

The TNF- α Gene *Nco* I Polymorphism Influences the Relationship Among Insulin Resistance, Percent Body Fat, and Increased Serum Leptin Levels

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Tumor necrosis factor- α (TNF- α), acting as a modulator of gene expression in adipocytes, is implicated in the development of insulin resistance and obesity. The aim of this study was to investigate whether the *Nco* I polymorphism of the TNF- α gene influences the relationship among insulin resistance, percent body fat, and serum leptin levels. A sample of 38 subjects (19 men, mean age 36.2 ± 1.9 years, BMI 28.8 ± 1.2 kg/m², range 22.2–35.7; and 19 women, age 34.9 ± 1.4 years, BMI 28.1 ± 0.8 kg/m², range 19–37.9) was divided into two groups on the basis of the *Nco* I genotype. Twenty-three subjects were (+/+) homozygotes for the presence of the *Nco* I restriction site that is associated with a guanine at position -308 of the TNF- α promoter. Of the other subjects, 12 were (+/-) heterozygotes and 3 (-/-) homozygotes for the absence of the restriction site, resulting from a guanine-to-adenine substitution at position -308 of the TNF- α promoter. This substitution (termed TNF-2) leads to higher rate of transcription of TNF- α than the wild-type allele TNF-1 *in vitro*. TNF-1 (+/+) and TNF-2 (+/- and -/-) groups of subjects were comparable in sex, age, BMI, waist-to-hip ratio, and several skinfold measurements. Basal serum insulin was greater (14.2 ± 2 vs. 9.2 ± 0.9 mU/l, $P = 0.041$) in the TNF-2 group in the presence of comparable serum glucose concentration. The integrated area under the curve of serum insulin concentrations, measured in response to a 75-g oral glucose challenge, and the percent body fat, measured by bioelectric impedance, were significantly increased in TNF-2 subjects (226.8 ± 33 vs. 139.4 ± 17.8 mU/l, $P = 0.032$; 33.6 ± 2.8 vs. $24.9 \pm 2\%$, $P = 0.01$). TNF-2 subjects also showed a decreased insulin sensitivity index, as determined by the frequently sampled intravenous glucose tolerance test with minimal model analysis (1.9 ± 0.4 vs. 3.05 ± 0.3 min⁻¹ · mU⁻¹ · l⁻¹, $P = 0.03$). These differences were more marked among women. Paralleling

the known relationship between insulin and leptin levels, serum leptin concentration was clearly increased in the TNF-2 group (19.6 ± 3.4 vs. 11.1 ± 1.5 ng/ml, $P = 0.03$). Therefore, (+/-) heterozygotes and (-/-) homozygotes may be more susceptible to developing insulin resistance and increased percent body fat. Results of the present study suggest that TNF- α /*Nco* I polymorphism may exacerbate the alterations in leptin levels normally found among insulin-resistant subjects. *Diabetes* 46:1468–1472, 1997

The cytokine TNF- α acting as a modulator of gene expression in adipocytes is implicated in the development of insulin resistance and obesity (1). Fat tissue is a significant source of endogenous TNF- α production, and the expression of this cytokine is elevated in human obesity in both adipose (2,3) and muscle tissues (4). Increased expression of TNF- α strongly correlates with the level of hyperinsulinemia (5) and the glucose disposal rate during euglycemic clamp technique (4).

Adipose tissue also produces leptin, a protein proposed to be an "adiposity signal," that acts in the brain to lower food intake and adiposity, as demonstrated by recent studies (6). Leptin levels increase in the fed state, when triglyceride is being stored in adipose cells, and decrease during nutritional deprivation (7,8). Defects in the production of leptin lead to overeating (6). In humans, as the degree of obesity seems to correlate closely with a rise in plasma leptin concentrations, the presence of leptin resistance has been hypothesized (8). Plasma leptin levels are also independently related to insulin resistance (9). Recently, leptin has been implicated in the development of insulin resistance in the human hepatocellular carcinoma cell lines HepG2 and Hep3B (10).

