (-23%) was matched by its appearance in the medium ($+22\%$). In contrast, under basal conditions, 22% was lost from the tissue and only 6% appeared in the medium. Summing medium and tissue [³⁵S]leptin

to calculate turnover (Fig. 3C), under basal conditions, $52 \pm 4\%$ of the newly synthesized leptin was lost from the system (tissue plus medium) and was presumably degraded after the 1 h chase period. However, in the presence of insulin, only $23 \pm 5\%$ of the newly synthesized leptin was degraded at 1 h ($n = 4$, each $P < 0.01$ compared with basal). Thus, in the presence of insulin and with protein synthesis blocked by CHX, the loss of [³⁵S]leptin during the first hour of chase was mainly accounted for by its appearance in the medium, consistent with the hypoth-

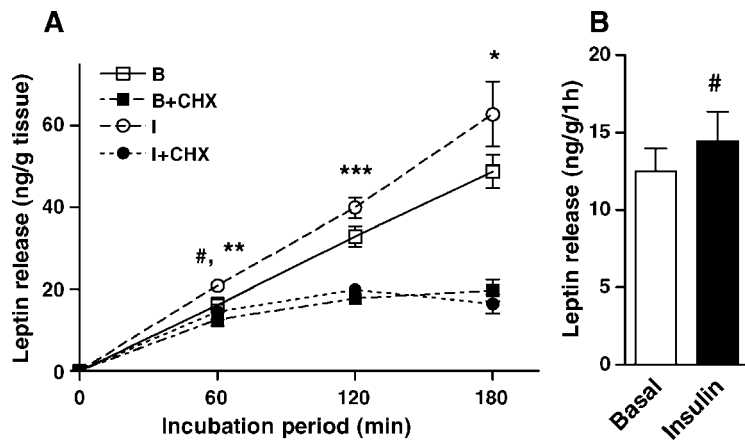


Fig. 2. Effects of cycloheximide (CHX) on insulin-stimulated leptin secretion. Adipose tissue fragments were incubated in MEM with 4% BSA, in the absence or presence of insulin (6 nM), with or without CHX (10 μ g/ml) for up to 3 h. A: Time course of leptin secretion in the basal (B), insulin (I), basal + CHX (B+CHX), and insulin + CHX (I+CHX) conditions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, insulin versus basal; # $P < 0.05$, insulin versus insulin + CHX. Each data point represent nine individual experiments. B: Insulin stimulates leptin secretion even in the presence of CHX at 1 h of incubation. # $P < 0.05$ ($n = 9$). Values shown are means \pm SEM.

esis that insulin stimulates leptin secretion concomitant with a depletion of tissue stores.

Although in the basal condition, medium [35 S]leptin gradually increased until 2 h of chase, in the presence of

insulin, leptin secretion reached its maximum at 1 h of chase and then declined over time. Thus, there was no difference in the amount of [35 S]leptin in the medium between basal and insulin treatment after 2 and 3 h of chase.

