Tumor Necrosis Factor-α Contributes to Obesity-related Hyperleptinemia by Regulating Leptin Release from Adipocytes

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Abstract

Cytokines, in particular tumor necrosis factor-alpha (TNF-α), have significant effects on energy metabolism and appetite although their mechanisms of action are largely unknown. Here, we examined whether TNF-α modulates the production of leptin, the recently identified fat-specific energy balance hormone, in cultured adipocytes and in mice. TNF-α treatment of 3T3-L1 adipocytes resulted in rapid stimulation of leptin accumulation in the media, with a maximum effect at 6 h. This stimulation was insensitive to cycloheximide, a protein synthesis inhibitor, but was completely inhibited by the secretion inhibitor brefeldin A, indicating a posttranslational effect. Treatment of mice with TNF-α also caused a similar increase in plasma leptin levels. Finally, in obese TNF-α–deficient mice, circulating leptin levels were significantly lower, whereas adipose tissue leptin was higher compared with obese wild-type animals. These data provide evidence that TNF-α can act directly on adipocytes to regulate the release of a preformed pool of leptin. Furthermore, they suggest that the elevated adipose tissue expression of TNF-α that occurs in obesity may contribute to obesity-related hyperleptinemia. (*J. Clin. Invest.* 1997. 100:2777–2782.) Key words: hormones • cytokines • secretion • mRNA • ELISA

Introduction

Several experimental studies have suggested that fat cells produce molecules that might affect energy metabolism systemically (1, 2). Among these are cytokines such as TNF-α and leptin, the recently identified product of the *ob* gene (3). These mediators are regulated during metabolic perturbations and changes in nutritional status, and many studies have causally linked them to a number of diseases such as obesity and non–insulin-dependent diabetes mellitus. Interestingly, both leptin and TNF-α decrease food intake and regulate other aspects of energy metabolism (4–7). They are correlated with percent body fat (5, 8–14), are disregulated in their expression in the obese state (9, 12, 15, 16), and modulate insulin sensitivity (17, 18).

All of the above raise the possibility that TNF-α and leptin might operate on the same pathway, or alternatively regulate the activities of each other. In fact, recent reports have demonstrated a surge in plasma leptin after lipopolysaccharide and TNF-α treatment in hamsters and mice, showing a possible link between TNF-α and leptin production (19, 20). In this study, we have used three different experimental models for analyzing the mechanism of leptin regulation by TNF-α. First, we used cultured adipocytes to characterize the mechanism of TNF-α stimulation of leptin secretion. Second, we administered TNF-α to normal mice to study the nature of TNF-α action as it relates to leptin secretion in vivo. Third, we have examined the long-term effects of lack of TNF-α on leptin secretion in obese mice with a targeted mutation in the TNF-α gene. These studies demonstrate that TNF-α regulates leptin expression and secretion by multiple mechanisms in cultured cells and animals.

Methods

Cell culture. 3T3-L1 preadipocytes were maintained in DME containing 10% calf serum, penicillin (200 U/ml), and streptomycin (50 μg/ml). Confluent cells (day 0) were differentiated into adipocytes by culturing in DME containing 10% FBS (complete media) plus 170 nM insulin, 100 nM dexamethasone, and 500 μM isobutylmethylxanthine for 2 d, followed by 2 d in complete media containing insulin alone. Cells were then cultured in complete media with regular changes for the remainder of the experiment.

TNF-α effects on leptin expression. For a time course of TNF-α treatment, adipocytes were fed fresh media on day 9. On day 12, conditioned media were harvested from cells that had been cultured for 0–24 h in fresh complete media containing no additions or 2.5 nM recombinant murine TNF-α (Life Technologies, Inc., Piscataway, NJ) and assayed for leptin. Total RNA was prepared from these cells and leptin mRNA was quantitated by Northern blotting. In other experiments, 12-d adipocytes were cultured for 9 h in complete media containing no additions or including 250 μM cycloheximide, 20 μM brefeldin A, 2.5 nM TNF-α, TNF-α plus cycloheximide, or TNF-α plus brefeldin A, and leptin in the media was assayed.

