Tumor Necrosis Factor α −238 and −308 Polymorphisms Do Not Associate With Insulin Resistance in Hypertensive Subjects

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It is well established that, as a group, patients with essential hypertension are characterized by insulin resistance. Previous studies have shown that a biallelic polymorphism in the tumor necrosis factor (TNF)α promoter position –308 and –238 might be involved in the insulin resistance state in diabetic and/or nondiabetic subjects. We determined these polymorphisms in 235 nondiabetic hypertensive subjects and 246 unrelated normotensive controls. Fasting plasma glucose, insulin, lipoprotein, leptin, and TNFα concentrations were measured, in addition to plasma glucose and insulin responses to a 75-g oral glucose tolerance test (OGTT). Insulin sensitivity was also determined by an insulin suppression test in 69 hypertensive and 76 normotensive individuals. The results showed no association of these genotypic distributions between hypertensive and normotensive individuals both at –308 (GG, GA, and AA were 80.9%, 17.9%, and 1.3% in hypertensives, 84.2%, 15.4%, and 0.4% in normotensives, $\chi^2 = 1.68$, $P = .432$) and at –238 (GG, GA, and AA were 98.3%, 1.7%, and 0% in hypertensives, 96.7%, 3.3%, and 0% in normotensives, $\chi^2 = 1.19$, $P = .276$) sites. These results did not change even after adjustment for values of age and body mass index (BMI). Anthropometric measurements, fasting plasma glucose, insulin, lipoprotein concentrations, glucose, and insulin responses to OGTT, TNFα, and leptin concentrations were similar between the genotype at the –308 site both in hypertensive and normotensive groups. Insulin sensitivity, either measured by an insulin suppression test or homeostasis model assessment (HOMA) index, did not differ between the genotype at the –308 site in subjects with hypertension or normotension. Fasting plasma TNFα (10.2 ± 0.5 pg/mL *v* 10.1 ± 0.5 pg/mL, $P = .928$) concentrations did not differ between hypertensive and normotensive subjects even after adjustment for body fat and BMI values. We conclude that TNFα promoter gene polymorphisms at position −238 and −308 do not play a major role in the pathogenesis of insulin resistance in Chinese subjects with or without hypertension.

TUMOR NECROSIS FACTOR (TNF)α is a cytokine, which was originally identified as the principal agent causing tumor necrosis in bacterially infected animals.[1] Later, it was established that TNFα, synthesized and secreted from adipose tissue,[2,3] plays a key role in the pathogenesis of obesity and the insulin-resistant state. TNFα can cause insulin resistance in vitro[4,5] and in vivo.[6-8] In addition, infusion of TNFα receptor fusion protein can neutralize endogenous TNFα and significantly improve peripheral insulin action in rats.[3] In human studies, adipose tissue TNFα mRNA levels show significant correlations with percent body fat, body mass index (BMI), and the level of hyperinsulinemia.[9-11] A recent study also demonstrated that circulating TNFα levels correlate well with the degree of insulin resistance in a group of newly diagnosed type 2 diabetic men.[12]

Previous studies have identified 2 biallelic polymorphisms in the TNFα promoter region, which is considered to play an important pathogenic role.[13-15] The first is at position −308, which involves the substitution of guanine (G) by adenine (A).[13] The second polymorphism is a G→A substitution at position –238, which was reported to associate with decreased insulin resistance among family members who carried this polymorphism.[14] Subsequent studies on the relationship between these polymorphisms and insulin resistance have shown inconsistent results.[15-18] Fernandez-Real et al[15] showed that this polymorphism at –308 influenced insulin sensitivity through increased body fat in a group of nondiabetic normotensive Spanish subjects. On the contrary, a difference was detected between polymorphism and insulin resistance at –238, but not –308 sites in another Caucasian population consisting of nondiabetic relatives of type 2 diabetics and control subjects with no family history of diabetes.[14] A recent study on type 2 diabetes mellitus patients showed no difference in the frequencies of allele at −308 compared with healthy control subjects.[16] Very recently, Walston et al[18] reported that TNFα polymorphism at –308 and –238 sites did not relate to any traits of obesity and insulin resistance in a group of nondiabetic subjects.