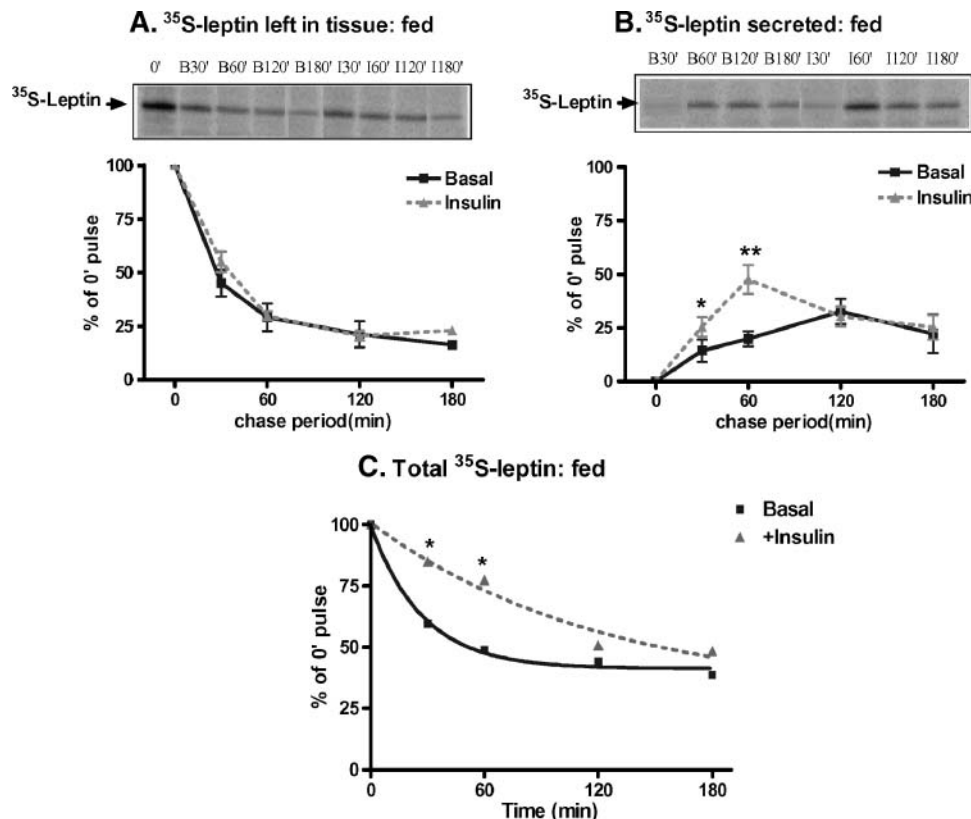


Fig. 3. Effect of insulin on leptin turnover in adipose tissue in the fed state. Adipose tissue from ad libitum-fed rats were pulse-labeled with [35 S]methionine/cysteine (0.5 mCi/ml) for 1 h. After removing unincorporated label by washing with warm saline, weighed portions of labeled tissue were divided into aliquots into tubes containing 3 ml of MEM (4% BSA, cold 45 mg/l methionine, and 78 mg/l L-cysteine) and chased in the absence (B, basal; solid lines) or presence of 6 nM insulin (I; dashed lines) for up to 3 h. One aliquot of tissue was homogenized immediately after labeling to measure initial rates of leptin biosynthesis. [35 S]leptin was immunoprecipitated from tissue lysate (0 min and at each chase time point) and chase medium and analyzed with fluorography after SDS-PAGE. Data are presented as percentages of the 0 min pulse level. Values shown are means \pm SEM of four individual experiments, except for the 30 min time point ($n = 2$). Representative fluorographs are shown at the top of the graphs. A: Leptin remaining in tissue during the chase period. B: Amount of leptin secreted during the chase period. C: Total amount of leptin (remaining in tissue and secreted into chase medium) during the chase period. * $P < 0.05$, ** $P < 0.01$, by Bonferroni paired t -test after one-way ANOVA.

The sum of leptin remaining in the tissue and that appearing in the medium at each time point (Fig. 3C) was also used to assess rates of leptin turnover. In the basal condition, the half-life of newly synthesized leptin was 50 ± 10 min, whereas in the presence of insulin, it was 150 ± 8 min ($n = 4$, $P < 0.01$).

No effect of insulin on leptin secretion and turnover in adipose tissue from starved rats

In contrast to the results from fed rats, in adipose tissue of starved rats, insulin did not affect the amount of [^{35}S]leptin that remained within the tissue (Fig. 4B) or the appearance of [^{35}S]leptin in the chase medium (Fig. 4A). In adipose tissue from starved rats, $30 \pm 6\%$ of the newly synthesized leptin was secreted at 2 h of chase (Fig. 4C). Under basal conditions, only $30 \pm 3\%$ of newly synthesized leptin was degraded in adipose tissue of starved rats, compared with $52 \pm 4\%$ in the fed state ($P < 0.05$, $n = 4$ for fed, $n = 3$ for starved after 60 min of chase). In the presence of insulin, the degradation of leptin was similar in the fed and starved groups, but the mechanisms differed. Thus, starvation, independent of acute insulin treatment, decreased the degradation of newly synthesized

leptin, whereas in the fed state, insulin stimulated leptin secretion indirectly, by decreasing its degradation.

