Relation Between Plasma Tumor Necrosis Factor-α and Insulin Sensitivity in Elderly Men With Non–Insulin-Dependent Diabetes Mellitus

Jan Nilsson, Stefan Jovinge, Audrey Niemann, Richard Reneland, Hans Lithell

Abstract—Human obesity is associated with an increased tumor necrosis factor-α (TNF-α) mRNA expression in adipose tissue. TNF-α decreases insulin-dependent glucose uptake by inhibiting autophosphorylation of the insulin receptor, suggesting that TNF-α may play a role in insulin resistance. In this study, we analyzed plasma levels of TNF-α in 40 70-year-old men with newly detected non–insulin-dependent diabetes mellitus and in 20 age-matched controls. Twenty of the patients had a moderate level of insulin resistance and 20 were severely insulin resistant. The plasma levels of TNF-α were higher in patients (4.00 ± 1.53 pg/mL in moderately insulin resistant and 4.91 ± 1.43 pg/mL in severely insulin resistant subjects) than in controls (3.27 ± 0.79 pg/mL, \( P < 0.001 \)). TNF-α was significantly related to body mass index, fasting glucose levels, and serum triglyceride levels and inversely related to the high density lipoprotein cholesterol level. The finding of an association between high plasma levels of TNF-α and several metabolic abnormalities characteristic for the insulin resistance syndrome suggests that TNF-α may be involved in the pathogenesis of non–insulin-dependent diabetes mellitus. (Arterioscler Thromb Vasc Biol. 1998;18:1199-1202.)

Key Words: tumor necrosis factor-α ● diabetes ● insulin resistance ● triglycerides ● HDL

Tumor necrosis factor-α (TNF-α) was originally identified as the factor responsible for hypertriglyceridemia in bacterially infected animals. It was later shown to be produced predominantly by macrophages and to function as an inducible cytokine with a wide range of important proinflammatory and immunoregulatory actions. Recent studies have demonstrated synthesis of TNF-α in muscle and adipose tissue also and have implicated TNF-α in the pathogenesis of insulin resistance (IR). Hotamisligil and coworkers found increased TNF-α mRNA expression in adipose tissue in obese fa/fa rats and showed that neutralization of TNF-α caused a significant increase in the peripheral uptake of glucose in response to insulin. TNF-α was later shown to inhibit insulin-stimulated glucose uptake in adipocytes by decreasing phosphorylation of the insulin receptor. Moreover, infusion of neutralizing, soluble TNF receptors results in a marked increase in insulin-stimulated autophosphorylation of the insulin receptor in obese, IR rats.

Increased adipose and muscle expression of TNF-α has been demonstrated in human obesity also. Adipose tissue TNF-α mRNA levels show significant correlations with percent body fat, body mass index (BMI), and the level of hyperinsulinemia. Reduction of body weight in obese subjects is associated with a decrease in adipose TNF-α mRNA expression as well as improved insulin sensitivity.

An increased IR is believed to be a key factor in non–insulin-dependent diabetes mellitus (NIDDM). To analyze the involvement of TNF-α in NIDDM-associated IR, we have used a highly sensitive ELISA to determine plasma levels of TNF-α in healthy controls and NIDDM patients with moderate and severe IR.

Methods

Subjects
The subjects in this study were recruited from a cohort of men with a median age of 70.8 years (range, 69.4 to 72.5) who participated in a health survey that was started in Uppsala, Sweden, in 1970. All men born in 1920 to 1924 and living in the municipality of Uppsala were invited to participate in this health survey: 2841 men were invited and 2322 agreed to take part in the study (participation rate, 82%). Twenty years later, the participants were invited to take part in a follow-up investigation. During the intervening 20 years, 422 had died and 199 had moved out of the Uppsala region. One thousand six hundred and eighty men were invited and 459 did not participate in this follow up, leaving 1221 men aged 70 years. All investigations started between 7:30 and 8:30 AM after an overnight fast. The oral glucose tolerance test (OGTT) and the clamp procedure took place on separate days within 1 week. BMI was calculated as the ratio of the weight in kilograms to the square of the height in meters. The waist and hip circumferences were measured midway between the lowest rib and the iliac crest and over the widest part of the hip, respectively. To investigate the role of TNF-α in IR, 3 groups of individuals from this cohort were included in the present study: (1) 20 normoglycemic healthy controls, (2) the 20 diabetics with the lowest insulin sensitivity, and (3) the 20 diabetics with the highest insulin sensitivity.
Oral Glucose Tolerance Test