More recently, it has been proposed that hypertension is part of a cluster of metabolic risk factors (a metabolic syndrome X) with insulin resistance or hyperinsulinemia as the common link.[19] Although all of these metabolic abnormalities are connected to the effect of TNFα, little is known about the role of the TNFα gene in regulating the insulin resistance syndrome in hypertensive patients. The purpose of this study, therefore, was to examine the relationship between the TNFα promoter polymorphism and insulin resistance in nondiabetic hypertensive subjects and normotensive controls.

MATERIALS AND METHODS

Study Subjects

We recruited 235 nondiabetic subjects with essential hypertension and 246 nondiabetic normotensive controls. Hypertensive patients were

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enrolled based on the criteria of systolic blood pressure ≥160 mm Hg and/or diastolic blood pressure ≥ 95 mm Hg on 2 consecutive measurements by Dinamap (Critikon model 8270, Tampa, FL) or on antihypertensive therapy. There was no apparent clinical evidence for secondary hypertension in any of the patients. Plasma creatinine and serum potassium were normal in all subjects. A total of 212 of the 235 (90.2%) hypertensive subjects were receiving antihypertensive medications. Antihypertensive agents such as diuretics, β-blockers, angiotensin-converting enzyme inhibitors, α-blockers, and calcium blockers were used in 54, 97, 89, 28 and 92 (not exclusively) of 235 hypertensive subjects, respectively. Normotensive healthy controls were enrolled from subjects who came for annual physical examinations. None of the study subjects were taking any drugs known to affect lipid or carbohydrate metabolism. None of the control subjects had first-degree relatives affected with diabetes. The protocol was approved by the Human Subjects Committee of Taichung Veterans General Hospital. Written informed consent was obtained from each subject before enrollment in this study.

Metabolic Tests

After an overnight fasting, blood was obtained from all subjects for determination of plasma glucose[20] and insulin[21] concentrations. Basal insulin resistance was determined using fasting insulin and glucose concentrations by homeostasis model assessment (HOMA).[22] A 75-g oral glucose tolerance test (OGTT) was performed to determine plasma glucose and insulin responses at baseline, 30, 60, 90, and 120 minutes in 177 (75.3%) hypertensive and 202 (82.1%) normotensive individuals. In addition, a modified insulin suppression test[23] was performed in 69 (29.4%) hypertensive and 76 (30.9%) normotensive subjects. Briefly, intravenous catheters were placed in both arms of the subjects after an overnight fast. Blood was sampled from 1 arm for the determination of plasma glucose and insulin concentrations, and the contralateral arm was used for the administration of test substances. Somatostatin was infused at 350 μg/h to suppress endogenous insulin secretion while insulin (25 mU/m^2/min) and glucose (240 mg/m^2/min) were administered simultaneously. Blood was sampled hourly until 2 hours into the study, and then every 10 minutes at 150, 160, 170, and 180 minutes. Insulin concentrations typically plateau after 30 minutes, whereas glucose concentrations plateau after 120 minutes. The values of individual glucose and insulin concentrations obtained from 150 to 180 minutes were averaged and considered to represent the steady-state plasma glucose (SSPG) and insulin (SSPI) concentrations. Because SSPI concentrations were similar in all subjects, the SSPG concentration provided a measurement of insulin-mediated glucose disposal, ie, the higher the SSPG concentration, the more insulin-resistant the person.

