

The Expression of Tumor Necrosis Factor in Human Adipose Tissue

Regulation by Obesity, Weight Loss, and Relationship to Lipoprotein Lipase

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Abstract

A previous study reported the increased expression of the cytokine TNF in the adipose tissue of genetically obese rodents. To examine this paradigm in humans, we studied TNF expression in lean, obese, and reduced-obese human subjects. TNF mRNA was demonstrated in human adipocytes and adipose tissue by Northern blotting and PCR. TNF protein was quantitated by Western blotting and ELISA in both adipose tissue and the medium surrounding adipose tissue. Using quantitative reverse transcriptase PCR (RT-PCR), TNF mRNA levels were examined in the adipose tissue of 39 nondiabetic subjects, spanning a broad range of body mass index (BMI). There was a significant increase in adipose TNF mRNA levels with increasing adiposity. There was a significant correlation between TNF mRNA and percent body fat ($r = 0.46, P < 0.05, n = 23$). TNF mRNA tended to decrease in very obese subjects, but when subjects with a BMI $> 45 \text{ kg/m}^2$ were excluded, there was a significant correlation between TNF mRNA and BMI ($r = 0.37, P < 0.05, n = 32$). In addition, there was a significant decrease in adipose TNF with weight loss. In 11 obese subjects who lost between 14 and 66 kg (mean 34.7 kg, or 26.6% of initial weight), TNF mRNA levels decreased to 58% of initial levels after weight loss ($P < 0.005$), and TNF protein decreased to 46% of initial levels ($P < 0.02$). TNF is known to inhibit LPL activity. When fasting adipose LPL activity was measured in these subjects, there was a significant inverse relationship between TNF expression and LPL activity ($r = -0.39, P < 0.02, n = 39$). With weight loss, LPL activity increased to 411% of initial levels. However, the magnitude of the increase in LPL did not correlate with the decrease in TNF. Thus, TNF is expressed in human adipocytes. TNF is elevated in most obese subjects and is decreased by weight loss. In addition, there is an inverse relationship between TNF and LPL expression. These data suggest that endogenous TNF expression in adipose tissue may help limit obesity in some subjects, perhaps by increasing insulin resistance and decreasing LPL. (*J. Clin. Invest.* 1995. 95:2111-2119.)

Key words: tumor necrosis factor • adipose tissue • lipoprotein lipase • obesity • quantitative reverse transcriptase polymerase chain reaction • gene expression

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Introduction

Obesity is a complex disorder that involves some degree of overconsumption (1, 2), coupled with a metabolic derangement. When presented with excess caloric intake, humans gain weight to variable degrees, and some subjects demonstrate remarkable resistance to weight gain (3, 4). In addition, insulin resistance accompanies the development of obesity to varying degrees, and this insulin resistance may serve to limit the degree of obesity (5). However, the relationship between adiposity and insulin resistance is poorly understood.

A recent study suggested a new mechanistic link between insulin sensitivity and obesity. The cytokine TNF is expressed in the adipose tissue of rats and mice and is expressed at a higher level in the adipose tissue from genetically obese rodents (6, 7). In addition, this overexpression of TNF is in part responsible for insulin resistance in rodent obesity. After infusion of anti-TNF binding protein, insulin sensitivity improves (7), owing to improved insulin receptor tyrosine kinase activity (8).

TNF has previously been associated with the acute phase response: it is a cytokine produced by macrophages in response to endotoxemia, inflammation, and cancer (9, 10). However, TNF has multiple actions in adipose tissue, which include a decrease in the activity of lipoprotein lipase (LPL)¹ (11, 12), a decrease in expression of the glucose transporter Glut4 (13), and an increase in hormone-sensitive lipase (14). The regulation of the expression of these and other genes could affect adipocyte insulin sensitivity and lipid accumulation. Therefore, the production of TNF by adipose tissue could be a local regulator of fat cell size, and the overproduction of TNF in adipocytes of obese animals could represent a form of "adipostat," i.e., a normal homeostatic mechanism designed to limit adipocyte size in the face of overconsumption (15, 16). These studies were intended to determine whether TNF is produced in human adipose tissue and whether it is regulated in a manner consistent with a possible role as an adipostat. To accomplish these aims, we studied TNF expression in lean subjects, obese subjects, and obese subjects after weight loss.

Methods

Subjects and sample acquisition. 39 subjects were recruited to undergo fat biopsies. All subjects were weight stable, were taking no medications likely to affect adipocyte metabolism, were nondiabetic, and did not exercise for at least 2 wk before the biopsies. For 2 d before the study, all subjects were placed on a standardized diet consisting of 35% fat,

1. *Abbreviations used in this paper:* BMI, body mass index; LPL, lipoprotein lipase; RT-PCR, reverse transcriptase PCR.

20% protein, and 45% carbohydrate, calorically adjusted for weight maintenance. In some subjects, body composition was measured using bioelectrical impedance (17). An incisional biopsy was performed in the postabsorptive state under local anesthesia from the lower abdominal wall, and 5–10 g of fat was obtained. Obese subjects were recruited before entering a weight loss program involving a low to very low calorie diet (520–800 calories/d; Health Management Resources, Boston, MA) and behavior modification. In some subjects, the same procedure was repeated after the subject had lost weight and maintained the weight loss for at least 3 mo. Before this second biopsy, subjects were weight stable and again consumed the same standardized eucaloric diet 2 d before the biopsies. Documentation of weight stability was accomplished by close follow-up and regular weighing of the patients. Of the 11 subjects who were studied before and after weight loss, 7 were reported on in a previous study (18). Non-obese subjects were healthy individuals recruited from the community. Data on some of these subjects have been reported previously (19, 20).

Cell and tissue preparation. To assess the distribution of TNF in adipose tissue cells, isolated adipocytes were prepared by digesting fresh adipose tissue with collagenase, as described previously (21). After filtering out large undigested pieces, adipocytes were separated by flotation, and the stromal-vascular cells were isolated as the pellet after a low speed spin (200 g for 20 min). To be sure that the TNF expressed in the total stromal-vascular fraction was not from resident macrophages, the stromal cells were placed into tissue culture and grown to confluence over 7 d. From each of these fractions (adipocytes, stromal-vascular cell pellet, and cultured stromal vascular cells), RNA was extracted and reverse transcriptase PCR (RT-PCR) was performed to detect TNF mRNA, as will be described.