Past studies have examined the transcriptional regulation of the TNF- α gene by viruses, lipopolysaccharide, and phorbol esters (phorbol myristic acid [PMA]) and have identified several regulatory elements upstream of the transcription start site (11). A biallelic polymorphism in the TNF- α promoter has been identified at position -308, involving the substitution of guanine (G) by adenine (A) (12). This polymorphism was termed TNF-2, as opposed to the wild-type TNF-1. When linked to a receptor gene and expressed in cultured cells, TNF-2 leads to a higher rate of transcription than the wild-type allele (13). On the other hand, adipose tissue gene expression is regulated by cytokines during infection. In a recent study, the administration of endotoxin to fasted hamsters increased the expression of leptin mRNA to

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ASF, abdominal skinfold thickness; BSF, biceps skinfold thickness; FSIGTT, frequently sampled intravenous glucose tolerance test; LPL, lipoprotein lipase; MAFA, mid-arm fat area; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; PMA, phorbol myristic acid; SSF, subscapular skinfold thickness; TNF, tumor necrosis factor; TNFR-2, TNF receptor 2; TSF, triceps skinfold thickness; WHR, waist-to-hip ratio.

levels similar to fed control animals, contributing to the anorexia of infection (14). Furthermore, leptin expression and secretion have been found to be regulated by TNF- α in cultured adipocytes (15). It is unknown whether obesity-induced increased expression of TNF- α may modulate leptin expression.

The etiology of human obesity is complex and multifactorial and, on the basis of twin studies, is thought to have a strong genetic component. Recent genetic studies using sibling pair analysis have shown linkage between a marker near the TNF- α locus and body fat content in Pima Indians (16).

We tested the hypothesis that TNF-2 allele results in increased activity or production of TNF- α at either the protein or message RNA level and contributes to differences in insulin sensitivity, body fat content, and serum leptin levels.

RESEARCH DESIGN AND METHODS

Subjects. Thirty-eight subjects (19 men, mean age 36.2 ± 1.9 years, BMI 28.8 ± 1.2 kg/m², range 22.2–35.7; and 19 women, age 34.9 ± 1.4 years, BMI 28.1 ± 0.8 kg/m², range 19–37.9) were studied. None of the subjects were taking any medication or had any evidence of metabolic disease other than obesity, and all reported that their body weight had been stable for at least 3 months before the study. All subjects were normotensive. The protocol was approved by the Hospital Ethics Committee.

Anthropometric measurement. All subjects were evaluated, in addition to BMI, through the following anthropometric parameters: triceps skinfold thickness (TSF), biceps skinfold thickness (BSF), subscapular skinfold thickness (SSF), abdominal skinfold thickness (ASF), and mid-arm fat area (MAFA). BMI was calculated as weight (in kilograms) divided by height (in meters) squared. TSF, BSF, SSF, and ASF were measured with a skinfold caliper (Holtain, Cambridge, U.K.) and MAFA was measured as described in the World Health Organization Monographic Series (17). Values for each variable were expressed as a percentage of the 50th percentile adjusted by sex and age, as obtained from a large sample of a healthy population living in the same area covered by our hospital (18). The percentage of body fat and fat-free mass were determined by bioelectric impedance analysis (Holtain BC Analyzer, U.K.).

Study protocol. An oral glucose tolerance test (OGTT) was performed according to the recommendations of the National Diabetes Data Group (19). After a 12-h overnight fast, glucose was ingested in a dose of 75 g, and blood samples were collected through a venous catheter from an antecubital vein at 0, 30, 60, 90, and 120 min for measurement of serum glucose and insulin. The glucose and insulin total areas under the curve during the OGTT were determined by the trapezoidal method.

Frequently sampled intravenous glucose tolerance test (FSIGTT). The subjects consumed a weight-maintaining diet containing at least 300 g of carbohydrate per day and refrained from exertion for 3 days before the test. The subjects also abstained from caffeine and alcohol for 72 h before the tests. All women had regular menstrual cycles. Frequently sampled intravenous glucose tolerance test (FSIGTT) and OGTT were performed on days 3–8 of two consecutive menstrual cycles. The experimental protocol started between 8:00 and 8:30 A.M. after an overnight fast. A butterfly needle was inserted into an antecubital vein, and patency was maintained with a slow saline drip.

Basal blood samples were drawn at -15 and -5 min, after which glucose (300 mg/kg body wt) was injected over 1 min starting at time 0. At 20 min, regular insulin (0.03 U/kg) was injected as a bolus. Additional samples were obtained from a contralateral antecubital vein at times 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 20, 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, and 180 min. Samples were rapidly collected via a three-way stopcock connected to the butterfly needle.

Analytical methods

Sample collection. A 10-ml sample of venous blood was collected in an EDTA vacutainer. Within 1 h of drawing, the buffy coat was separated from the blood by centrifugation at 800–900g for 10 min. Genomic DNA was isolated from the buffy coat using QiaAMP spin columns (Qiagen, Chatsworth, CA). There was 100 ng of DNA used for the polymorphism analysis by the polymerase chain reaction (PCR).

