Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance

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Adipose tissue tumor necrosis factor (TNF) and interleukin (IL)-6, which may cause obesity-related insulin resistance. We measured TNF and IL-6 expression in the adipose tissue of 50 lean and obese subjects without diabetes. Insulin sensitivity (S_i) was determined by an intravenous glucose tolerance test with minimal-model analysis. When lean (body mass index (BMI) < 25 kg/m^2) and obese (BMI 30–40 kg/m^2) subjects were compared, there was a 7.5-fold increase in TNF secretion (P < 0.05) from adipose tissue, and the TNF secretion was inversely related to S_i (r = −0.71, P < 0.001). IL-6 was abundantly expressed by adipose tissue. In contrast to TNF, plasma (rather than adipose) IL-6 demonstrated the strongest relationship with obesity and insulin resistance. Plasma IL-6 was significantly higher in obese subjects and demonstrated a highly significant inverse relationship with S_i (r = −0.42, P < 0.02). IL-6 was significantly associated with plasma nonesterified fatty acids (NEFA) which are not well understood.

Obesity represents an expansion of adipose tissue mass, and one explanation for obesity-related insulin resistance is the production of factors by adipose tissue that render some subjects more insulin resistant than others. Numerous adipocyte secretory products have recently been described that play a role in carbohydrate and lipid metabolism (14, 21, 23). One such adipocyte secretory product is tumor necrosis factor (TNF)-α. A new role for TNF was proposed in 1993 with the description of TNF expression by adipose tissue and the elevated expression of TNF in obese, insulin-resistant rodents and humans (17, 20, 24). Although it is unclear how adipose TNF expression may cause insulin resistance (36), TNF is known to impair insulin receptor signaling (18). TNF also inhibits lipoprotein lipase (LPL) and stimulates lipolysis in adipocytes (34), and the resulting increase in circulating nonesterified fatty acids (NEFA) would be expected to contribute to insulin resistance (7).

Another adipocyte secretory product that may be involved in insulin resistance is interleukin (IL)-6, which is a cytokine secreted by many cells, including adipocytes and adipose stromal cells (11, 15). Like TNF, IL-6 inhibits the expression of LPL, but, unlike TNF, IL-6 does not stimulate lipolysis (13, 16). IL-6 secretion is increased in the adipocytes of obese subjects (29) and may be important either as a circulating hormone or as a local regulator of insulin action.

Although many studies have examined the role of TNF in insulin resistance, relatively few of these have been in humans, and none has examined cytokine expression in detail along with the measurement of insulin resistance. In this study, we examined the expression of TNF and IL-6 in human adipose tissue from nondiabetic subjects with varying degrees of obesity and insulin resistance. We found that TNF secretion from human adipose tissue and circulating plasma IL-6 were both highly associated with obesity-associated insulin resistance.
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Table 1. Characteristics of the study subjects

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age, yr</th>
<th>BMI, kg/m²</th>
<th>Fat, %</th>
<th>TG, mg/dl</th>
<th>LDL, mg/dl</th>
<th>HDL, mg/dl</th>
<th>FBG, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women</td>
<td>39</td>
<td>40 ± 1.6</td>
<td>36 ± 1.4</td>
<td>43 ± 1.2</td>
<td>118 ± 12</td>
<td>125 ± 5</td>
<td>53 ± 2</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>Men</td>
<td>11</td>
<td>42 ± 3.3</td>
<td>33 ± 3.8</td>
<td>27 ± 4.9</td>
<td>143 ± 24</td>
<td>124 ± 11</td>
<td>45 ± 1.8</td>
<td>100 ± 3</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM. BMI, body mass index; TG, triglyceride; LDL and HDL, low- and high-density lipoprotein, respectively; FBG, fasting blood glucose.