RNA extraction and Northern blotting. Adipose tissue was frozen at -80°C , and RNA was extracted according to the method of Chomczynski and Sacchi (22). RNA quality was verified by ethidium bromide staining of rRNA bands on a minigel. Northern blotting was performed as described previously (20). After analysis on a 1% agarose gel, the RNA was transferred to nylon and blotted with the random-primed (23) ^{32}P -labeled human LPL cDNA (24) or human TNF cDNA (25). Northern blots were performed with either total RNA or poly(A)-enriched RNA (Poly(A) tract mRNA isolation system; Promega Corp., Madison, WI).

Measurement of TNF protein. TNF was measured in the medium surrounding adipose tissue, as well as in the tissue homogenate, by Western blotting. For medium TNF, adipose tissue pieces (0.5 g) were minced and incubated in 2 ml of serum-free medium 199 (Gibco Laboratories, Grand Island, NY) for 4 h at 37°C . The medium was then removed and concentrated by Speedvac (Savant Instruments, Inc., Farmingdale, NY) centrifugation. 5–20 μl was analyzed by Western blotting, as will be described. For tissue TNF, 0.5 g of tissue was homogenized in 1 ml of a PBS solution containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors. This solution was reduced in volume to 200 μl by Speedvac centrifugation. Equal quantities of total protein (between 5 and 20 μl of this solution) were then analyzed under denaturing conditions on a 15% SDS-polyacrylamide gel. After transfer to nitrocellulose, the membrane was blotted with a 1:2,000 dilution of rabbit anti-TNF antiserum (generously provided by Dr. Carl Grunfeld, University of California, San Francisco), followed by biotinylated anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) and streptavidin-peroxidase (Promega Corp.). The blot was developed with a chemoluminescent peroxidase substrate (Promega Corp.).

TNF protein was also measured by ELISA. Each well of a 96-well plate was coated with 100 μl of a monoclonal anti-TNF antibody (2.5 $\mu\text{g}/\text{ml}$; Olympus Corp., Lake Success, NY). After blocking with PBS-albumin, samples and standards (human TNF α ; 1–64 $\mu\text{g}/\text{ml}$) were added and incubated overnight at 4°C . Samples for ELISA were homogenized in PBS containing 0.5% Tween 20 and protease inhibitors. After sample addition, a 1:800 dilution of rabbit anti-TNF antiserum (Genzyme Corp., Boston, MA) was added to each well, followed by an alkaline phosphatase-coupled anti-rabbit antibody. All washes used PBS containing 0.5% Tween 20. The reaction was then developed,

and the samples were quantitated using the standard curve. Data were expressed as picograms of TNF per microgram of DNA, and DNA was measured as described previously (26).

To demonstrate that the expression of data in relation to total tissue DNA was valid after weight loss, measurement of cell number and tissue DNA was performed in seven subjects before and after weight loss, when a decrease in adipocyte size (and therefore an increase in cell number per gram of tissue) was expected. In response to weight loss, mean cell number per gram of adipose tissue increased by 71%, from 2.3×10^6 to 3.93×10^6 cells per g; mean DNA per gram of adipose tissue increased to a similar degree, by 97%, from 476 to 936 $\mu\text{g}/\text{g}$ ($P = \text{NS}$).

Measurement of TNF mRNA levels using RT-PCR. To measure TNF expression, a quantitative competitive PCR assay was developed, similar to the assays described previously (27–29). 0.4 μg of total RNA from adipose tissue extracts was added to increasing quantities of a cRNA construct that contained primer sites for human TNF. The development of this cRNA construct was described previously (30). The cRNA contained nucleotides 217–568 of the TNF α cDNA, with 49 bp deleted (nucleotides 349–397). PCR with the cRNA generated a product that was 49 nucleotides shorter than the PCR product generated from native human TNF mRNA. The primer sites for PCR were located at nucleotides 217–236 and 426–445 of the TNF cDNA. Because the primer sites spanned introns in the TNF gene, quantitation of the reaction was not affected by the presence of genomic DNA. After reverse transcription, PCR was performed by the “hot start” method (31, 32), using wax plugs (AmpliWax; Perkin Elmer, Branchburg, NJ) to improve reproducibility and precision of reaction initiation. PCR was performed for 35 cycles at 60°C . The resulting ethidium bromide-stained gel was imaged using an Imagestore 5000 scanner and analyzed using the Gelbase/Gelblot software (Ultraviolet Products, Ltd., San Gabriel, CA). The ratio of TNF product/cRNA standard was plotted against the number of copies of cRNA added, to yield the equivalence point between cRNA and TNF mRNA. Data were expressed as the number of copies per microgram of total RNA (number of copies refers to the number of copies of the cRNA added).

Measurement of LPL. LPL activity and mRNA levels were performed as described previously (18–20). For total adipose tissue LPL activity, the sum of heparin-releasable and -extractable LPL was used, as described previously (19). LPL activity was standardized to cell number, and cell number was determined according to the method of DiGirolamo et al. (33).

Statistics. Data were analyzed nonparametrically, using the Wilcoxon matched paired sign rank test for paired data and the Mann-Whitney test for nonpaired data. Linear regressions were performed using the Spearman rank, order correlation test, except for the analyses of body composition, for which linear regression was used.