Leptin quantitation. Leptin concentrations in cell culture media and plasma were determined using a sandwich ELISA. An affinity-purified polyclonal rabbit antibody raised against recombinant mouse leptin produced in *Escherichia coli* was bound to microtiter plates as the capture antibody to which leptin samples were immobilized. Bound leptin was detected using the above leptin antibody that had been biotinylated, plus alkaline phosphatase–conjugated streptavidin (Pierce Chemical Co., Rockford, IL). Alkaline phosphatase activity was determined using Attophos (JBL Scientific, Inc., San Luis Obispo, CA) as a substrate and the fluorescent product quantitated with a Cytofluor II plate reader (Perspective Biosystems, Inc., Cambridge, MA). Antigen purified to apparent homogeneity from murine leptin recombinant baculovirus–infected insect cell media was used as a standard.

RNA analysis and leptin mRNA half-life measurements. Total RNA was isolated from cultured adipocytes using Trizol according to the supplier (Life Technologies, Inc.) and was electrophoresed in formaldehyde containing agarose gels, blotted, and hybridized to ^{32}P-labeled mouse leptin cDNA in Church buffer (21). Total RNA from tissues

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was prepared and analyzed as described (13). Leptin mRNA signal from washed blots was visualized and quantitated by either autoradiography and densitometry or with Betascope imaging (Betagen, Inc., Witham, MA). Blots were additionally probed with a ^{32}P-labeled actin cDNA to normalize differences in gel loading. For half-life measurements, adipocytes were cultured for 2–16 h in complete media with no additions or containing 50 ng/ml actinomycin D, 0.5 nM TNF-α, or both. Leptin mRNA levels were determined by Northern blot analysis and normalized to 18S rRNA.

Administration of TNF-α to mice and sampling of plasma and tissues. 4–6-wk-old C57BL/6J male mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at least 1 wk before experimental procedures. After a 4-h fast, blood samples were collected from all mice for basal measurements. Animals were then divided into weight-matched groups and each group received intraperitoneal injections of 0.2 ml PBS containing 0, 0.1, or 1 μg recombinant mouse TNF-α (Genzyme Corp., Cambridge, MA). The animals were killed 3 and 6 h later, and epididymal white fat pads and intrascapular brown adipose tissue were dissected and immediately frozen in liquid nitrogen for subsequent analysis. Plasma was collected immediately and aliquots were frozen at −80°C for leptin protein measurements.

Generation of obese TNF-α–deficient mice and plasma and tissue collection. Male mice homozygous for a targeted null mutation at the TNF-α locus (22) and their wild-type littermates (C57BL/6 × 129 genetic background after three rounds of backcrossing to C57BL/6) were housed in a barrier-free facility and placed on a high-fat, high-carbohydrate diet ad libitum (diet F3282; Bio-Serv, Inc., Frenchtown, NJ) at 4 wk of age and were followed for the next 12 wk (22, 23). Parallel groups of animals were left on standard rodent chow to serve as controls. The detailed characterization of these animals has been reported elsewhere (23). Blood samples were collected after a 6-h fast at 4, 8, and 16 wk of age. At 18 wk of age, animals were killed after a 6-h fast and the epididymal fat pads were dissected and immediately frozen in liquid nitrogen.

Measurement of leptin protein in tissues. Protein extracts from the tissue samples were prepared as described previously (24). 50 or 75 μg of protein extracts for obese or lean animals, respectively, was electrophoresed on a 10–20% SDS-PAGE gradient gel (Integrated Separation Systems, Natick, MA). After electrophoresis, the proteins were transferred to nitrocellulose membranes and protein immunoblot analysis was performed using a 1:500 dilution of a polyclonal anti–mouse leptin as the primary antibody, followed by horseradish peroxidase–conjugated anti–rabbit IgG antibody (Promega, Madison, WI) for detection.