METHODS

**Subjects.** Fifty subjects were recruited for these studies. This research was approved by the Institutional Review Board, and all subjects gave informed consent. All subjects were weight stable at the time of the study. Subjects initially underwent an oral glucose tolerance test using 75 g of glucose, and blood glucose was measured fasting and at 2 h. Subjects with diabetes (fasting blood sugar >126 mg/dl, 2-h glucose >200 mg/dl) were excluded. Of the 50 subjects, 15 had impaired glucose tolerance based on a 2-h glucose of 140–200 mg/dl, and three of these subjects had impaired fasting glucose based on a fasting glucose of 110–126 mg/dl.

Subjects then underwent a frequently sampled intravenous glucose tolerance test (FSIVGTT) and an adipose tissue biopsy. The FSIVGTT and the biopsy were performed at least 3 days apart.

Characteristics of the subjects that comprised this study are shown in Table 1. Blood lipids were measured using standard clinical assays, and plasma NEFA were measured using a colorimetric assay (Waco Chemical, Richmond, VA). Of the 50 subjects studied, 39 were women and 8 were African-American. The subjects ranged from lean to very obese, and insulin sensitivity (S₁; using the S₁ index from the FSIVGTT) varied considerably. Some subjects demonstrated moderate dyslipidemia, but no subject demonstrated fasting triglycerides >400 mg/dl. Body composition was determined using bioelectric impedance (38).

**S₁ measurements.** The measurement of in vivo S₁ was performed in the fasting state with the minimal-model analysis of the FSIVGTT (4, 5). We used the classic tolbutamide-modified test, which has been validated against the euglycemic clamp in humans (6, 41). In brief, catheters were placed for glucose injection and blood sampling. Four basal blood samples were obtained, and the patient was given an intravenous glucose bolus (11.4 g/m²) at time 0. At 20 min after the glucose injection, patients were given an injection of tolbutamide (125 mg/m²), again followed by frequent blood sampling, according to the standard protocol. Together, 4 basal and 27 postglucose blood samples were taken, the last one at 240 min. Glucose was measured in a glucose analyzer by use of the glucose oxidase method, and insulin was measured using radioimmunoassay. These measurements were performed in the Endocrinology Laboratory of the Indiana University School of Medicine (Indianapolis, IN). The S₁ was calculated using the MINMOD program (4) and was expressed in microunits per milliliter per minute.

**Adipose tissue biopsy.** Abdominal subcutaneous adipose tissue (~10 g) was removed from each patient by incision, which avoids trauma to fat cells and minimizes the amount of blood in contact with the fat cells. Some of the tissue was immediately frozen in liquid N₂ for later RNA extraction, whereas the rest of the tissue was placed into cold DMEM for other assays.

**Adipose tissue cytokine secretion.** TNF and IL-6 may function in an autocrine or paracrine manner; hence, we wished to measure the local secretion of these cytokines into the medium. Immediately after the biopsy, adipose tissue pieces of ~500 mg were minced and placed into serum-free DMEM (pH 7.4, 10 mM HEPES) at 37°C for varying times. Figure 1 illustrates the secretion of TNF and IL-6 into the medium of three subjects. There was little secretion of either cytokine into the medium for the first 60 min, followed by an increase in secretion over the next 60 min. Medium cytokine levels continued to increase for up to 24 h. To compare TNF and IL-6 secretion among different subjects, we measured cytokine levels in the medium after 2 h at 37°C. All data were normalized to adipose DNA content to control for differences in fat cell size. In general, IL-6 secretion from adipose tissue was much higher than TNF. In all subjects studied, the TNF level in the medium at 2 h was 0.78 ± 0.14 pg/μg DNA, and the IL-6 level in the medium was 9.8 ± 1.8 pg/μg DNA.

**Measurement of TNF and IL-6.** Adipose tissue TNF protein was measured using an ELISA (R&D Systems, Minneapolis, MN). This assay demonstrates an 8% intra-assay and a 15% interassay variation. This ELISA method was used to measure TNF in fasting plasma as well as TNF secretion by adipose tissue (see Relationship between TNF and obesity). TNF mRNA levels were measured by competitive RT-PCR, as described by us previously (24). IL-6 was measured in fasting plasma and secreted from adipose tissue using an ELISA assay (R&D Systems). This assay demonstrates intraday and interassay variations of <5%.