Results

As described previously (7), TNF is expressed in the adipose tissue of rodents. To demonstrate the presence of TNF mRNA in human adipose tissue, Northern blotting and PCR were performed. Figure 1 demonstrates the 1.6-kb TNF mRNA in adipose tissue. For this Northern blot, 1 μg of poly(A)⁺ RNA was loaded onto the gel and blotted with the ^{32}P -labeled cDNA for TNF, and the film was exposed for 1 wk. Next to the lane for TNF is the image for LPL from the same sample after blotting with the ^{32}P -labeled cDNA for LPL and exposure to film for 1 d. As illustrated by the relative intensities of the images, human adipose tissue expressed TNF at a level much lower than that of LPL mRNA. To demonstrate the presence of TNF in both adipose tissue and adipocytes, RNA was extracted from cells or tissue, followed by RT-PCR, using the primers and conditions described in Methods. Results of PCR with various tissues and cells are shown in Fig. 2. The anticipated PCR product of 229

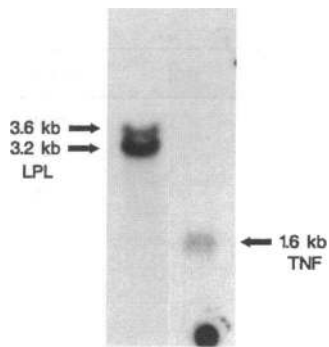


Figure 1. Northern blot of TNF in human adipose tissue. RNA was extracted from a sample of subcutaneous human adipose tissue. 1 μ g of poly(A)-enriched RNA was prepared and blotted with the 32 P-labeled cDNAs for LPL and TNF, as indicated. The film exposure time was 1 d for LPL and 7 d for TNF.

bases was obtained with RT-PCR of isolated human adipocytes (from a collagenase digestion), whole human adipose tissue, and LPS-stimulated THP-1 cells, which are human monocyte/macrophages. To determine whether TNF was expressed in stromal-vascular cells, adipose tissue was digested with collagenase, and stromal-vascular cells were prepared as described in Methods. As shown in Fig. 2, TNF was detected in these cells. To be sure that the TNF expressed in the total stromal-vascular fraction was not from resident macrophages, stromal cells were placed into tissue culture and grown to confluence over 7 d. As shown in Fig. 2, these cultured stromal cells also expressed

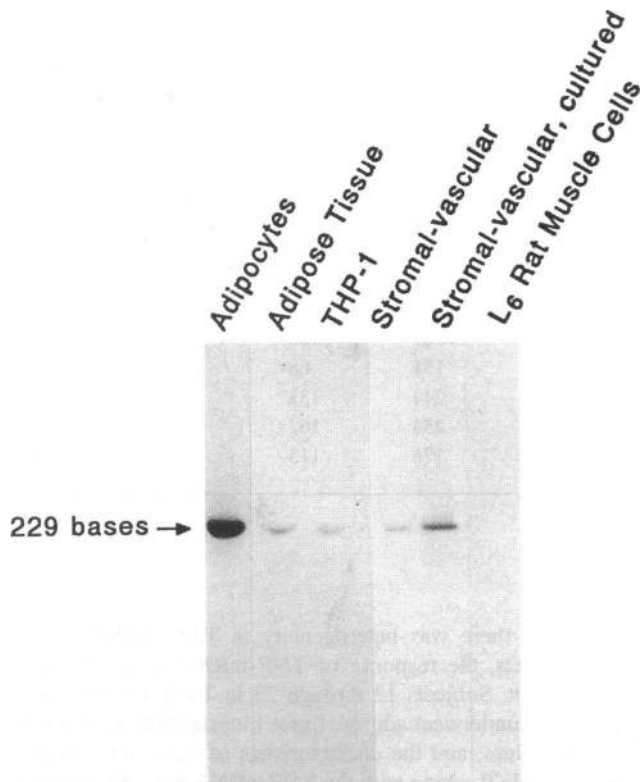


Figure 2. Demonstration of TNF mRNA by RT-PCR. Total RNA was extracted and RT-PCR was performed, as described in Methods. The anticipated PCR product at 229 bases was demonstrated in isolated human adipocytes, whole human adipose tissue, and LPS-stimulated THP-1 cells. In addition, the 229-base product was found in the stromal-vascular pellet after collagenase digestion of human adipose tissue and in the cultured stromal-vascular cells. TNF was not detected in L₆ cells.

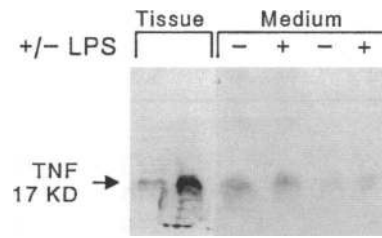


Figure 3. Western blotting of TNF from human adipose tissue. Subcutaneous adipose tissue was cultured in serum-free medium 199 for 4 h in the presence and absence of LPS, as indicated by the + and -. The medium surrounding the adipose tissue was then removed, and the tissue was extracted, as described in Methods, followed by Western blotting with a polyclonal anti-TNF antibody.

Figure 3. Western blotting of TNF from human adipose tissue. Subcutaneous adipose tissue was cultured in serum-free medium 199 for 4 h in the presence and absence of LPS, as indicated by the + and -. The medium surrounding the adipose tissue was then removed, and the tissue was extracted, as described in Methods, followed by Western blotting with a polyclonal anti-TNF antibody.

TNF. Because TNF was detected in isolated human adipocytes and in cultured stromal cells, these data demonstrate that the TNF detected by PCR was from the adipocytes and stromal cells and not simply from macrophages trapped in the capillaries of adipose tissue.

To determine whether the TNF protein was synthesized and secreted by adipocytes, Western blotting was performed. Figure 3 is a Western blot for TNF of the medium surrounding human adipose tissue pieces and from adipose tissue homogenates. A specific band was identified by the anti-TNF antibodies at the anticipated molecular mass of 17 kD, and this band comigrated with the purified TNF α standard (data not shown). TNF was detected in the medium from adipose tissue pieces, as well as in the tissue homogenate. In addition, adipocyte TNF was not stimulated by LPS, suggesting that adipocyte TNF expression is regulated differently from macrophage TNF (Fig. 3).

To examine the effects of obesity on TNF expression, subjects were recruited for adipose tissue biopsy, and the clinical characteristics of these subjects are summarized in Table I. These subjects were clinically and ethnically heterogeneous. Of the 39 subjects in this study, 24 were women, 6 were African-Americans, 11 were lean volunteers (BMI < 28 kg/m², or normal percent body fat), 7 were very obese (BMI > 45 kg/m²), and the remaining subjects were overweight to varying degrees. Among the lean subjects, one was a male with a BMI of 29.7 kg/m², but a normal body fat of 20%, and one was a woman with a BMI of 26.9 kg/m², but slightly elevated body fat (34.6%). All subjects had a normal fasting blood glucose, and none were hyperlipidemic.