An OGTT was performed by measuring the concentrations of plasma glucose and insulin immediately before and at 30, 60, 90, and 120 minutes after challenge with 75 g anhydrous dextrose. Plasma insulin was assayed by using an enzymatic-immunological assay (Enzymum, Boehringer Mannheim) performed in an ES300 automatic analyzer (Boehringer Mannheim). Plasma glucose was measured by the glucose dehydrogenase method (Gluc-DH, Merck). Diabetes and impaired glucose tolerance were diagnosed according to the National Diabetes Data Group criteria; ie, diabetes was diagnosed if the 120-minute and 1 or more of the 30- to 90-minute glucose values were $\geq 11.1$ mmol/L and impaired glucose tolerance was diagnosed when the fasting glucose value was $< 7.8$ mmol/L and 1 or more of the 30- to 90-minute glucose values were $\geq 11.1$ mmol/L and the 120-minute value was between 7.8 and 11.1 mmol/L.

Insulin Sensitivity

Insulin sensitivity was measured by the euglycemic hyperinsulinemic clamp procedure as described by DeFronzo et al. with slight modification. Insulin (Actrapid Human, Novo) was infused at a rate of 56 mU/min per square meter of body surface area instead of at 40 mU/min per square meter of body surface area. Hepatic glucose output was inhibited by 88% to 95% in diabetics as well as nondiabetics. Plasma glucose was assayed in duplicate in a Beckman glucose analyser IIr (Beckman Instruments). Glucose disposal (M) was calculated as the amount of glucose (in milligrams) infused per minute and per unit of body weight (kilograms). The insulin sensitivity index (M/I) was calculated by dividing M by the mean plasma insulin concentration (I, in milliunits per liter) during the last 60 minutes of the insulin/glucose infusion and multiplying by 100 to represent M at a plasma insulin level of 100 mU/L (the values for M/I are given in milligrams per kilogram per minute per 100 mU insulin). The M/I compensates for differences in insulin levels attained during the clamp and can therefore be considered a more accurate index of peripheral insulin sensitivity than is the glucose disposal rate.

Lipid and Lipoprotein Measurements

Cholesterol and triglyceride concentrations in serum were assayed by enzymatic techniques (Instrumentation Laboratories) in a Monarch 2000 centrifugal analyzer. HDLs were separated by precipitation with MgCl2/phosphotungstate. LDL cholesterol was calculated by using the Friedewald formula. Serum nonesterified fatty acids were measured by an enzymatic colorimetric method (Wako Chemical GmbH) applied for use in the Monarch 2000 centrifugal analyzer. The laboratory at the Department of Geriatrics is an accredited reference laboratory of the Centers for Disease Control and Prevention (Atlanta, Ga).

Analysis of TNF-α

Plasma TNF-α levels were measured by using an ELISA for human TNF-α (Quantikine, R&D Systems). This is a newly developed, sensitive sandwich ELISA with a lower detection limit of 0.5 pg/mL. The ELISA used in the present study detects both free TNF-α and TNF-α bound to its soluble receptor (data in the manufacturer’s protocol booklet). The intra-assay coefficient of variation for analysis of TNF-α in serum is $< 8\%$, and the average recovery of the assay was 95% for serum and 96% for EDTA-plasma. The correlation coefficient for duplicate determinations of the same sample was 0.93 (n=92).

Statistical Analysis

Conventional methods were used for calculation of means and SDs. Coefficients of skewness were calculated to test deviations from a normal distribution, and logarithmic transformation was performed when appropriate to allow hypothesis testing with parametric methods. Differences in continuous variables between groups were tested by ANOVA and the Scheffe F test. Pearson correlation coefficients were calculated to estimate relations between different variables. The interactions between the group factor and BMI, waist-hip ratio, M/I, fasting insulin, fasting glucose, serum cholesterol, serum triglycerides, HDL cholesterol, and blood pressure were assessed to examine whether there were dissimilar associations between these variables and TNF-α in the 3 groups. None of these interactions were significant, so all correlations reported are based on the group as a whole.

Results

Forty 70-year-old men with NIDDM and 20 healthy, age-matched controls were included in the study. The NIDDM subjects were selected to include 20 patients with severe IR as assessed by the euglycemic hyperinsulinemic clamp technique (M/I, 1.4±0.4) and 20 patients with a moderate level of IR (M/I, 5.1±1.6). The M/I of the controls was 9.9±0.6. The basic characteristics of the study groups are presented in the Table.