Degradation of exogenously added ^{125}I -leptin

To further investigate the mechanism by which starvation regulates leptin degradation, we studied the fate of exogenously added ^{125}I -leptin. We reasoned that during our pulse-chase studies, secreted leptin could be degraded secondary to the nonspecific release of proteases from the minced adipose tissue or endocytosed and then degraded intracellularly by adipocytes or other cells within adipose tissue. The former possibility was excluded by the finding that ^{125}I -leptin was not degraded during a 3 h incubation at 37°C in adipose tissue conditioned (data not shown) or control medium. After incubation of ^{125}I -leptin with tissue fragments, however, up to 50% was degraded after a 3 h incubation. Adipose tissue from fed rats degraded more leptin ($\sim 200\%$ more at 1 h, $\sim 20\%$ more at 3 h) than tissue from starved rats (each $P < 0.05$ by two-way ANOVA) (Fig. 5). The time lag in the rate of degradation between 1 and 3 h probably represents the time required for diffusion into the tissue fragments. Insulin did not affect the

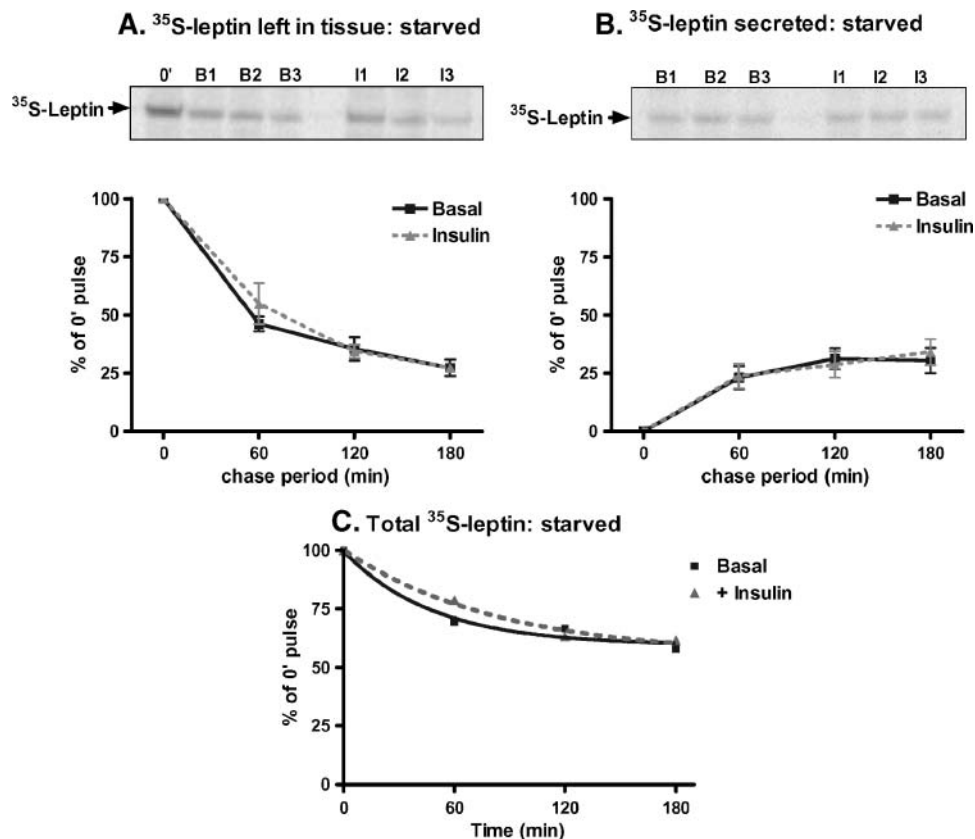


Fig. 4. Effect of insulin on leptin turnover in adipose tissue in the starved state. Adipose tissue from overnight-starved and fed (~ 14 h) rats was used to determine the insulin effect on leptin secretion as described in Materials and Methods. Three individual experiments were repeated. Representative autoradiographs are shown at the top of the graphs. A: Leptin remaining in tissue during the chase period. B: Amount of leptin secreted during the chase period. C: Total amount of leptin (remaining in tissue and secreted into chase medium) during the chase period. Solid lines represent the basal (B; without insulin) condition, and dashed lines represent insulin treatment (I). Values shown are means \pm SEM.