The competitive RT-PCR method for measuring human TNF mRNA is illustrated in Fig. 4. RNA was extracted from adipose tissue that had been frozen at -80°C, and quantitative RT-PCR was performed as described in Methods. As shown in Fig. 4, PCR products derived from TNF cRNA and TNF mRNA were separated on the gel, and the ratio of the two products increased linearly with addition of cRNA to the reaction. The equivalence point, at which the amount of added cRNA product was equal to the amount of mRNA product, was determined and expressed as number of copies per microgram of total RNA (number of copies refers to the number of copies of the cRNA added). The adipose tissue samples used in Fig. 4 were from one of the subjects (described below) who was studied before and after weight loss.

Adipose tissue TNF mRNA levels were measured in all 39 subjects using competitive RT-PCR. Figure 5 A illustrates the relationship between TNF mRNA and BMI. There was a wide distribution of TNF mRNA values among the subjects. The highest levels of TNF mRNA tended to be in subjects with

Table I. Clinical Characteristics of the Subjects

| Subject | Age | Sex | Weight | BMI | Body fat | TG | Chol | LDLc | HDL | Glucose |
|---------|-----|-----|--------|-------------------|----------|-------|-------|-------|-------|---------|
| | | | kg | kg/m ² | % | mg/dl | mg/dl | mg/dl | mg/dl | mg/dl |
| 1 | 35 | F | 55 | 22.3 | NA | 55 | 155 | 95 | 49 | 77 |
| 2 | 47 | M | 82 | 24.5 | NA | 67 | 185 | 112 | 60 | 86 |
| 3 | 32 | M | 76 | 24.0 | 16.1 | 88 | 155 | 73 | 64 | 74 |
| 4 | 35 | M | 97 | 28.1 | 16.5 | 100 | 160 | 107 | 33 | 79 |
| 5 | 26 | M | 81 | 29.7 | 20.0 | NA | NA | NA | NA | 89 |
| 6 | 38 | M | 75 | 23.0 | NA | 97 | 157 | 90 | 48 | 86 |
| 7 | 45 | F | 54 | 20.2 | 20.2 | 78 | 203 | 101 | 86 | 72 |
| 8 | 32 | M | 77 | 22.4 | 13.6 | 75 | 158 | 85 | 58 | 85 |
| 9 | 29 | F | 62 | 21.5 | 28.8 | NA | NA | NA | NA | 75 |
| 10 | 31 | F | 76 | 24.1 | 32.7 | NA | NA | NA | NA | 72 |
| 11 | 27 | F | 71 | 26.9 | 34.6 | 31 | 129 | | NA | 96 |
| 12 | 36 | M | 107 | 33.9 | NA | 46 | 232 | 173 | 50 | 70 |
| 13 | 58 | F | 139 | 47.4 | 40.8 | 142 | 240 | 170 | 41 | 95 |
| 14 | 24 | F | 123 | 42.9 | NA | 89 | 177 | 116 | 43 | 79 |
| 15 | 56 | F | 96 | 31.8 | 39.7 | 157 | 235 | 163 | 41 | 99 |
| 16 | 46 | F | 82 | 28.6 | 34.3 | 118 | 247 | 175 | 48 | 83 |
| 17 | 54 | F | 116 | 42.4 | 44.1 | 118 | 247 | 175 | 48 | 83 |
| 18 | 39 | M | 150 | 43.0 | NA | 106 | 205 | 149 | 35 | 83 |
| 19 | 33 | M | 115 | 33.3 | NA | 89 | 205 | 158 | 29 | 81 |
| 20 | 43 | M | 175 | 52.5 | NA | 115 | 252 | 198 | 30 | 104 |
| 21 | 43 | M | 153 | 52.8 | NA | 133 | 166 | 106 | 34 | 94 |
| 22 | 46 | F | 117 | 38.0 | NA | 75 | 182 | 120 | 46 | 86 |
| 23 | 39 | F | 152 | 57.6 | NA | 355 | 226 | 121 | 34 | 118 |
| 24 | 29 | F | 85 | 31.0 | NA | 62 | 210 | 116 | 82 | 65 |
| 25 | 21 | F | 91 | 29.6 | NA | 28 | 193 | 107 | 80 | 96 |
| 26 | 53 | M | 144 | 47.1 | NA | 153 | 207 | 145 | 31 | 115 |
| 27 | 45 | F | 83 | 28.5 | NA | 51 | 205 | 125 | 70 | 77 |
| 28 | 45 | F | 92 | 38.3 | 38.8 | 120 | 172 | | NA | 56 |
| 29 | 54 | M | 85 | 30.3 | 26.5 | NA | NA | NA | NA | NA |
| 30 | 45 | F | 121 | 44.6 | 41.7 | 144 | 270 | 152 | 89 | 89 |
| 31 | 49 | F | 118 | 43.3 | 41.4 | 121 | 218 | 155 | 39 | 113 |
| 32 | 32 | M | 105 | 30.5 | 27.9 | 162 | 207 | 136 | 39 | 65 |
| 33 | 46 | F | 150 | 49.5 | NA | 132 | 134 | 108 | NA | 93 |
| 34 | 39 | F | 93 | 31.3 | 40.9 | 55 | 178 | 113 | 54 | 82 |
| 35 | 28 | F | 88 | 28.0 | 37.3 | 62 | 189 | 128 | 49 | 91 |
| 36 | 27 | F | 113 | 47.9 | 45.5 | 50 | 151 | 68 | 73 | 80 |
| 37 | 42 | M | 103 | 33.4 | 31.3 | 299 | 244 | 138 | 26 | 89 |
| 38 | 45 | F | 91 | 36.0 | 40.1 | 139 | 234 | 162 | 44 | 75 |
| 39 | 26 | F | 103 | 42.8 | 44.1 | 108 | 176 | 115 | 39 | 64 |

TG, triglyceride; Chol, cholesterol; NA, data not available.

mild to moderate obesity (BMI 27–45); lower levels were in subjects with a BMI > 45. When considering all subjects, there was no statistically significant relationship between obesity (using BMI) and TNF. However, if the very obese subjects (BMI > 45) were eliminated, TNF mRNA was significantly associated with BMI ($r = 0.37, P < 0.05, n = 32$). For 23 of the 39 subjects, body composition data were available, and these data are listed in Table I. The relationship between TNF mRNA and body fat was stronger than the relationship between TNF and BMI. As shown in Fig. 5 B, TNF mRNA increased significantly with percent body fat ($r = 0.46, P < 0.05$). A similar correlation was observed between TNF and total body fat in kilograms ($r = 0.41, P < 0.05$; data not shown).