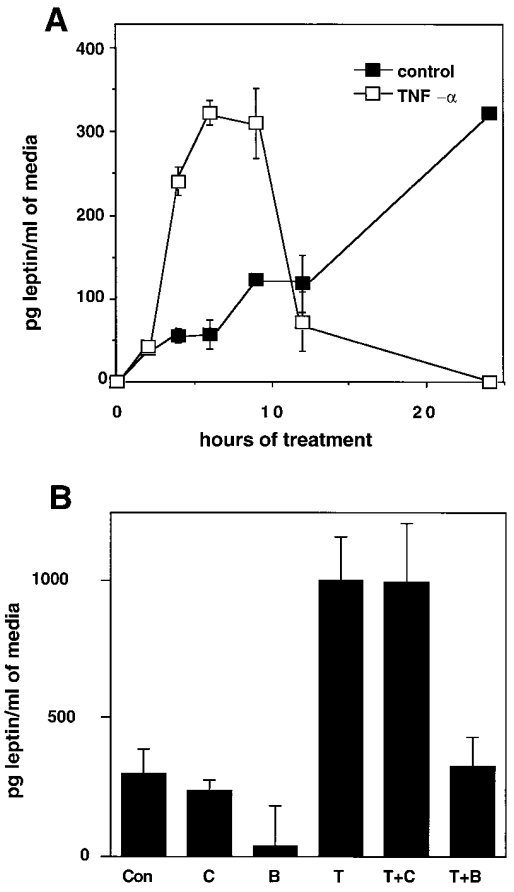

Figure 1. The effect of TNF-α treatment on leptin expression in 3T3-L1 adipocytes. (*A*) Time course of leptin accumulation in the media from TNF-α– and vehicle-treated adipocytes. After 11 d of differentiation, adipocytes were cultured in the presence or absence of 2.5 nM TNF-α. Shown is the amount of leptin present in the media at various times after TNF-α or vehicle addition to the media. (*B*) Leptin levels in the media from 3T3-L1 adipocytes cultured for 9 h in complete media with no additions (*Con*) or containing 250 μM cycloheximide (*C*), 20 μM brefeldin A (*B*), 2.5 nM TNF-α (*T*), 2.5 nM TNF-α plus 250 μM cycloheximide (*T*+*C*), or 2.5 nM TNF-α plus 20 μM brefeldin A (*T*+*B*). The mean and range of leptin released into the media of duplicate samples are shown in both panels *A* and *B*.

Results

TNF-α regulates leptin expression posttranslationally in cultured adipocytes. A survey of a variety of effectors revealed that TNF-α modulated the release of leptin from 3T3-L1 adipocyte cell cultures. Fig. 1 *A* shows a time course of leptin accumulation in the media in response to TNF-α treatment. A complex pattern of regulation was observed; a pronounced stimulation of leptin accumulation occurred early (5.5-fold higher than control by 6 h), followed by a rapid decline to undetectable levels by 24 h. Untreated cells showed a steady increase in leptin accumulation in the media throughout the same time period. Additional factors including thrombin, epidermal growth factor, platelet-derived growth factor, and angiotensin II showed no significant modulation of leptin during a 24-h treatment (data not shown) indicating the specificity of the TNF-α effect.

To address the mechanism of TNF-α stimulation, the sensitivity of leptin release to brefeldin A and cycloheximide in the presence and absence of TNF-α was determined. In adipocytes treated for 9 h, TNF-α stimulated leptin levels greater than threefold compared with control both in the presence and absence of cycloheximide. In contrast, brefeldin A completely blunted the TNF-α–mediated stimulation (Fig. 1 *B*). Thus, the increase in leptin in the media of TNF-α treated cells is due to a posttranslational stimulation of leptin secretion.