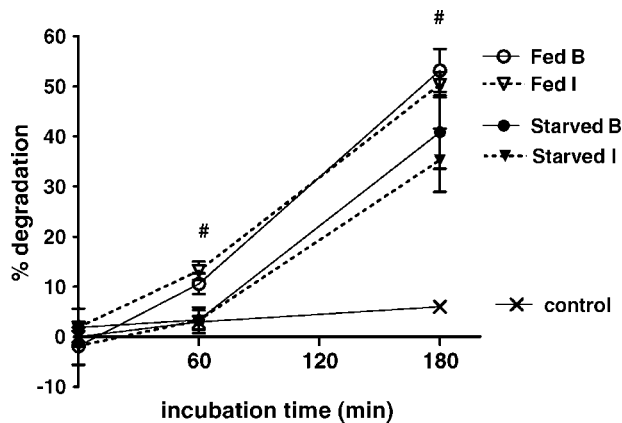


Fig. 5. ¹²⁵I-leptin degradation during adipose tissue incubation. ¹²⁵I-leptin was incubated for up to 3 h with adipose tissue fragments. ¹²⁵I-leptin degradation was determined after TCA precipitation. A control incubation (no tissue) was run in parallel. Data are presented as percentages of the initial value in each experiment. Values shown are means \pm SEM. #, main effect of feeding effect by 2-way ANOVA, $P < 0.05$. B, basal; I, insulin.

rates of degradation during tissue incubations by fed or starved adipose tissue (NS by two-way ANOVA).

Lysosomal and proteosomal pathways contributed to leptin degradation

To further study the mechanism by which leptin was degraded, pulse-chase experiments were performed in the presence of the proteosomal inhibitor MG 132 or the lysosomal inhibitor chloroquine (Fig. 6). Inclusion of chloroquine during the 1 h labeling period had no effect on [³⁵S]methionine/cysteine incorporation into leptin or total protein. MG 132 produced a small ($\sim 8\%$) but statistically insignificant increase in the incorporation of [³⁵S]methionine/cysteine into leptin as well as total TCA-precipitable protein during a 1 h pulse; thus, the effect was not specific for leptin.

Inhibition of the proteosomal pathway with MG 132 did not affect the rate of loss of leptin from the cells or the amount secreted after 1 h. After 3 h of chase in the presence of MG 132, there was no effect on medium leptin, but there was more labeled leptin remaining in the tissue at the end of 3 h. Thus, at the end of 3 h, $45 \pm 6\%$ compared with $32 \pm 3\%$ of the labeled leptin remained in the system in the presence of MG 132 and controls, respectively ($P < 0.05$, $n = 4$). Inhibition of lysosomal degradation with chloroquine did not affect the appearance rate of [³⁵S]leptin in the medium or the rate of decline in tissues during the first hour of chase. In controls, medium [³⁵S]leptin declined or did not change from 1 to 3 h of chase, whereas in the presence of chloroquine, it increased in both experiments (23–89% increase from 1 to 3 h; $n = 2$). Thus, in the presence of chloroquine, 48–59% of the newly synthesized leptin remained in the system after 3 h of changes compared with 32% in controls. Together with our finding that ¹²⁵I-leptin was degraded through a cellular process, these experiments indicate that chloroquine prevents the reuptake and degradation of newly synthesized leptin

through the lysosomal pathway and thereby affects the accumulation of leptin in the medium over time.

DISCUSSION

We demonstrated with metabolic labeling studies that feeding modulated leptin production and release at multiple posttranscriptional steps. The increased leptin translation in adipose tissue of fed compared with starved 12–14 week old rats occurs without any change in leptin mRNA and appears to be a major mechanism that increases tissue leptin stores as well as release. In addition, pulse-chase studies show that insulin stimulated the secretion of preformed leptin from adipose tissue of fed rats. In the absence of an acute insulin stimulus, however, degradation becomes a significant fate of newly synthesized leptin, and both lysosomes and proteosomes are involved. Together, these data suggest that the regulated release of leptin from an intracellular pool may contribute to the increases in serum leptin observed after meals and in response to insulin.