Fasting blood was drawn for measurements of plasma cholesterol[24] and triglyceride[25] concentrations. In addition, high-density lipoprotein (HDL) cholesterol level was determined in the supernatant of plasma after magnesium chloride-phosphotungstic precipitation of apolipoprotein B-containing lipoproteins.[26] The low-density lipoprotein (LDL) cholesterol concentration was estimated by the formula of Friedewald et al.[27]

The fasting plasma leptin concentration was measured by a commercial radioimmunoassay (Linco Research, St Louis, MO).[28] The intraassay coefficient of variation was 3.4% and the interassay coefficient of variation was 5.6%. Plasma concentrations of TNFα were determined using commercially available chemiluminescent enzyme immunometric assay kits (IMMULITE, EURO/Diagnostic Products, Gwynedd, UK) according to the manufacturer's instructions. IMMULITE TNFα is a solid-phase, 2-site chemiluminescent immunometric assay. The solid phase, a polystyrene bead, is coated with a monoclonal antibody specific for TNFα. Briefly, 100 μL plasma and alkaline phosphastase-conjugated polyclonal anti-TNFα antibody were incubated at 37°C for 60 minutes with intermittent agitation. TNFα in the plasma was bound to form an antibody sandwich complex. Unbound conjugate was removed by a centrifugal wash. The chemiluminescent substrate, which was added and incubated for another 10 minutes underwent hydrolysis to yield an unstable intermediate and sustained emission of light. The bound complex and thus also the photon output was measured by luminometer and was found to be proportional to the concentration of TNFα in the plasma. The assay range for plasma samples was 1.7 pg/mL to 1,000 pg/mL. The intra- and interassay variances were 3.5% and 6.5%, respectively.

Body fat was measured using the bioelectrical impedance method (Model BIA-101, RJL System, Detroit, MI).[29] The data were analyzed by an established program, and the percentage of fat was calculated.

Analysis of TNFα Polymorphisms

Both TNFα polymorphisms (−238 and −308) were detected as previously described[14] using primers containing a single base pair mismatch adjacent to the polymorphic site to create a restriction site into the wild-type nucleotide sequences after amplification. Briefly, 100 ng of the extracted DNA was used as a template. The reaction was performed in a final volume of 50 μL containing 3 mmol/L $MgCl_2$, 0.2 mmol of each deoxynucleoside triphosphate (dNTP) (Promega, Madison, WI), 20 pmol of each primer, and 2.5 U of Taq DNA polymerase (Promega). DNA was amplified during 35 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 59°C, and a 45-second extension at 72°C. Before that, 1 cycle of a 5-minute denaturation at 94°C, 1 minute annealing at 59°C, and a 45-second extension at 72°C was performed. After completion of all cycles, a final cycle of a 1-minute denaturation at 94°C, 1 minute annealing at 59°C, and a 10-minute extension at 72°C was included. Polymerase chain reaction (PCR) products were digested at 37°C for 4 hours with *Msp* I and *Nco* I restriction enzymes (New England Biolabs, Beverly, MA) and electrophoresed on a 3% agarose gel to detect the −238 and −308 polymorphisms, respectively. *Msp* I restriction fragment length polymorphism that produced 3 bands of different sizes: a 152-bp fragment (A, restriction site absent) and a set of 133-bp and 19-bp fragments (G, restriction site present). *Nco* I restriction fragment length polymorphism that produced 3 bands of different sizes: a 222-bp fragment (A, restriction site absent) and a set of 202-bp and 20-bp fragments (G, restriction site present).

Statistical Analysis

Results are expressed as mean ± SEM. The χ^2 test was used to analyze genotypic distribution and allele frequencies. Odds ratio (OR) was calculated, using age and BMI as covariate, in a logistic regression analysis. We present ORs for GA and AA genotypes, with GG genotype as the reference. Unpaired *t* test was used to compare differences in anthropoemetric and biochemical variables between genotypes at position –308 only, because very few subjects carried polymorphism at the –238 site. Multiple regression analysis was performed to adjust for effect of age, BMI, and body fat on several metabolic variables. Glucose and insulin areas were defined as the areas under the glucose and insulin curves, respectively (0, 1, and 2 hours). Values of plasma TNFα, leptin, and HOMA index were logarithm-transformed as required to improve normality. All statistical analyses were conducted using a Macintosh computer with the Statview 4.0 Statistical Package (Statview 4.0, SAS Institute, Cary, NC).