Although there was heterogeneity in TNF mRNA levels among subjects, the response of TNF mRNA to weight loss was consistent. Subjects 12 through 23 in Table I were obese subjects who underwent adipose tissue biopsies both before and after weight loss, and the characteristics of these subjects are shown in Table II, along with the TNF mRNA data. The second biopsy was performed after the subject had maintained the reduced weight for at least 3 mo, and all subjects were stabilized on the weight maintenance diet before the second biopsy. The mean initial BMI of these 11 subjects was 41 ± 2 , and the average weight loss was 34.7 kg, or 26.6% of initial weight. As illustrated in Fig. 6, the reduction in weight resulted in a consistent reduction in TNF mRNA levels to $58 \pm 7\%$ of the initial

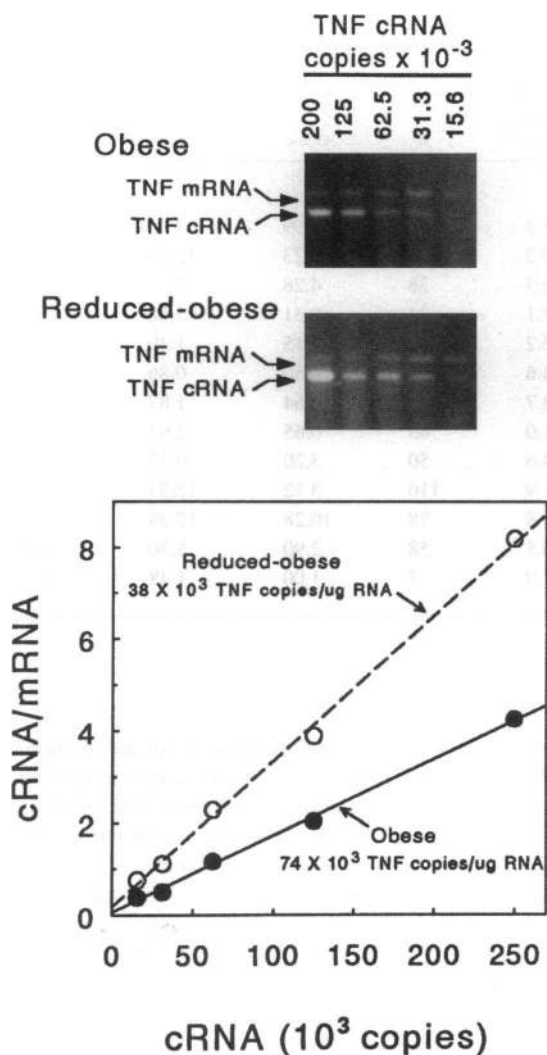


Figure 4. Use of competitive RT-PCR for measurement of TNF mRNA levels. Total RNA from adipose tissue (0.8 μg) samples was added to increasing quantities of a cRNA construct that contained primer sites for human TNF. The cRNA contained a 49-bp internal deletion, such that PCR with the cRNA generated a product that was 49 nucleotides shorter than the PCR product generated from native human TNF mRNA. The ratio of TNF product/cRNA standard was plotted against the number of copies of cRNA added, to yield the equivalence point between cRNA and TNF mRNA. Data were expressed as the number of copies per microgram of total RNA (number of copies refers to the number of copies of the cRNA added). This figure shows gels of RT-PCR from an obese subject before and after weight loss. The TNF mRNA and cRNA are clearly distinguishable, and the plot demonstrates the difference in equivalence point after weight loss.

TNF mRNA level. Before weight loss, adipose TNF mRNA was $161.9 \pm 48.1 \times 10^{-3}$ copies per μg of RNA, and after weight loss, TNF mRNA was reduced to $96.5 \pm 31.9 \times 10^{-3}$ copies per μg of RNA ($P < 0.005$). The magnitude of reduction in TNF mRNA levels did not correlate with the amount of weight lost or the degree of initial obesity.

To determine whether the fall in TNF mRNA levels resulted in a fall in TNF protein levels in adipose tissue, Western blotting and ELISA were performed. As shown in Fig. 7, TNF protein levels in adipose tissue (by ELISA) were 0.59 ± 0.09 pg per μg

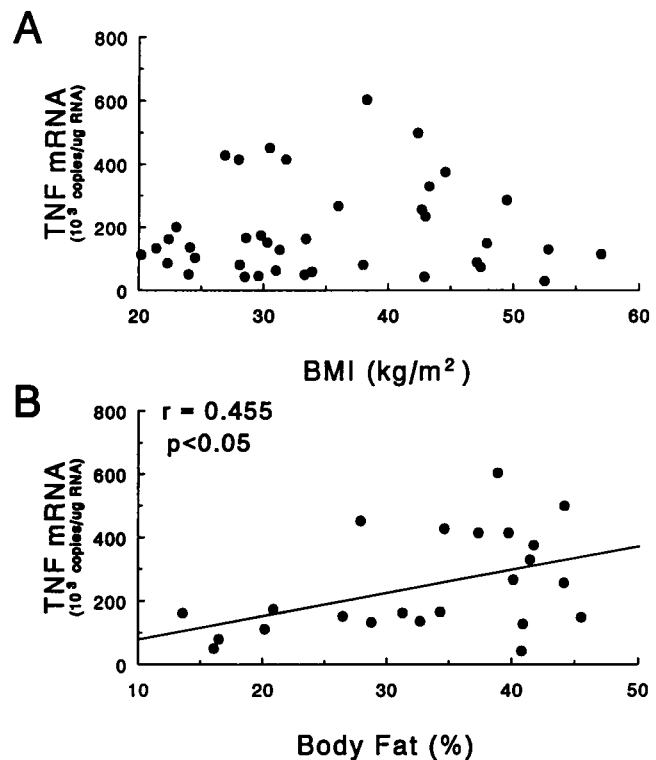


Figure 5. Relationship between TNF mRNA and BMI. (A) TNF mRNA levels (using RT-PCR) for all 39 subjects are plotted in relation to the subjects' BMI. (B) TNF mRNA levels are plotted in relation to the percent body fat in 23 subjects.