TNF- α gene polymorphism analysis. A transition polymorphism G to A in the -308 position of the gene was detected. There was 100 ng of the extracted DNA used as a template. The primers used were as follows: 5'-AGGCAATAG-GTTTTGAGGGCCAT-3' and 5'-TCCTCCCTGCTCC GATTCCG-3'. The reaction was carried out in a final volume of 50 μ l containing 3 mmol/l MgCl₂, 0.5 mmol of each dNTP (Boehringer Mannheim, Mannheim, Germany), 0.2 μ mol of each primer, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). DNA was amplified during 35 cycles with 1 min denaturation at 94°C, 1 min annealing at 60°C,

and 1 min extension at 72°C. Before that, 1 cycle of 3-min denaturation at 94°C, 1-min annealing at 60°C, and 1-min extension at 72°C was performed. After all the cycles, a final cycle of 1-min denaturation at 94°C, 1-min annealing at 60°C, and 5-min extension at 72°C was included. PCR products were digested with 10-fold excess *Nco* I restriction enzyme at 37°C for 2–4 h and electrophoresed on a 2.5% agarose gel. *Nco* I restriction fragment length polymorphism was detected by ethidium bromide staining, which revealed a two-allele polymorphism that produced three bands of different sizes: a 107-bp fragment corresponding to the T2 allele (restriction site absent) and a set of 87 and 20 bp corresponding to the T1 allele (restriction site present).

Other analyses. The serum glucose level during the FSIGTT was measured in duplicate by the glucose oxidase method with a glucose analyzer 2 (Beckman, Brea, CA). The coefficient of variation was 1.9%. The serum insulin level during the FSIGTT was measured in duplicate by monoclonal immunoradiometric assay (IRMA, Medgenix Diagnostics, Fleunes, Belgium). The lowest limit of detection was 4.0 mU/l. The intraassay coefficient of variation was 5.2% at a concentration of 10 mU/l and 3.4% at 130 mU/l. The interassay coefficients of variation were 6.9 and 4.5% at 14 and 89 mU/l, respectively.

Serum leptin concentrations were measured by radioimmunoassay in samples obtained after an overnight fast (Linco Research, St. Charles, MO). The lower limit of detection was 0.5 ng/ml. Coefficients of variation intra- and interassay were <7% and <8%, respectively. The radioimmunoassay for leptin does not present cross-reaction with human proinsulin, insulin, or glucagon. Plasma TNF- α was measured using IRMA (Medgenix Diagnostics, Fleunes, Belgium).

Total serum cholesterol was measured through the reaction of cholesterol esterase/cholesterol oxidase/peroxidase (20) using a BM/Hitachi 747. VLDL cholesterol was measured after ultracentrifugation at 45,000g. HDL cholesterol was quantified after precipitation with polyethylene glycol at room temperature (21). Total serum triglycerides were measured through the reaction of glycerol-phosphate-oxidase and peroxidase (22). VLDL triglycerides were measured after ultracentrifugation at 45,000g.

Data analysis. Data from the FSIGTT were submitted to computer programs that calculate the characteristic metabolic parameters by fitting glucose and insulin to the minimal model that describes the time course of glucose and insulin concentrations. The glucose disappearance model, by accounting for the effect of insulin and glucose on glucose disappearance, provides the parameter S_1 (glucose sensitivity index) (10⁻⁴ per minute per microunit per milliliter, a measure of the effect of insulin concentrations above the basal level to enhance glucose disappearance) and S_2 (glucose effectiveness) (per minute), defined as the effect of glucose itself, at basal insulin, to promote its own disposal through uptake by mass action into the tissues and through suppression of endogenous glucose production. The estimation of model parameters was performed according to the MINMOD computer program (23).

Statistical methods. Descriptive results of continuous variables are expressed as means \pm SE. Before statistical analysis, normal distribution and homogeneity of the variables were tested. Parameters that did not fulfill these tests were log-transformed. We used the χ^2 test for comparisons of proportions and unpaired *t* tests for comparisons of quantitative variables. Levels of statistical significance were set at $P < 0.05$. Statistical analysis was performed with the BMDP statistical package (BMDP Statistical Software, Cork, Ireland).