RESULTS

Genotypes and allele frequencies of −308 and −238 A/G polymorphisms of TNFα promoter region are shown in Tables 1 and 2. None of these distributions of genotypes deviated from Hardy-Weinberg equilibrium. There were no differences in genotypes or allele frequencies between hypertensive and nor-

Table 1. Genotype and Allele Frequencies of TNFα Promoter at −308 Between Hypertensive Patients and Normotensive Controls

Genotypes/Allele	Hypertensives (%)	Normotensives (%)	OR (95% CI)*
No.	235	246	
Genotype			
GG	190 (80.9)	207 (4.2)	1.00
GA	42 (17.9)	38 (15.4)	1.04 (0.94-1.16)
AA	3 (1.3)	1 (0.4)	1.26 (0.81-1.96)
	$\chi^2 = 1.68$	$df = 2$, $P = .432$	
G	422 (89.8)	452 (91.9)	
A	48 (10.2)	40 (8.1)	
	$\chi^2 = 1.25$	$df = 1$, $P = .263$	

Abbreviation: CI, confidence interval.

*OR: Odds ratio with GG as reference group, adjusted for age and BMI, using logistic regression analysis.

motensive groups. When GG was considered as a reference, the age, BMI-adjusted ORs for GA and AA was 1.04 (95% confidence interval [CI], 0.94 to 1.16) and 1.26 (95% CI, 0.81 to 1.96), respectively at position −308 (Table 1). When GG was considered as a reference, the age, BMI-adjusted OR for GA was 0.83 (95% CI, 0.65 to 1.07) at position −238 (Table 2).

The anthropometric characteristics and biochemical findings of hypertensive patients according to TNFα genotypes at the −308 site are shown in Table 3. Mean age, sex distribution, BMI, amount of body fat, mean blood pressure, fasting plasma glucose levels, insulin levels and lipids values, leptin levels, and TNFα levels did not differ between those with or without the *Nco* I restriction site. Plasma glucose and insulin responses to a 75-g oral glucose challenge, as well as the degree of insulin resistance, as assessed by HOMA index, showed no difference between subjects with GG and subjects with a combination of GA plus AA. Another independent method used to evaluate insulin resistance in a subgroup of study individuals showed that SSPG concentrations were not significantly different between subjects with and without cutting site.

The anthropometric and biochemical variables of normotensive subjects according to TNFα genotypes at position –308 are shown in Table 4. There were no differences in sex distribution, BMI values, amount of body fat, mean blood pressure, fasting plasma glucose levels, insulin levels, lipids concentrations,

Table 2. Genotype and Allele Frequenccies of TNFα Promoter at −238 Between Hypertensive Patients and Normotensive Controls

Genotypes/Allele	Hypertensives (%)	Normotensives (%)	OR (95% CI)*
No.	235	246	
Genotype			
GG	231 (98.3)	238 (96.7)	1.00
GA	4 (1.7)	8 (3.3)	0.83 (0.65-1.07)
AA	0	0	
	$\chi^2 = 1.19$	$df = 2$, $P = .276$	
Allele			
G	466 (99.1)	484 (98.4)	
A	4 (0.9)	8 (1.6)	
	$\chi^2 = 1.17$	$df = 1$, $P = .279$	

Abbreviation: CI, confidence interval.

*OR: Odds ratio with GG as reference group, adjusted for age and BMI, using logistic regression analysis.

Table 3. Anthropometric and Metabolic Characteristics (mean ± SEM) of Hypertensive Patients According to Genotype of TNFα Promoter at −308