of DNA in the obese subjects and 0.25 ± 0.05 pg per μg of DNA in the reduced-obese subjects ($P < 0.02$). In the reduced-obese subjects, TNF protein levels in adipose tissue were $46 \pm 12\%$ of the initial TNF protein level. Thus, there was a decrease in TNF protein level of approximately the same magnitude as the fall in TNF mRNA level.

TNF is known to inhibit LPL in certain cells and tissues (11, 34). In addition, other studies have demonstrated increases in LPL activity with weight loss (18, 35). To determine whether there was any relationship between TNF expression and LPL activity in the adipose tissue of these subjects, linear regression analyses were performed on TNF mRNA levels and LPL activity. Figure 8 illustrates the relationship between fasting TNF mRNA levels and LPL activity in all 39 subjects from Table I. Overall, subjects with the highest TNF expression generally had the lowest levels of LPL activity, and the relationship appeared to be curvilinear. Using a Spearman rank order regression, this relationship was statistically significant ($r = -0.39$, $P < 0.02$). If the subjects were divided into those with TNF mRNA levels more or less than 200×10^3 copies per μg of RNA, subjects with the lower adipose TNF expression had 2.5 times more adipose tissue LPL activity (Fig. 8).

As previously described, TNF mRNA and protein levels decreased with weight loss. In addition, LPL activity was higher in the reduced-obese subjects (Table II), as described in previous studies (18, 35). Although the changes in LPL occurred in a direction opposite to the changes in TNF, the magnitudes of the changes were different, such that there was no significant correlation between change in LPL and change in TNF.

Table II. Effects of Weight Loss on TNF and LPL

| | Reduced-obese | | | | TNF mRNA* | | | Adipose LPL* | | |
|------|---------------|-------------------|-------------|----|-----------|---------------|-----|--------------|---------------|------|
| | Weight | BMI | Weight lost | % | Obese | Reduced-obese | % | Obese | Reduced-obese | % |
| | kg | kg/m ² | kg | % | | | | | | |
| 1 | 82 | 26 | 24.7 | 23 | 58.8 | 21.3 | 36 | 0.99 | 2.00 | 203 |
| 2 | 74 | 25 | 64.9 | 47 | 73.7 | 38.2 | 52 | 7.23 | 12.58 | 174 |
| 3 | 98 | 34 | 25.4 | 21 | 42.9 | 16.3 | 38 | 4.28 | 2.77 | 65 |
| 4 | 76 | 25 | 20.0 | 21 | 415.3 | 137.1 | 33 | 0.31 | 5.08 | 1622 |
| 5 | 68 | 24 | 14.0 | 17 | 166.1 | 136.2 | 82 | 0.15 | 1.39 | 935 |
| 6 | 94 | 35 | 21.4 | 19 | 499.8 | 374.6 | 75 | 0.50 | 0.86 | 173 |
| 7 | 99 | 28 | 51.4 | 34 | 234.8 | 103.7 | 44 | 0.64 | 1.87 | 293 |
| 8 | 87 | 25 | 27.7 | 24 | 49.4 | 31.0 | 63 | 0.65 | 2.03 | 312 |
| 9 | 130 | 39 | 45.0 | 26 | 29.1 | 14.6 | 50 | 3.20 | 9.37 | 293 |
| 10 | 87 | 30 | 65.7 | 43 | 129.6 | 141.9 | 110 | 3.72 | 12.33 | 332 |
| 11 | 95 | 31 | 21.6 | 18 | 80.9 | 46.8 | 58 | 10.28 | 12.38 | 120 |
| Mean | 90 | 29 | 34.7 | 27 | 161.9 | 96.5 | 58 | 2.90 | 5.70 | 411 |
| SEM | 5 | 1 | 5.6 | 3 | 48.1 | 31.9 | 7 | 1.00 | 1.48 | 140 |

* TNF mRNA: 10³ copies per μg of RNA; LPL activity: nEq/min per 10⁶ cells.

Discussion

A recent paper described the production of TNF by adipose tissue in rodents (7). When compared with lean littermates, rodents with endogenous obesity (*ob/ob*, *db/db*, *tub/tub*, and *fa/fa*) express five- to tenfold more TNF mRNA and twofold more TNF protein in adipose tissue, whereas rodents with ac-

quired obesity (treatment with monosodium glutamate) demonstrate no change in TNF expression. In an attempt to reverse the insulin resistance in these animals, a soluble TNF-binding protein was infused into *fa/fa* rats daily for 3 d. When insulin sensitivity was assessed using a euglycemic clamp, insulin-stimulated glucose uptake was increased two- to threefold by the infusion of the TNF-binding protein. These observations suggested that local secretion of TNF was elevated in the adipose tissue of genetically obese rodents and was etiologic in the insulin resistance of obesity.