RESULTS

The subjects were divided into two groups according to the absence (homozygous for the TNF-1 allele) or presence of the TNF-2 allele. Twenty-three subjects were (+/+) homozygotes for the presence of the *Nco* I restriction site. Twelve of the TNF-2 subjects were heterozygous for TNF-2 (TNF-1/TNF-2), and three were homozygous. Main anthropometric measurements are shown in Table 1. TNF-1 and TNF-2 groups of subjects were comparable in sex, age, BMI, waist-to-hip ratio (WHR), and several anthropometric parameters, including subscapular, biceps, triceps, and abdominal skinfolds (data not shown). There was a slight nonsignificant tendency toward increased central fat in the TNF-2 group. Basal serum insulin was greater in the TNF-2 group in the presence of comparable serum glucose concentration (Table 2). The integrated area under the curve of serum insulin concentrations after OGTT and the percent body fat were significantly increased in TNF-2 subjects (Table 2). TNF-2 subjects also showed a decreased insulin sensitivity index (1.9 ± 0.4 vs. 3.05

TABLE 1
Anthropometric characteristics

	TNF-1	TNF-2	Difference between groups (<i>P</i>)
<i>n</i>	23	15	—
Men/women	12/11	7/8	NS
Age (years)	36.4 ± 1.6	37.1 ± 1.7	NS
Weight (kg)	81.5 ± 4.2	83.5 ± 2.8	NS
BMI (kg/m ²)	29.2 ± 1.1	30.3 ± 1.2	NS
Waist (cm)			
Men	99.8 ± 3.6	97.9 ± 4.1	NS
Women	87.8 ± 4.6	89.8 ± 4.6	NS
Hip (cm)			
Men	98.5 ± 3.5	96.5 ± 3.7	NS
Women	96.5 ± 3.6	94.6 ± 2.7	NS
WHR			
Men	1.01 ± 0.014	1.02 ± 0.016	NS
Women	0.906 ± 0.02	0.949 ± 0.02	0.1
Fat mass (kg)	20.9 ± 2.4	28.2 ± 2.7	0.06
Body fat (%)	24.9 ± 2	33.6 ± 2.8	0.015
Fat free mass (kg)	58.5 ± 2.4	54.8 ± 2.7	NS

Data are means ± SE. Comparison of anthropometric measurements among carriers of TNF-1 or TNF-2 genotypes.

± 0.3 min⁻¹ · mU⁻¹ · l⁻¹, *P* = 0.03). These differences were not mirrored by differences in serum cholesterol (total or HDL and VLDL fractions, and total or VLDL triglycerides). Paralleling the known relationship between insulin and leptin levels, serum leptin concentration was almost 40% greater in the TNF-2 group (Table 2).

DISCUSSION

We have found that percent body fat content, insulin sensitivity, and leptin levels differ among subjects otherwise similar in BMI, total fat content, WHR, and plasma TNF- α levels, depending on the presence of a transition polymorphism G to A in the -308 position of the TNF- α gene. Whether these genetic variants result in modified bioactivity, in different

paracrine effects at the local adipose or muscle tissues, or in differences in the production of the protein or message RNA level is unknown. In Raji cells, in vitro TNF-2 allele causes a six- to sevenfold increase in constitutive and PMA-induced transcription rate compared with the wild-type TNF-1 (13). Because the rate of transcription is an important regulator of TNF- α expression, genetic variations within regulatory regions of the TNF- α gene could contribute to altered expression of the cytokine in adipose cells.

In a recent study of Pima Indians, a marker (TNF α 24) located 10 kb from TNF- α was linked to percent body fat, but not to BMI or body fatness (16). The lack of correlation between TNF- α expression and WHRs suggests that the former is not tightly linked to differences in regional fat deposition (3). Among French-Canadians, a recessive allele was proposed to contribute to higher fat mass and percent body fat (24). In our study, subjects with TNF-1 and TNF-2 alleles were comparable in BMI, WHRs, and several anthropometric parameters. A nonsignificant tendency toward increased central fat (mainly among women) was observed in TNF-2 carriers, although a better measurement of intra-abdominal fat would be needed. However, differences in percent body fat were detected. In a recent population-based study in our area, WHRs were found to be significantly higher than those previously reported in other populations (25). One of the possible shortcomings of this study is the small number of homozygotes for the TNF-2 allele. Whether the differences in percent body fat are causally related to the TNF-2 allele awaits further study in a greater sample of subjects. The frequency of the TNF-2 allele (0.16) (12) is similar to that found among 155 healthy control subjects in our population (0.13) (C.G., J.M.F.-R., J.V., unpublished observations). The existence of such a marker should enhance the ability to quickly distinguish these patients for both diagnostic and therapeutic purposes. This factor should be taken into account during the design of clinical trials aiming to interfere with TNF action.