In contrast to the biphasic regulation of leptin protein, Northern blot analysis showed a steady decline in leptin mRNA throughout the duration of TNF-α treatment beginning at the earliest time point (2 h) and decreasing to nearly undetectable levels by the end of the experiment (Fig. 2 *A*). Leptin mRNA levels in untreated adipocytes remained essentially constant throughout the time course. The mechanism of the decline in mRNA was addressed by quantitating leptin mRNA from control and TNF-α–treated cells cultured in the presence and absence of the RNA synthesis inhibitor actinomycin D (ActD) (Fig. 2 *B*). TNF-α treatment caused an accelerated turnover of

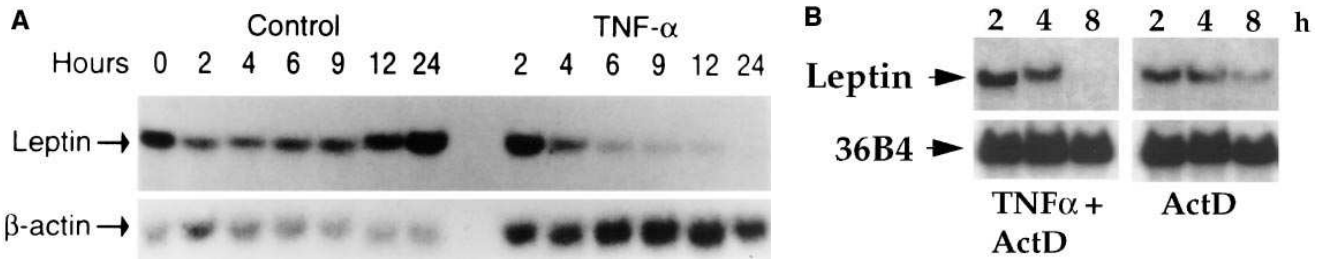

Figure 2. TNF-α effects on leptin mRNA levels in 3T3-L1 adipocytes. (*A*) Northern blot of total RNA (20 μg each) from the time course of control and TNF-α–treated cells described in Fig. 1 *A* were probed with radiolabeled mouse leptin and β-actin cDNAs. (*B*) Northern blot of total RNA (20 μg each) from 3T3-L1 adipocytes cultured for the indicated times in complete medium containing 0.5 nM TNF-α plus 50 ng/ml actinomycin D (*ActD*) or actinomycin D alone. The blot was probed with radiolabeled leptin and 36B4 cDNAs.

leptin mRNA (compare leptin signal in TNF-α plus ActD vs. ActD alone). This suggests that the decline of leptin mRNA in TNF-α treated cells is due, at least in part, to mRNA destabilization. In this regard, earlier studies have demonstrated TNF-α destabilization of other adipocyte mRNAs including those for GLUT-1, GLUT-4, and C/EBPα (25).

TNF-α regulation of leptin in vivo. To test whether similar regulation of leptin also occurs in whole animals, C57BL/6 mice were administered TNF-α intravenously, and plasma samples were collected at 0, 3, and 6 h thereafter for quantitation of leptin. A single dose of TNF-α (1 μg/animal) caused a sharp rise in plasma leptin levels, ~ 14-fold greater than control within 3 h of administration, while mice given 0.1 μg had leptin levels approximately twice that of controls at 6 h (Fig. 3 *A*). A decline in serum leptin at 6 h was observed in those mice receiving the higher dose. In contrast, a Northern blot analysis of white adipose tissue RNA from the same animals showed only a modest increase in leptin mRNA due to TNF-α treatment and no change was observed in brown adipose tissue (Fig. 3 *B*). Thus, the 14-fold stimulation of plasma leptin is consistent with the posttranslational stimulation of leptin secretion observed in vitro. However, additional effects of TNF-α resulting in decreased leptin catabolism in vivo cannot be ruled out. The difference in TNF-α regulation of leptin mRNA in vivo compared with cultured adipocytes could be related to differences in the dose and kinetics of exposure of adipocytes to the cytokine in these two experimental systems. It may also reflect the limitations of the 3T3-L1 culture system in studying this aspect of leptin biology.