This study was designed to determine whether the aforemen-

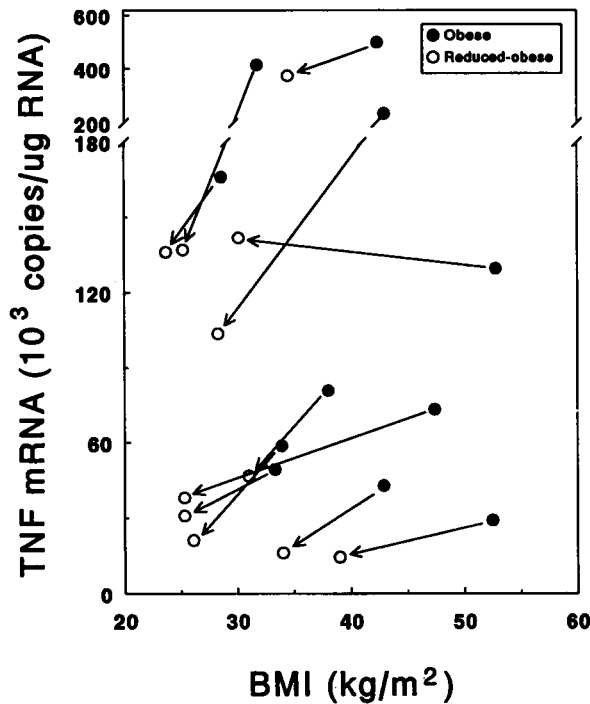


Figure 6. Effect of weight loss on TNF mRNA levels. For the 11 subjects who lost weight, TNF mRNA levels are shown before and after weight loss and in relation to the subjects' BMI.

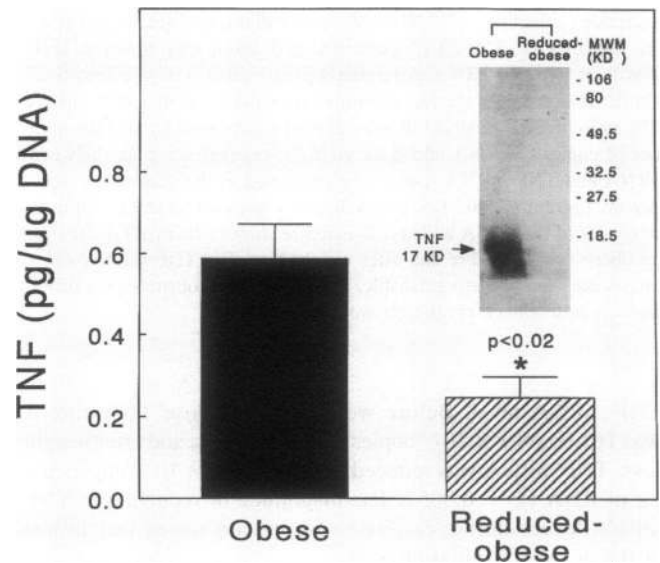


Figure 7. Effect of weight loss on TNF protein level. TNF protein level was measured by ELISA in obese and reduced-obese subjects, as described in Methods. A Western blot for TNF from a representative subject is shown in the inset.

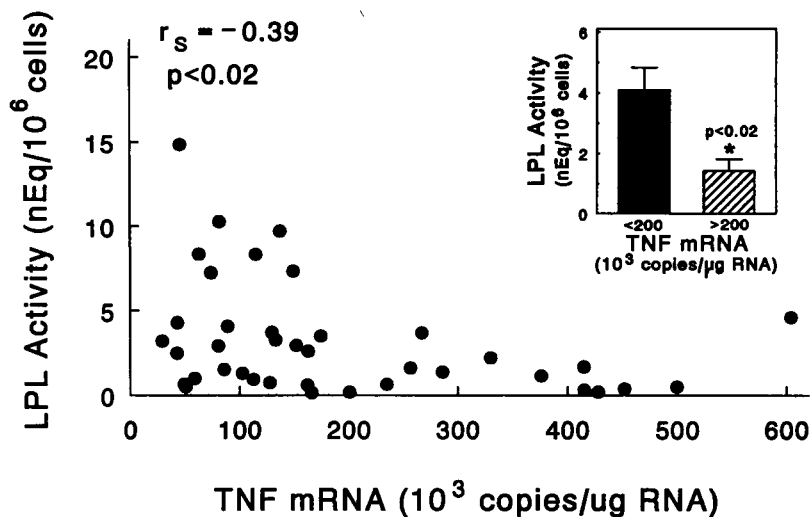


Figure 8. Relationship between TNF mRNA and LPL activity. The TNF mRNA level for each subject (before any weight loss) is plotted against the total adipose tissue LPL activity. A curvilinear relationship is evident. (Inset) Comparison of LPL activity in subjects with TNF mRNA levels < 200 vs $> 200 \times 10^3$ copies per μg of RNA.

tioned observations in rodents were relevant to humans. To accomplish this aim, we measured TNF mRNA and protein levels in the adipose tissue of obese, reduced-obese, and lean humans. TNF mRNA expression by adipose tissue was documented by Northern blotting and RT-PCR, and quantitative RT-PCR was established for precise measurement of TNF mRNA. In addition, we demonstrated that the TNF protein was synthesized and secreted by adipose tissue, using both Western blotting and ELISA.

TNF mRNA levels were examined in the adipose tissue of 39 subjects covering a broad spectrum of adiposity. Although there was a significant positive relationship between percent body fat and TNF mRNA expression, there was considerable interindividual variability. Of particular interest was the relatively low level of TNF mRNA in subjects with a BMI $> 45 \text{ kg/m}^2$. Although no significant relationship was observed between TNF expression and BMI when all subjects were included, the relationship became significant when the very obese subjects (BMI > 45) were excluded. A further analysis was conducted on 11 subjects who underwent weight loss and weight stabilization. Although the level of TNF mRNA in obese subjects varied, both TNF mRNA and protein decreased significantly in response to weight loss. Thus, these data demonstrate that TNF expression is variably increased with increasing adiposity and is decreased with decreased adiposity.

The relatively lower TNF mRNA levels in the very obese subjects were intriguing, and there are several possible explanations for these data. If indeed adipose TNF expression is involved in adipocyte lipid accumulation and insulin sensitivity, then a lower TNF level in some subjects would suggest a failure of TNF to function as an "adipostat." This would then lead to efficient adipose lipid accumulation and a lack of resistance to the development of obesity. It should be noted, however, that TNF mRNA levels decreased with weight loss, even in these massively obese subjects with low TNF mRNA levels, suggesting some level of regulation by adiposity. Another explanation of these data could be the selection of nondiabetic subjects for this study. If adipose TNF were to increase with progressive obesity, then most subjects would eventually become sufficiently insulin resistant to manifest diabetes. By excluding diabetics, we may have excluded subjects that had the most vigorous increase in TNF with weight gain. Such an explanation,

however, presupposes that adipose tissue TNF expression can cause whole body insulin resistance in humans, as has been demonstrated in rodents (7).