The serum TNF-α level was 3.27±0.29 pg/mL in controls, which is comparable to the levels previously demonstrated in healthy, middle-aged men. TNF-α levels were elevated by 23% in NIDDM patients with moderate IR and by 51% in NIDDM patients with severe IR (Figure 1, P<0.001 as determined by ANOVA). TNF-α levels were significantly correlated with body weight ($r=0.32$, P<0.05) and BMI ($r=0.32$, P<0.05; Figure 2) but not with the waist-hip ratio ($r=0.10$, NS). There were also significant correlations between TNF-α and fasting glucose ($r=0.43$, P<0.001; Figure 3) as well as between TNF-α and basal insulin levels ($r=0.39$, P<0.005; Figure 4).

The basal TNF-α level was also correlated with the area

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**Basic Characteristics of the Study Groups**

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=20)</th>
<th>NIDDM, IR Moderate (n=20)</th>
<th>NIDDM, IR Severe (n=20)</th>
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</thead>
<tbody>
<tr>
<td>BMI</td>
<td>22.8±2.2</td>
<td>25.5±2.8</td>
<td>31.4±5.5</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.92±0.08</td>
<td>0.95±0.04</td>
<td>0.99±0.05</td>
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<td>Fasting glucose, mmol/L</td>
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<td>6.3±1.1</td>
<td>7.6±1.5</td>
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<tr>
<td>Fasting insulin, pmol/L</td>
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<td>9.7±3.5</td>
<td>21.5±10.5</td>
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<tr>
<td>M/I ratio</td>
<td>9.9±0.6</td>
<td>5.1±1.6</td>
<td>1.4±0.4</td>
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<tr>
<td>Cholesterol, mmol/L</td>
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<td>5.5±1.2</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>1.6±0.4</td>
<td>1.4±0.6</td>
<td>2.0±1.0</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.57±0.39</td>
<td>1.21±0.38</td>
<td>1.09±0.22</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>142±11</td>
<td>150±17</td>
<td>159±22</td>
</tr>
</tbody>
</table>

Values are mean±SD.

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**Figure 1.** Plasma levels of TNF-α in control and NIDDM patients with moderate and severe IR. Values are mean±SD.
under the curve for glucose during the OGTT ($r=0.37$, $P<0.005$).

There was a significant correlation between TNF-α and the degree of IR as assessed by the M/I value ($r=-0.50$, $P<0.0001$; Figure 5). To test whether the relation was independent of BMI and waist-hip ratio, these variables were entered as independent variables together with TNF-α in a multiple regression model with M/I as the dependent variable. This adjustment decreased the strength of the relationship only slightly (partial $r=0.43$, $P<0.0005$).

Significant correlations also existed between TNF-α and serum cholesterol ($r=0.26$, $P<0.05$), triglycerides ($r=0.36$, $P<0.005$), and HDL cholesterol ($r=-0.43$, $P<0.001$). There was no correlation between TNF-α and blood pressure levels or between TNF-α and serum nonesterified fatty acids.

**Discussion**

Previous studies in humans have demonstrated increased adipose expression of TNF-α mRNA in nondiabetic subjects with obesity-dependent IR, in normoglycemic subjects with increased IR, and in NIDDM patients.$^9$–$^{11}$ The previously available TNF-α ELISA and bioassays were not sensitive enough to allow determination of the low levels of circulating TNF-α present in the subjects included in those studies. Until now, this circumstance has made it difficult to analyze the role of TNF-α in large populations. We used a newly developed, highly sensitive ELISA for TNF-α that detects plasma levels as low as 0.5 pg/mL. Analysis with the use of this assay in 60 individuals with normal, moderate, or severe IR reveals a significant correlation between circulating TNF-α and the degree of IR as assessed by the euglycemic hyperinsulinemic clamp technique. These observations are interesting for 2 reasons. First, they confirm at the protein level the previous finding of a relation between increased TNF-α expression and IR in humans. Second, they suggest that the increased local production of TNF-α in adipose and muscle tissue is associated with an increased release of TNF-α into the circulation.