	GG	GA and AA	*P*
No.	190	45	
Male/female	104/86	24/21	.865
Age (yr)	56 ± 1	53 ± 1	.101
BMI (kg/m^2)	26.7 ± 0.3	26.8 ± 0.5	.848
Body fat (kg)	14.8 ± 0.5	13.0 ± 1.0	.135
Mean BP (mm Hg)	125 ± 5	122 ± 3	.766
Fasting plasma			
Glucose (mg/dL)	111 ± 3	113 ± 5	.755
Insulin (μU/mL)	11 ± 1	11 ± 1	.966
Triglyceride (mg/dL)	177 ± 9	165 ± 12	.569
Cholesterol (mg/dL)	183 ± 3	174 ± 7	.145
LDL-C (mg/dL)	103 ± 3	95 ± 6	.194
HDL-C (mg/dL)	45 ± 1	46 ± 2	.467
Leptin (ng/mL)	11.5 ± 1.1	17.0 ± 4.3	.078
TNFα (pg/mL)	10.1 ± 0.5	11.2 ± 1.5	.513
Glucose response*			
(mg · dL^{-1} · h^{-1})	363 ± 8	357 ± 7	.750
Insulin response*			
(μU · dL^{-1} · h^{-1})	153 ± 7	166 ± 9	.493
HOMA index	3.2 ± 0.2	3.3 ± 0.5	.850
SSPG (mg/dL)†	212 ± 11	182 ± 31	.332
SSPI (μU/mL)†	45 ± 2	46 ± 4	.778

Abbreviations: SSPG, steady state plasma glucose concentrations; SSPI, steady state plasma insulin concentrations.

*N = 147 in GG and 30 in GA and AA genotypes.

†N = 59 in GG and 10 in GA and AA genotypes.

leptin levels, and TNFα levels between those with or without the *Nco* I restriction site except that younger age was found in the GA and AA group. Plasma glucose and insulin responses to OGTT, as well as the degree of insulin resistance, as measured by HOMA index, showed no difference in subjects with or without A alleles. SSPG concentrations in a subgroup of study individuals were not different between those with and without cutting site. The anthropometric and biochemical variables at position –238 were not analyzed, because very few subjects carried polymorphism at this site.

After adjusting for age and BMI, patients with hypertension had higher mean blood pressure, fasting plasma glucose levels, insulin levels, triglyceride levels, and LDL cholesterol concentrations, as well as lower HDL cholesterol concentrations, greater glucose and insulin responses to OGTT, and higher SSPG concentrations when compared with control subjects (data not shown). Fasting plasma TNFα (10.2 ± 0.5 pg/mL *v* 10.1 ± 0.5 pg/mL, $P = .928$) levels were similar between hypertensive and normotensive individuals. After adjusting for BMI and body fat amount, there were no differences in fasting plasma TNFα or leptin levels between hypertensive patients and normotensive controls.

DISCUSSION

It is well established that, as a group, patients with essential hypertension are characterized by insulin resistance syndrome, namely hyperinsulinemia, hypertriglyceridemia, and low HDL cholesterol concentrations.[19,30] Although the cause and mech-

Table 4. Anthropometric and Metabolic Characteristics (mean ± SEM) of Normotensive Subjects According to Genotype of TNFα Promoter at −308

	GG	GA and AA	*P*
No.	207	39	
Male/female	96/111	20/19	.574
Age (yr)	47 ± 1	42 ± 1	.019
BMI (kg/m^2)	23.9 ± 0.2	23.9 ± 0.5	.977
Body fat (kg)	12.3 ± 0.4	11.6 ± 0.9	.522
Mean BP (mm Hg)	102 ± 1	98 ± 2	.149
Fasting plasma			
Glucose (mg/dL)	98 ± 2	100 ± 5	.631
Insulin (μU/mL)	9 ± 0	8 ± 1	.736
Triglyceride (mg/dL)	139 ± 7	155 ± 21	.445
Cholesterol (mg/dL)	170 ± 2	174 ± 6	.583
LDL-C (mg/dL)	93 ± 2	95 ± 6	.692
HDL-C (mg/dL)	49 ± 1	47 ± 2	.419
Leptin (ng/mL)	8.3 ± 0.7	7.2 ± 0.9	.548
TNFα (pg/mL)	10.4 ± 0.5	8.7 ± 0.6	.195
Glucose response*			
(mg · dL^{-1} · h^{-1})	319 ± 6	288 ± 7	.132
Insulin response*			
(μU · dL^{-1} · h^{-1})	121 ± 6	123 ± 14	.881
HOMA index	2.1 ± 0.1	2.0 ± 0.2	.635
SSPG (mg/dL)†	164 ± 10	144 ± 22	.466
SSPI (μU/mL)†	42 ± 1	46 ± 4	.278

Abbreviations: SSPG, steady state plasma glucose concentrations; SSPI, steady state plasma insulin concentrations.