**Statistics.** All data are expressed as means ± SE. To analyze data between groups, a one-way ANOVA was performed, and secondary analysis was performed with the Student’s t-test with Bonferroni correction. Analysis of trends was performed using linear regression after log transformation. The Wilcoxon signed-rank test was used for the paired data in Table 2.
RESULTS

Relationship between TNF and obesity. To better define the effects of obesity on TNF expression, we measured TNF mRNA in the adipose tissue from each subject, along with plasma TNF and TNF secretion from the adipose tissue. Subjects were divided into four BMI groups representing lean subjects (BMI <25 kg/m²) and subjects with increasing degrees of obesity. A: plasma TNF was measured and expressed as pg/ml, along with TNF secreted into the medium of the adipose tissue expressed as pg/μg DNA, as described in METHODS. B: TNF mRNA levels were measured using RT-PCR, as described in METHODS. *P < 0.05 vs. BMI 30–40 and BMI >40.

IL-6 expression with obesity and insulin resistance. The adipose tissue fragments secreted relatively high levels of IL-6. When IL-6 expression was examined in the same BMI groups, as described in the preceding section for TNF, there was a tendency for an increase in IL-6 secretion from adipose tissue with increasing BMI and increasing body fat (Fig. 4B); however, these changes were not statistically significant. Plasma IL-6, however, was strongly associated with increasing obesity (Fig. 4A). In lean subjects (BMI <25), plasma IL-6 was 0.73 ± 0.23 pg/ml and increased about fourfold to 2.86 ± 0.61 pg/ml in the most obese subjects (BMI >40, 40 kg/m²) (P < 0.05 vs. <30% group).

TNF expression and insulin sensitivity. As expected, there was a significant relationship between obesity and insulin sensitivity. As described previously by others (22), the relationship between BMI and SI is curvilinear and best represented by a log/log transformation, and in our subjects, BMI and SI were significantly related (r = 0.65, P < 0.001). Because SI varies considerably among nonobese subjects with normal glucose tolerance, we did not divide SI into subgroups but instead examined TNF expression over the spectrum of SI. There was no significant relationship between either plasma TNF or TNF mRNA levels and SI (data not shown). However, there was a significant decrease in TNF secretion with increasing SI (Fig. 3), such that most of the insulin-sensitive subjects (SI >5) had lower levels of TNF secretion, and most of the insulin-resistant subjects (SI <2) had the highest levels of TNF secretion.
In a similar manner, plasma IL-6 was lower in subjects with low percent body fat. Plasma IL-6 was 0.84 ± 0.19 pg/ml (n = 10) in subjects with <30% body fat and was 2.05 ± 0.38 (n = 14) and 2.58 ± 0.44 (n = 18) pg/ml in subjects with 30–45 and >45% fat, respectively (P < 0.05). The relationship between S_I and plasma IL-6 was examined in the same manner as described for TNF. In contrast to TNF, adipose-secreted IL-6 demonstrated no significant relationship with S_I (r = −0.04, P = NS). However, there was a highly significant relationship (r = −0.71, n = 38, P < 0.001) between plasma IL-6 and S_I, as shown in Fig. 5. Plasma IL-6 was 3.0 ± 0.53 pg/ml in the most insulin-resistant subjects (S_I <2) and was 0.82 ± 0.19 pg/ml in the most insulin-sensitive subjects (S_I >5, P < 0.05).

One mechanism by which TNF may cause insulin resistance is through an increase in adipocyte lipolysis, leading to a rise in plasma NEFA. Hence, the relationship between cytokine expression and plasma NEFA was examined. The only significant relationship with plasma NEFA levels was with plasma IL-6 and adipose TNF secretion. As shown in Fig. 6, there were significant increases in plasma NEFA levels in subjects with higher levels of plasma IL-6 (r = 0.54, P < 0.001). There was also a significant association between plasma NEFA and TNF secretion (r = 0.35, n = 37, P < 0.05), although this association was less robust than the association with IL-6.