Leptin expression in obese TNF-α–deficient mice. To address the role of TNF-α in leptin secretion in vivo more directly, we have generated obese animals with no functional copy of the TNF-α gene. Mice with a targeted null mutation in the TNF-α gene (TNF-$\alpha^{-/-}$) and their control litter mates (TNF-$\alpha^{+/+}$) were placed on a high-fat and high-caloric diet (50% of the total calories in the form of fat, 5,286 kcal/kg; Bio-Serv, Inc.) at 4 wk of age. Both TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice gradually developed significant obesity as compared with mice kept on standard rodent diet. At 16 wk of age, obese mice were ~ 45% heavier than lean animals and there were no significant weight differences between the obese TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice (23). In all animals on high-fat diet, the circulating leptin levels increased as the body weights increased (Fig. 4 *A*). However, the rate of increase in plasma leptin was significantly less pronounced in TNF-$\alpha^{-/-}$ animals when compared with TNF-$\alpha^{+/+}$ animals. At 16 wk of age, obese TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice had 18.8±2.4 and 9.8±2.2 ng/ml ($P < 0.05$) leptin in their plasma, respectively. To understand the mechanism of this decrease in plasma leptin levels in TNF-$\alpha^{-/-}$ animals, we examined the amount of leptin mRNA and protein in white adipose tissue (Fig. 4, *B* and *C*, respectively). Leptin mRNA expression in obese TNF-$\alpha^{-/-}$ animals was slightly, but

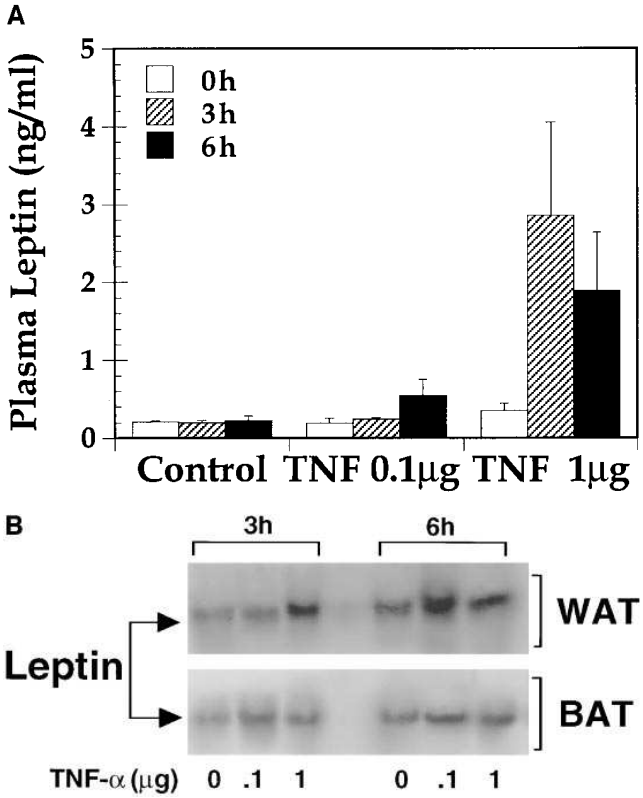

Figure 3. Regulation of leptin expression in vivo by TNF-α administration. (*A*) C57BL/6 mice were administered 0.1 or 1 μg of TNF-α (~ 3 and 30 μg/kg, respectively) or saline control intravenously, and plasma samples were taken at 0, 3, and 6 h. Shown are the mean plasma leptin levels±SD of six mice for each determination. (*B*) Northern blot of white (*WAT*) and brown (*BAT*) adipose tissue total RNA from the 3- and 6-h time points described in *A*. The blot was probed with radiolabeled leptin cDNA.