*N = 166 in GG and 36 in GA and AA genotypes.

†N = 65 in GG and 11 in GA and AA genotypes.

anisms of insulin resistance and hyperinsulinemia in the development of hypertension remain speculative, genetic factors are assumed to play a certain role.[30,31] Recent interest has focused on the role of the TNFα gene in association with the insulin-resistant state. Norman et al[32] found that a marker located 10 kb from TNFα was linked to percent body fat, but not to BMI or body fatness in Pima Indians. Wilson et al[13] discovered a biallelic polymorphism in the TNFα promoter position −308, which involves the substitution of guanine by adenine. Very recently, it was demonstrated that this TNFα promoter polymorphism may contribute to differences in leptin levels through increased percent body fat and insulin sensitivity in a group of 38 Spanish nondiabetic subjects.[15] However, a subsequent report showed that the TNFα promoter gene at −238, but not −308, related to insulin resistance in nondiabetic relatives of type 2 diabetes family and control subjects.[14] In a group of 424 nondiabetic subjects, Walston et al[18] found that neither TNFα at –238 nor –308 sites were associated with phenotypic traits of obesity and insulin resistance. Very recently, Koch et al[33] reported that these 2 polymorphisms were not associated with insulin sensitivity and insulin secretion in young healthy relatives of type 2 diabetic patients. To the best of our knowledge, this is the first study to investigate the association between TNFα promoter gene and insulin resistance in hypertensive patients. Our results demonstrated that the genotypic distribution and allele frequencies of the TNFα promoter region at positions −238 and −308 were not different between hypertensive patients and normotensive controls. These results did not change even after adjustment for values of age and BMI. In addition, metabolic variables, including degree of insulin resistance and plasma leptin levels, were not different between genotypes at the −308 site either in hypertensive or normotensive individuals. In accord with our findings, 2 independent groups from Japanese populations showed that polymorphisms at positions −308 and −238 of the TNFα promoter region did not relate to the insulin resistance syndrome.[34,35]

The allele frequencies of mutant type obtained in this study were lower than those reported in Caucasians[14-16,18,33] and in Chinese type 1 diabetics and nondiabetic controls,[17] but higher than those in Japanese subjects.[34,35] The reason for these discrepancies between studies is not clear, but 4 possibilities can be considered. First, there may be differences in the study design and case selections. However, we had adjusted our results by age and BMI carefully. Second, there may be differences in the methods of measuring insulin sensitivity. We used 2 independent methods, HOMA index and insulin suppression test, and obtained similar results. A third possibility is a racial difference, ie, difference in genetic and environmental backgrounds. Fourth, the sample size was not sufficient to clarify the effect.

Previous investigations[9-11] have found increased TNFα expression in adipose and muscle tissues in human obesity. TNFα inhibits insulin-mediated glucose in adipocytes by decreasing phosphorylation of the insulin receptor.[4,36] In fact, circulating TNFα levels were found to have good correlation with the degree of insulin sensitivity in a group of newly diagnosed type 2 diabetic subjects.[12] Pfeiffer et al[37] also showed that plasma TNFα levels are elevated in men, but not in women with type 2 diabetes mellitus. On the other hand, it was reported that the circulating TNFα level did not relate to insulin sensitivity in offspring of type 2 diabetic patients.[38] These divergent results could be due to previous TNFα assays that were less sensitive[12] or to the paracrine fashion of TNFα's action.[39] In addition, circulating TNFα has been reported be associated with a soluble receptor that may inhibit its biologic activity,[40] suggesting that TNFα action is primarily a local one. The current findings are inconsistent with our previous observation[41] that circulating TNFα concentrations did not differ between hypertensive and normotensive subjects despite the fact that the former group is more insulin resistant.

We conclude that the TNFα promoter gene polymorphism at positions −238 and −308 does not likely play a major role in the development of hypertension in the Chinese population. In addition, it appears to have no association with the insulin resistance syndrome either in hypertensive patients or normotensive subjects.

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