Our studies of leptin mass and metabolically labeled leptin both showed that insulin directly increased leptin release even when new protein synthesis was blocked with CHX, in agreement previous studies (13, 14). Thus, at least in the moderately large fat cells from the 12–14 week old rats studied here, the insulin-stimulated release of stored leptin may contribute to increases in serum leptin. Recent immunohistochemical and ultrastructural studies suggest that leptin is packaged within low-density secretory vesicles and is secreted through regulated exocytosis (15, 18, 25). Furthermore, Roh et al. (18) as well as Barr et al. (15) showed that insulin depletes this leptin store within 1 h, consistent with our result that insulin stimulated leptin secretion in the presence of CHX, with its maximum at 1 h. In contrast, Levy and Stevens (19) observed that insulin and glucose did not increase leptin release in the presence of CHX, concluding that these secretagogues increase leptin secretion by increasing leptin synthesis; however, they did not directly measure leptin biosynthesis. Here, with pulse-chase experiments, we directly demonstrate that insulin stimulates leptin secretion as early as within 30 min of insulin treatment. It is possible that the different nutritional status of animals or composition of incubation media (e.g., the presence of amino acids) (26, 27) might contribute to these discrepant results. Nonetheless, the demonstration that even when protein synthesis is inhibited, insulin stimulates the secretion of newly synthesized leptin provides evidence for the existence of a regulated pathway of secretion in adipocytes, as shown previously for adipisin, adipocyte complement related protein 30 (ACRP30), and LPL (28–30).

Our results indicate that the insulin stimulation of leptin secretion depends on nutritional status. In contrast with results with fed rats, insulin did not affect leptin release from adipose tissue in starved rats that had lower tissue leptin content. In agreement with the current results, Walker et al. (27) showed that the plasma leptin

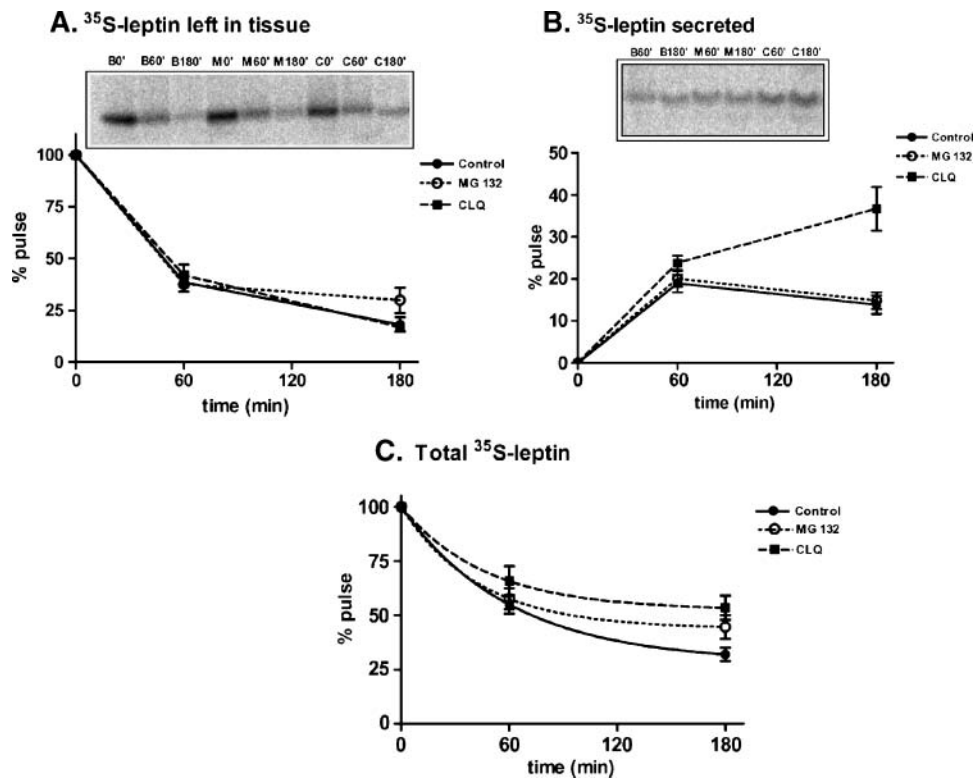


Fig. 6. Effects of proteosomal and/or lysosomal inhibitors on leptin turnover. Adipose tissue from ad libitum-fed rats was pulse-labeled for 1 h and chased for up to 3 h with vehicle control, MG 132 (M; 5 or 10 μ M), or chloroquine (CLQ or C; 20 μ M). [35 S]leptin was immunoprecipitated from tissue lysate (0 min and at each chase time point) and chase medium and analyzed with fluorography after SDS-PAGE. Data are presented as percentages of the 0 min pulse level in each experiment. Data shown are means \pm SEM of four independent experiments comparing MG 132 and control. A chloroquine condition was included in two of these experiments. Representative fluorographs are shown at the top of the graphs. A: [35 S]leptin remaining in tissue at the initial pulse (0 min) and during the chase. B: Amount of [35 S]leptin in the medium at each chase time point. C: Total amount of [35 S]leptin (remaining in tissue and secreted into chase medium) during the chase period.