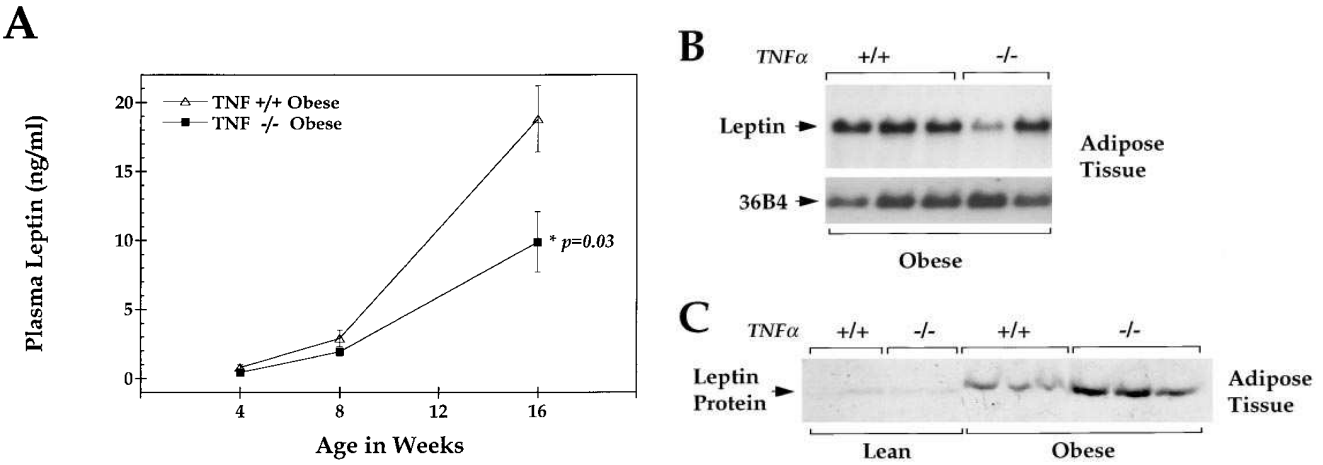

Figure 4. Leptin expression in TNF-α–deficient and wild-type mice. (*A*) Leptin levels in plasma from obese TNF-α–deficient (*TNF −/− Obese*) and wild-type (*TNF +/+ Obese*) mice maintained on a high-fat diet were determined at 4, 8, and 16 wk of age. Shown are the mean±SD plasma leptin concentrations in 10 mice from each age group. (*B*) Northern blot of white adipose tissue total RNA from 18-wk-old obese TNF-$\alpha^{-/-}$ and TNF-$\alpha^{+/+}$ mice maintained on a high-fat diet. The blot was probed with radiolabeled leptin and 36B4 cDNAs. (*C*) Immunoblot showing leptin levels in adipose tissue extract from lean TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice maintained on a normal chow diet, and from obese TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice maintained on a high-fat diet.

not significantly, lower compared with the obese wild-type mice. In contrast, the adipose tissue leptin protein levels were significantly higher in the obese TNF-$\alpha^{-/-}$ mice than obese TNF-$\alpha^{+/+}$ mice. Therefore, despite the presence of elevated leptin in their adipose tissue compared with TNF-$\alpha^{+/+}$ animals, the TNF-$\alpha^{-/-}$ mice had lower levels of circulating plasma leptin.

Discussion

We present evidence here that TNF-α regulates leptin production posttranslationally. TNF-α treatment of cultured adipocytes results in an acute stimulation of leptin release into the media that is cycloheximide insensitive and brefeldin A sensitive. In addition, the rapid disappearance of leptin from adipocyte media after the TNF-α–stimulated release suggests that an extracellular TNF-α–dependent degradation of leptin or a cellular leptin reuptake mechanism takes place. Administration of TNF-α to C57BL/6 mice resulted in a rapid rise in plasma leptin levels reaching 14-fold higher levels than control by 3 h. These in vitro and in vivo effects could not be accounted for by changes in mRNA since leptin mRNA declined in TNF-α–treated adipocytes and was only modestly elevated or unchanged in TNF-α–treated mice. This suggests that TNF-α treatment causes a release of leptin due to a secretagogue-like mechanism. We believe this is the first direct evidence for the existence of regulatable, preformed pools of leptin in adipocytes and suggests that there are mechanisms to rapidly increase plasma leptin levels without requiring de novo synthesis. It remains to be seen whether additional effectors can mobilize leptin in a similar manner.