Cytokines and insulin resistance independent of obesity. Insulin resistance is exacerbated by obesity, leading to a significant relationship between S_I and BMI. Therefore, we examined the relationship between adipose cytokine expression and S_I without the confounding effects of BMI. To factor out obesity, we identified subjects who were of the same BMI but who were discordant for S_I. We compared the cytokine expression of subjects with insulin resistance (S_I <2.0) with that of subjects with less insulin resistance (S_I >3.0) who were matched for BMI (±5 kg/m²), age (±10 yr), and gender. Using these criteria, we were able to match nine subjects with S_I <2.0 with nine subjects with S_I >3.0. As shown in Table 2, these subjects were well matched for age and BMI, and there were significant differences in S_I by virtue of subject selection. No differences were noted between plasma TNF or adipose
IL-6 expression. However, the insulin-resistant subjects had significantly higher levels of plasma IL-6 as well as significantly higher levels of adipose TNF secretion ($P < 0.05$). In these matched subjects, TNF secretion and plasma IL-6 were two- to threefold higher in the insulin-resistant subjects.

Previous studies have demonstrated that IL-6 and TNF interact with each other in both 3T3-L1 adipocytes and mice (3, 16). We examined TNF and IL-6 expression from each subject’s adipose tissue to determine whether there was any relationship between IL-6 and TNF expression. As shown in Fig. 7, there was a strong linear relationship between the secretions of IL-6 and TNF from the adipose tissue ($r = 0.81$, $P < 0.0001$). On the other hand, there was no significant relationship between plasma IL-6 and plasma TNF (data not shown).

**DISCUSSION**

Since the initial description of TNF expression by adipose tissue, several lines of evidence have suggested that TNF overproduction by adipose tissue may be involved in the pathogenesis of the insulin resistance of obesity. TNF mRNA levels were high in obese, insulin-resistant rodents, and the infusion of a soluble TNF binding protein into insulin-resistant rats improved insulin sensitivity and improved the defect in insulin receptor and insulin receptor substrate-1 autophosphorylation in fat and muscle (18, 20). Recent studies using genetic manipulations resulting in knockout or deletion of TNF or TNF receptor have confirmed the importance of TNF in rodent insulin resistance (9, 19, 40), although one such study (37) found no role for TNF or the TNF receptor in insulin resistance.

Relatively few studies have examined the relationship between TNF and insulin resistance in humans. Studies by us (24) and others (1, 17) demonstrated elevated levels of adipose TNF mRNA and protein in obese subjects and a decrease in TNF with weight loss. No study has examined the relationship between SI and TNF, although one study noted a significant correlation between TNF mRNA levels and fasting insulin (17), and several studies observed a decrease in TNF after weight loss (12, 17, 24). High TNF secretion from human adipose tissue was associated with decreased $[^3H]$glucose incorporation into lipids (26).

It is not clear whether TNF functions locally or circulates in a sufficiently high concentration to influence distant targets. Plasma TNF has been measured, and several studies have observed increased plasma TNF levels in obese subjects and in subjects with hyperinsulinemia or insulin resistance (10, 42, 43). Plasma TNF was elevated in male diabetic subjects compared with male controls, but no such relationship was observed in women (35). In an attempt to bind plasma TNF and reverse insulin resistance in humans, diabetic or insulin-resistant subjects have been given an injection of anti-TNF binding protein. In both studies, there was no improvement in insulin resistance (31, 33).

The role of IL-6 in insulin resistance has been much less studied. IL-6 is secreted by many cells, including adipocytes and adipose stromal cells (11, 15) and is increased after a meal (32). Like TNF, IL-6 inhibits the expression of LPL, but unlike TNF, IL-6 does not stimulate lipolysis (13, 16). Linking IL-6 to insulin resistance are studies demonstrating increased IL-6 secretion in the adipocytes of subjects with obesity (29) and diabetes (2).