response to glucose infusion is lower in the starved compared with the fed state and that the magnitude of the response is determined by insulin. It is possible that the insulin-stimulated uptake and metabolism of glucose is decreased in adipose of starved rats (31), and this may contribute to the lower secretory response to insulin in the starved state. Another possibility is that in the fed state, leptin is sorted into secretory vesicles whose exocytosis is stimulated by insulin, whereas in the starved state, leptin is sorted into vesicles that undergo constitutive exocytosis. Partial sorting of adipocyte proteins into regulated secretory vesicles has been observed (28, 30).

Inhibitor studies indicated that both lysosomal and proteosomal mechanisms contributed to the degradation of newly synthesized leptin. When proteosomal degradation was blocked, \sim 8% more [35 S]methionine/cysteine was incorporated into leptin during a 1 h labeling period, but the effect was nonspecific (i.e., paralleled by an increased incorporation of the label into total protein). MG 132 also led to a lower loss of [35 S]leptin within the tissue after 3 h of chase but did not affect [35 S]leptin in the medium. Thus, at least under our *in vitro* conditions, the proteosomal pathway contributes to the intracellular deg-

radation of newly synthesized leptin, most likely as a means of eliminating misfolded leptin protein. Other studies have also shown that the proteosomal pathway is involved in leptin degradation when its intracellular transport/secretion is blocked either by a defective mutation (32) or by vanadate treatment (33).

Inhibition of lysosomal degradation did not affect the initial rate of leptin degradation in adipose tissue of fed rats, but clearly it increased the accumulation of newly synthesized leptin in the medium from 1 to 3 h. These data indicate that reuptake and lysosomal degradation limit the net secretion of leptin under our *in vitro* conditions. Consistent with this idea, adipose tissue of fed rats also degraded 50% of exogenously added 125 I-leptin, and this was not attributable to the leakage of proteases from the minced tissue. The reuptake and degradation of leptin may be mediated by leptin receptors that are found in adipocytes as well as other cell types present in adipose tissue (25). Uotani et al. (34) showed that overexpression of both short and long forms of leptin receptors could mediate the degradation of 125 I-leptin by a lysosomal mechanism in CHO cells. Thus, it is possible that nutritionally induced variations in the expression of leptin

receptors may regulate leptin production by modulating leptin binding, secretion, reuptake, and degradation. We speculate that this reuptake mechanism may also modulate leptin secretion in vivo if the transit of leptin out of the adipose tissue is delayed by low blood flow or binding to the extracellular matrix or the adipocyte cell surface.

Considering that the proteosomal and lysosomal inhibitors each decreased leptin degradation by ~25%, it is likely that 20% of degradation must be attributable to another mechanism, including possible extracellular degradation within the tissue fragment. Alternatively, some [³⁵S]leptin might be lost during tissue washes, or there may be a delay in the access of the inhibitors to their intracellular sites of action.

In adipose tissue of fed rats, insulin increased the secretion of newly synthesized leptin in association with a decline in total degradation (sum of medium plus tissue). However, insulin did not increase the degradation of exogenously added leptin. In addition, the degradation of exogenous leptin was greater in adipose tissue of fed than fasted rats. Together, these results indicate that it is unlikely that insulin directly regulates leptin degradation. Vannier and Ailhaud (30) and others (35, 36) observed similar results for LPL: a large fraction of intracellular LPL is normally degraded, but this is prevented with heparin-stimulated LPL release. Thus, we suggest that insulin likely decreases leptin degradation by accelerating leptin secretion. In the presence of insulin, medium [³⁵S]leptin peaked at 1 h, followed by a gradual decline that was likely attributable to the eventual reuptake and lysosomal degradation.