The available data on rodent obesity suggest that increased TNF expression is most associated with genetic models of obesity and insulin resistance and is not present in monosodium glutamate-induced obesity, in which insulin resistance is minimal (6, 7). Although there is considerable epidemiological evidence for a genetic component to human obesity (36), multiple genes are likely involved, and one would not expect human obesity to resemble precisely a monogenic form of rodent obesity. If TNF expression is important in the pathogenesis of human obesity, it may be important in only a subfraction of obese humans. If TNF expression normally increases with obesity as a mechanism to limit further fat cell enlargement, then a low or normal level of TNF in the face of severe obesity may be a defect in an important counterregulatory mechanism.

Obesity is associated with insulin resistance and hyperinsulinemia, which is then reversed upon weight loss (37). Although these clinical observations are well established, the mechanism of obesity-related insulin resistance is not well understood. One explanation for insulin resistance revolves around the production of a locally active substance to prevent further adipocyte lipid accumulation (15, 16). As suggested previously (5), adipose tissue insulin resistance could have evolved as a mechanism to prevent excess adiposity. One way in which insulin resistance could limit adiposity is through changes in LPL. LPL hydrolyzes the triglyceride core of VLDL and chylomicrons into nonesterified fatty acids and glycerol and hence is an important determinant of adipose triglyceride storage (38). Adipose and muscle LPLs are inversely regulated (39, 40), such that the adipose/muscle LPL ratio is high during simple hyperinsulinemia, leading to a partitioning of lipid into adipose tissue. Progressive insulin resistance leading to non-insulin-dependent diabetes mellitus would be expected to reverse the high adipose/muscle LPL ratio present during simple hyperinsulinemia and would tend to prevent further adipose tissue expansion.

There is much data on the relationship between TNF and LPL. TNF/cachectin was initially described as an endotoxin-stimulated substance that was secreted by macrophages and that inhibited LPL in 3T3-L1 adipocytes (34). Because of the importance of LPL in lipogenesis in adipose tissue, TNF was

suggested to be central to the etiology of cachexia and the hyperlipidemia of cancer and endotoxemia. A number of studies have injected, infused, or transfected TNF into rodents to determine whether TNF causes weight loss or lowered LPL. Overall, the data indicate that TNF will cause weight loss in animals, but only in the context of continuous secretion at very high levels (41–44). In a study that controlled for TNF-induced anorexia, TNF injections yielded small changes in LPL in rats, but these changes in LPL were not responsible for the hyperlipidemia that ensued (12). Further studies found that TNF inhibited LPL in whole adipose tissue pieces, but not in isolated adipocytes (11, 45, 46), suggesting that TNF may stimulate the production of another substance that affects LPL.

In the present study, LPL activity was measured in the adipose tissue of these subjects, and a significant relationship was found between adipose LPL activity and TNF expression. In particular, subjects with the lowest levels of TNF expression had levels of LPL activity that were 2.5-fold higher than those in subjects with the highest TNF expression. Nevertheless, it is likely that many factors regulate LPL, and these data do not suggest that TNF is the primary source of LPL regulation. As described herein and previously (18, 35), LPL activity increases with weight loss. Although the increase in LPL with weight loss in these 11 subjects was consistent, as was the decrease in TNF, the magnitude of these changes did not correlate with each other, suggesting that other factors, besides adipocyte TNF expression, are involved in regulating LPL in the reduced-obese state.

The regulation of LPL is complex. In adipose tissue, LPL activity has been shown to correlate with serum insulin levels and the degree of insulin sensitivity under many circumstances (19, 47–50). However, obesity and weight loss represent important exceptions to this correlation. In obese hyperinsulinemic subjects, there is an increased amount of LPL in adipose tissue (19, 35, 51), but with weight loss and a reduction in hyperinsulinemia, adipose LPL increases further (18, 35). In vitro studies also point to the complexity of LPL regulation by insulin. Although insulin increases LPL in rat adipocytes (52, 53), human adipocyte LPL does not increase after exposure to physiologic insulin concentrations (21, 54, 55). Together, these studies are consistent with the existence of some additional local regulatory factor that affects LPL, such as TNF, but that is regulated independently.

Could TNF be a locally produced factor that causes insulin resistance and acts as a natural adipostat? The decrease in TNF expression with weight loss and the relationship between TNF and LPL are consistent with such a hypothesis. Although adipocyte TNF expression may be part of a homeostatic mechanism that controls fat cell size, other proteins are likely involved as well. Previous studies have demonstrated an increase in hormone-sensitive lipase activity in adipocytes in response to TNF (14, 56). Increased adipose tissue lipolysis has been demonstrated in obese subjects (57, 58). Such an increase in lipolysis would be expected to decrease fat cell size, and the increase in plasma nonesterified fatty acids would then provide an alternative fuel for muscle, leading to resistance to insulin-mediated glucose uptake (59). Another potential target of adipose tissue TNF is Glut4, the insulin-responsive glucose transporter found in adipose tissue and muscle. Several groups have demonstrated a decrease in Glut4 expression in response to TNF using 3T3-L1 or 3T3-F442A adipocytes (7, 13). In addition, decreased

Glut4 protein and mRNA levels have been demonstrated in adipocytes of obese subjects (60).

In summary, we have demonstrated that TNF is produced and secreted by human adipose tissue and is regulated by weight loss in obese subjects. In addition, there was an inverse correlation between adipose TNF expression and adipose LPL activity. These data are consistent with the hypothesis that the local production of TNF plays an important role in regulating adipocyte metabolism and may be a homeostatic mechanism that functions to limit adipocyte enlargement, perhaps through the local action of increasing insulin resistance.

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