In the studies described herein, we measured TNF and IL-6 gene expression at several levels from the adipose tissue of lean and obese subjects and related this expression to SI, a reliable measure of insulin sensitivity. Both IL-6 and TNF were expressed and secreted by human adipose tissue, although IL-6 levels were much higher in both adipose tissue and plasma. The most consistent relationship between cytokine expression and obesity-related insulin resistance involved increased TNF secretion from adipose tissue and increased plasma IL-6 levels. Elevated TNF and IL-6 expression was found in subjects who were only moderately obese (BMI > 30) and increased progressively with decreasing SI. The relationship between
plasma IL-6 and $S_I$ was very strong, with a highly significant inverse correlation and a fivefold difference between the most insulin-resistant and most insulin-sensitive subjects. Thus both TNF and IL-6 were associated with both obesity and insulin resistance; however, it was the adipose-secreted form of TNF and the plasma level of IL-6 that displayed the strongest relationships.

The subjects in this study were heterogeneous with regard to degree of obesity, gender, and race, and it is possible that a study using a more focused group of subjects would yield different results. However, we observed no consistent effect of gender or race on cytokine expression in these subjects. This study also relied on plasma cytokine levels and cytokine secretion from adipose tissue, and these measurements may not be reflective of cytokine biological effects at the tissue level.

Because obesity and insulin resistance are related to each other, we wished to determine whether TNF and IL-6 expression were related to insulin resistance independently of obesity. As described in Table 2, we paired insulin-resistant subjects with more-insulin-sensitive subjects and matched them for BMI and age. By use of this analysis, high levels of TNF secretion and plasma IL-6 were both significantly associated with insulin resistance. Thus the expression of these cytokines was associated with insulin resistance independently of obesity.

There are differences in the expression of TNF and IL-6 that may be important in understanding their functions. IL-6 was secreted at high levels from adipose tissue, and there was a significant arteriovenous difference in IL-6 across the adipose tissue bed, whereas there was no arteriovenous difference with TNF (29). We found no relationship between plasma TNF and obesity or insulin resistance, although other studies have noted increased plasma TNF with obesity (2, 10, 42, 43). IL-6 and TNF may interact with each other, as suggested by the strong correlation between TNF secretion and IL-6 secretion in this study and by previous studies that demonstrated increased IL-6 expression in response to TNF (3, 16). Together, these data suggest that TNF functions locally at the level of the adipocyte in a paracrine fashion, perhaps stimulating the secretion of NEFA, IL-6, or other circulating substances. On the other hand, plasma IL-6 circulates at high levels and may be more important systemically and perhaps represents a hormonal factor that induces muscle insulin resistance.

It is noteworthy that two studies have tried, and failed, to reverse insulin resistance with an injection of anti-TNF binding proteins (31, 33). On the basis of the studies described herein, we can speculate on several possible reasons for the failure of anti-TNF therapy in humans. If TNF functions in a paracrine or autocrine fashion in adipose tissue, then the anti-TNF binding proteins may not reach the microcirculation in sufficient concentration to prevent TNF-mediated effects. In addition, our data raise the possibility that IL-6 is the major circulating component of obesity-related insulin resistance.

The development of insulin resistance with increasing adiposity suggests that an adipocyte product may be important in insulin resistance. Both TNF and IL-6 are adipocyte products that are overexpressed in obese insulin-resistant subjects, and we have shown that the secretion of these cytokines is interrelated. Some of these cytokines may function systemically, others may function locally, and still others may function to increase the secretion or synthesis of other adipocyte factors or to act as an adjuvant to the actions of other insulin resistance factors. One such insulin resistance factor is NEFA, which are closely associated with insulin resistance (28, 39). TNF stimulates lipolysis in adipocytes (34); hence, it is possible that TNF functions at the level of the adipocyte to stimulate lipolysis. Although IL-6 is not known to stimulate lipolysis (13, 16), we found a significant relationship between plasma IL-6 and plasma NEFA levels, whereas the relationship between TNF expression and plasma NEFA was much less robust.

These studies provide the first comprehensive analysis of IL-6 expression in obese, insulin-resistant humans and add to the data on TNF expression. Together, these studies suggest that obesity-related insulin resistance represents a complex syndrome, mediated by a number of adipocyte secretory products, which ultimately lead to defects in insulin action in other target organs.

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