Paradoxically, starvation decreased rather than increased relative rates of leptin degradation, apparently allowing preservation of the leptin that is synthesized. In addition, adipose tissue from starved rats was not responsive to the ability of insulin to increase the secretion of newly synthesized leptin. It is not clear whether this is merely secondary to the lower translation and smaller pool of intracellular leptin in the starved animals or is a specific alteration in insulin signaling to the secretory machinery.

Compared with results from young rats (6–7 weeks old) from our previous report, the older rats (12–14 weeks old) used in this study were heavier and had larger fat cells. As expected, plasma leptin levels (4.5-fold), tissue leptin protein content (5-fold), and leptin mRNA expression levels (3- to 5-fold) were higher in older/obese rats (unpublished observations). Larger adipocytes from older/obese rats contained more than five times the amount of leptin stores compared with smaller fat cells from younger rats that we had studied previously. The content of leptin in human adipose tissue also correlates well with adiposity, body mass index, and fat cell size (our unpublished observation). High leptin release from a larger intracellular pool may contribute to the higher basal secretion and the rapid increases in leptin release after meals. Furthermore, the insulin-triggered release of preformed leptin may mediate the high leptin pulse amplitude in obese humans (37).

In the current study, we did not observe any effect of in vitro insulin treatment on relative rates of leptin biosyn-

thesis. However, in younger animals studied in our laboratory during the same period, there was a robust effect of insulin in adipocytes of younger, 6–7 week old rats [2-fold in fed: 274 ± 40 (basal) vs. 539 ± 65 (insulin) arbitrary units ($P < 0.05$, $n = 4$); 3-fold in starved: 60 ± 19 (basal) vs. 181 ± 48 (insulin) arbitrary units ($P < 0.01$, $n = 4$)] (16). Thus, age or fat cell size appears to be a determinant of the insulin effect of leptin translation. In a previous study, we also observed a small (1.6-fold) but consistent stimulation of relative rates of leptin biosynthesis in rats of the same age studied here (17). However, those animals were housed in a different vivarium, with a different light/dark cycle (4:00 AM to 4:00 PM dark), so they were likely more food-deprived when they were studied at 10 AM.

The lack of an insulin effect on leptin biosynthesis in the older/more obese rats used in this study was specific, because insulin was able to increase relative rates of LPL biosynthesis. Thus, we speculate that insulin acts on leptin and LPL biosynthesis through different signaling mechanisms. In adipose tissue from young rats, insulin increases leptin synthesis through the phosphatidylinositol-3-kinase/mammalian target of rapamycin (mTOR) pathway (13, 14). Although Kraemer et al. (38) found that inhibitors of phosphatidylinositol-3-kinase and mTOR also decrease insulin-stimulated LPL activity, the involvement of other signaling pathways in the insulin stimulation of LPL synthesis cannot be excluded.

Inhibition of leptin translation appears to be the major mechanism decreasing leptin storage and release during starvation. Leptin mRNA has a very long 3' untranslated region with potential AU-rich sequences that are implicated in translational control. We found that the leptin 3' untranslated region suppresses the expression of reporter constructs (16) and that insulin derepresses leptin translation. We also showed that adrenergic activation inhibited the ability of insulin to increase leptin biosynthesis (17). Thus, the marked suppression of leptin translation during starvation likely involves a decrease in insulin levels as well as an increase in catecholamines.

In summary, we have demonstrated multilevel regulation of leptin production in rat adipose tissue by feeding/starvation and insulin at the biosynthesis, storage, degradation, and secretion steps. Starvation did not affect leptin mRNA but decreased tissue leptin content and release by decreasing leptin biosynthesis. Furthermore, in 12–14 week old mice that are resistant to the ability of insulin to increase relative rates of leptin translation, insulin can stimulate leptin release by increasing leptin secretion from intracellular stores. In addition, our data suggest that a process of secretion, reuptake, and degradation of leptin has the potential to regulate net leptin release. In summary, results of metabolic labeling studies are consistent with the hypothesis that insulin-induced changes in leptin secretion per se contribute to feeding effects on serum leptin. If the mechanism applies to humans, the results suggest that higher leptin secretion from large fat cells in response to secretagogues may explain the higher pulse amplitude in obese individuals. This short-term leptin regulation is likely to be an important means of the meal-

to-meal adjustments in circulating leptin that are critical to the maintenance of energy homeostasis. **FA**

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