Importantly, these results were supported by measuring plasma leptin and leptin protein and mRNA in adipose tissue from wild-type and TNF-α–deficient mice, both of which were obese from being maintained on a high-fat diet. While both strains had elevated plasma leptin levels compared with nonobese mice, TNF-α–deficient animals had significantly lower circulating leptin compared with wild-type animals. In contrast, leptin levels in adipose tissue of TNF-α–deficient mice were higher than those of the wild-type animals. No significant differences were observed in leptin mRNA levels. This is consistent with the posttranslational stimulation of leptin secretion by TNF-α observed in cultured cells and the rise in plasma leptin that occurred after TNF-α administration to mice. In addition, while experimental doses of TNF-α were used in treatments of cultured adipocytes and control mice, the results seen in TNF-α–deficient mice demonstrate that physiologically relevant levels can regulate leptin release. Thus, the elevated TNF-α expression in adipose tissue that occurs in obesity is likely an important factor contributing to the overall energy balance equation that regulates leptin output in the anabolic state.

The ability of preformed pools of leptin to be rapidly mobilized may be pertinent to the recently described rapid changes in serum leptin levels seen in humans (26). Acute changes in leptin levels were observed that are indicative of a pulsatile release of leptin with a mean periodicity of $\sim$ 30 min. A posttranslationally regulated release mechanism could account for such rapid changes in plasma leptin although the role, if any, of TNF-α in this is unknown. It will be interesting to determine if TNF-α production also exhibits pulsatility similar to other functionally related cytokines (27). Finally, TNF-α is a potent anorectic in experimental animal models (28, 29) and the results presented here suggest that this might be mediated acutely by stimulating leptin production.

There are numerous functional similarities between leptin and TNF-α and they appear to regulate a number of common pathways. For example, both TNF-α (30) and leptin (31) inhibit the expression of the rate-limiting enzyme for long chain fatty acid synthesis, acetyl CoA carboxylase, in cultured differentiating adipocytes. In addition, elevated leptin expression and increased plasma leptin levels are associated with obesity and are presumably part of an adipostat mechanism (4–7). Leptin and TNF-α expression in adipose tissue are similarly disregulated in the obese state and have anorectic properties in

some animal models and additional effects on energy metabolism (29, 32). In both humans and experimental models, leptin and TNF-α expression correlate with percent body fat (5, 8–14). The expression of both molecules increases during weight gain and decreases upon weight loss (9, 12, 15, 16). The obesity-related increase in both leptin and TNF-α production positively correlates with hyperinsulinemia and increased insulin resistance (5, 8, 9, 12, 14). More direct measures of insulin sensitivity have also indicated leptin and TNF-α as potential mediators of insulin resistance in obesity (17, 18). The ability of leptin and TNF-α to regulate insulin secretion from the pancreatic β cells might also contribute to the abnormalities in glucose homeostasis in obesity (33, 34). At the molecular level, both TNF-α and leptin inhibit insulin receptor signaling in cultured cells (35–37). Finally, exogenous modulators of insulin action have similar influence on leptin and TNF-α expression. The best example of this is the downregulation of both leptin and TNF-α mRNA expression by thiazolidinediones, a family of antidiabetic, insulin-sensitizing agents (11, 38–41). These similarities indicate extensive overlap in the function of these two cytokines and, thus, it is not surprising to find that one regulates the other.

In light of the above findings, the present results suggest that TNF-α stimulation of leptin production plays a role in two distinct but metabolically related processes. TNF-α–mediated leptin release from adipose tissue may be part of the adipostat mechanism that relates circulating leptin concentrations to adipocyte triglyceride stores. Alternatively, by increasing the local concentration of leptin in the vicinity of the adipocyte, TNF-α may potentiate its insulin resistance effects due to an autocrine or paracrine effect of leptin on these same insulin signaling pathways.

In summary, in vitro and in vivo evidence has been presented showing that leptin secretion is regulated by TNF-α posttranslationally. This secretagogue-like activity is consistent with the existence of regulatable pools of leptin and may contribute in part to the insulin resistance properties of TNF-α as well as the adipostatic mechanisms of leptin.

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