The question whether circulating TNF-α is biologically active remains to be fully answered. Available bioassays for TNF-α activity do not detect levels $<$10 pg/mL in plasma, making it impossible to correlate plasma TNF-α bioactivity with the TNF-α protein levels found in controls and NIDDM patients. Circulating TNF-α has been reported to associate with a soluble receptor that inhibits its biological activity,$^{16}$ suggesting that the action is primarily a local one. If TNF-α has a dual role as a regulator of both metabolic and immune

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**Figure 2.** Relation between BMI and plasma TNF-α. Controls (■), NIDDM with moderate IR (●), and NIDDM with severe IR (▲) are shown.

**Figure 3.** Relation between TNF-α and fasting glucose. Controls (■), NIDDM with moderate IR (●), and NIDDM with severe IR (▲) are shown.

**Figure 4.** Relation between TNF-α and fasting insulin. Controls (■), NIDDM with moderate IR (●), and NIDDM with severe IR (▲) are shown.

**Figure 5.** Relation between TNF-α and insulin sensitivity index (M/I; see Methods). Controls (■), NIDDM with moderate IR (●), and NIDDM with severe IR (▲) are shown.
processes, then it also appears reasonable that the systemic effects of TNF-α produced in adipose tissue and muscle are restricted. The ELISA used in the present study detects both free TNF-α and TNFα bound to its soluble receptor.

The effect of TNF-α on IR is believed to be due to its ability to inhibit insulin-dependent autophosphorylation of the insulin receptor and the phosphorylation of insulin receptor substrate-1, the major substrate of the insulin receptor in vivo.6,7 Accordingly, increased expression of TNF-α in muscle and adipose tissue will result in a decrease of insulin-dependent uptake of glucose in these tissues.

In obese individuals, there is a significant relation between BMI and adipose tissue TNF-α mRNA levels.10 Similarly, there was a significant correlation between BMI and plasma TNF-α levels in the current study. Weight reduction is associated with decreased TNF-α mRNA expression in adipose tissue.9,10 It has been speculated that TNF-α may act as an “adipostat” that protects fat cells against lipid overloading.11 The regression analyses showed that plasma levels of TNF-α are more closely related to the level of IR itself than to BMI. This does, however, not necessarily mean that it is IR per se rather than BMI that regulates TNF-α expression.

Genetic variation within regulatory elements of the TNF-α promoter represents another possible cause of differences in tissue TNF-α expression. A polymorphism at the −308 position of the TNF-α receptor has been shown to influence the rate of gene transcription but shows no association with NIDDM.17

High plasma levels of TNF-α were found to be associated with increased triglyceride levels and a low HDL cholesterol. This relation is also found in patients with early-onset coronary heart disease,19 another group with an overrepresentation of IR. Similarly, there is a significant correlation between adipose tissue TNF-α mRNA expression and plasma triglycerides in obese individuals.8 Infusion of TNF-α increases triglyceride levels,18 and cytokines are known to mediate hypertriglyceridemia in bacterial infections.19 There are several mechanisms by which TNF-α may influence triglyceride levels. It inhibits adipose tissue lipoprotein lipase activity20 and induces dissociation of lipoprotein lipase from endothelial cells.21 An increase in triglycerides may also be the result of a TNF-α–mediated inhibition of adipocyte insulin receptors, leading to enhanced release of free fatty acids used for the synthesis of VLDL in the liver. Infusion of TNF-α also results in decreased HDL cholesterol levels. The mechanisms by which TNF-α influences the metabolism of HDL cholesterol are less clear and may be secondary to the effects on triglycerides rather than to a direct effect on HDL synthesis or catabolism.

There are some limitations to the current study that need to be considered. For one, we do not know whether the TNF-α detected in plasma originates from muscle and adipose tissue. Parallel analysis of TNF-α levels in plasma and TNF-α mRNA expression in adipose tissue could provide some additional information in this respect. The fact that IR is associated with increased expression of TNF-α in both adipose tissue and plasma provides some indirect evidence for an association. The possibility that the increased levels of TNF-α in IR subjects reflects an inflammatory activation should also be considered. All subjects included in this study were free of symptoms of infection. Moreover, previous studies in patients with early-onset coronary heart disease showed no relation between TNF-α levels and the levels of acute-phase reactants such as orosomucoid and haptoglobin.5

It should also be kept in mind that it remains to be demonstrated whether a similar relation between TNF-α levels and insulin sensitivity also exists in younger men and in women. In conclusion, the current observations add further support to the hypothesis that TNF-α is involved in the etiology of IR and NIDDM.

References

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