Obesity is associated with insulin resistance and hyperinsulinemia. The increased expression of TNF- α in muscle and adipose tissues of obese subjects induces insulin resistance through its ability to produce serine phosphorylation of

TABLE 2
Comparison between TNF-1 and TNF-2 polymorphisms

	TNF-1	TNF-2	Difference between groups (<i>P</i>)
<i>n</i>	23	15	—
Fasting glucose (mmol/l)	5.45 ± 0.19	5.28 ± 0.18	NS
Fasting insulin (mU/l)	9.2 ± 0.9	14.2 ± 2	0.041
Fasting leptin (ng/ml)	11.1 ± 1.5	19.6 ± 3.4	0.038
Plasma TNF- α (pg/ml)	16.4 ± 2.3	14.2 ± 2.8	NS
Total cholesterol (mmol/l)	5.1 ± 0.21	5.08 ± 0.21	NS
HDL cholesterol (mmol/l)	1.17 ± 0.07	1.12 ± 0.06	NS
VLDL cholesterol (mmol/l)	0.39 ± 0.07	0.35 ± 0.06	NS
Total TAG (mmol/l)	1.3 ± 0.15	1.4 ± 0.21	NS
Glucose area (mmol/l)	18 ± 1.16	17.8 ± 1.4	NS
Insulin area (mU/l)	139.4 ± 17.8	226.8 ± 33	0.032
<i>S</i> ₁ (min ⁻¹ · mU ⁻¹ · l ⁻¹)	3.05 ± 0.39	1.9 ± 0.4	0.031
<i>S</i> _G (min ⁻¹)	0.018 ± 0.002	0.016 ± 0.001	NS

Data are means ± SE. Comparison of biochemical, hormonal, and metabolism parameters among subject carriers of TNF-1 or TNF-2 genotypes. Insulin area, integrated area under the curve of serum insulin concentrations, measured in response to a 75-g oral glucose challenge; *S*_G, glucose effectiveness index; *S*₁, insulin sensitivity index; TAG, total serum triglycerides.

insulin receptor substrate 1, decreasing the tyrosine kinase activity of the insulin receptor (26). TNF- α expression in obesity may be envisioned as a mechanism to limit further fat cell enlargement through the induction of adipose tissue insulin resistance (27). This insulin resistance could have evolved as a tool to prevent excess adiposity through changes in lipoprotein lipase (LPL). A high adipose-to-muscle LPL ratio, as observed during simple hyperinsulinemia, leads to partitioning of lipid into adipose tissue (28). Progressive insulin resistance would be expected to reverse the high adipose-to-muscle LPL ratio and would tend to prevent further adipose tissue expansion (2). It is tempting to speculate that locally active TNF- α is expressed in subjects with TNF-2 allele, which induce insulin resistance, hyperinsulinemia, and higher adipose LPL activity, resulting in greater percent body fat. Conversely, increased percent body fat may result in a greater production of TNF- α that leads to insulin resistance. Whether percent body fat causes, or is the consequence of, differences in TNF expression has been rarely studied (2). In general, TNF expression is variably increased with increased adiposity and is decreased with decreased adiposity (2). Mutations within regulatory elements of the TNF- α gene were not associated with an increase in the prevalence of NIDDM in one study (29). However, insulin resistance was not evaluated.

The lack of significant differences in serum cholesterol (total or HDL and VLDL fractions) or total and VLDL triglycerides, despite different insulin sensitivity, may be attributable to the selection of subjects. By excluding diabetic subjects, we may have excluded subjects that had the most vigorous insulin resistance, mirrored in the lipid levels.

Differences in percent body fat and insulin sensitivity, despite similar plasma TNF- α levels, do not exclude different TNF- α expression. TNF- α is not an endocrine factor produced by adipose tissue and targeted at muscle (30). TNF- α probably interacts with the insulin-signaling pathway in a relatively inaccessible microenvironment, or even intracellularly. Further studies on bioactivity and availability of local TNF production are required.

Interestingly, since the initial submission of this manuscript, Hotamisligil et al. (31) have found twofold more TNF receptor 2 (TNFR-2) mRNA in fat tissue and sixfold more soluble TNFR-2 in circulation in obese premenopausal females relative to lean control subjects (31).

We have found differences in leptin levels between TNF-1 and TNF-2 alleles. Plasma leptin levels are elevated in most overweight individuals in parallel with increased plasma insulin levels (8). Insulin is a potent modulator of *ob* gene expression in differentiated adipose cells (32), and this expression is regulated by changes in the physiological state (i.e., fasting/feeding and diabetes) in which the concentration of circulating insulin is altered in vivo (33). Insulin resistance is an independent predictor of serum leptin levels (9). Again, differences in leptin levels may be simply the consequence of increased percent body fat or insulin resistance or both, which in turn may be induced by differences in local TNF- α production. Furthermore, the increased leptin levels normally found in human obesity would partially be explained by the increased expression of TNF- α if it regulates the expression of leptin.

In summary, the TNF-2 allele may contribute to differences in leptin levels through increased percent body fat and insulin sensitivity.

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