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Circulating Tumor Necrosis Factor-α Concentrations in a Native Canadian Population with High Rates of Type 2 Diabetes Mellitus*

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ABSTRACT

Recent research suggests that tumor necrosis factor-α (TNFα) may play an important role in obesity-associated insulin resistance and diabetes. We studied the relationship between TNFα and the anthropometric and physiological variables associated with insulin resistance and diabetes in an isolated Native Canadian population with very high rates of type 2 diabetes mellitus (DM).

A stratified random sample (n = 80) of participants was selected from a population-based survey designed to determine the prevalence of type 2 DM and its associated risk factors. Fasting blood samples for glucose, insulin, triglyceride, leptin, and TNFα were collected; a 75-g oral glucose tolerance test was administered, and a second blood sample was drawn after 120 min. Insulin resistance was estimated using the homeostasis assessment (HOMA) model. Systolic and diastolic blood pressure (BP), height, weight, and waist and hip circumferences were determined, and percent body fat was estimated using biological impedance analysis. The relationship between circulating concentrations of TNFα and the other variables was assessed using Spearman correlation coefficients, analysis of covariance, and multiple linear regression.

The mean TNFα concentration was 5.6 pg/mL (SD = 2.18) and ranged from 2.0–12.9 pg/mL, with no difference between men and women ($P = 0.67$). There were moderate, but statistically significant, correlations between TNFα and fasting insulin, HOMA insulin resistance (HOMA IR) waist circumference, fasting triglyceride, and systolic BP (r = 0.23–0.34; all $P < 0.05$); in all cases, coefficients for females were stronger than those for males. Individuals with normal glucose tolerance had lower log TNFα concentrations than those with impaired glucose tolerance or type 2 DM (both $P = 0.03$, adjusted for age and sex), although differences were not significant after adjustment for HOMA IR (both $P > 0.25$). Regression analysis indicated that log HOMA IR and log systolic BP were significant independent contributors to variations in log TNFα concentration (model $r^2 = 0.32$). We conclude that in this homogeneous Native Canadian population, circulating TNFα concentrations are positively correlated with insulin resistance across a spectrum of glucose tolerance. The data suggest a possible role for TNFα in the pathophysiology of insulin resistance. (*J Clin Endocrinol Metab* **84:** 272–278, 1999)

INSULIN resistance appears to be an important component in the pathophysiology of a number of chronic diseases, including type 2 diabetes mellitus (DM) (1, 2), hypertension (3, 4), cardiovascular disease (3, 4), and hyperlipidemia (3, 4). The etiology of insulin resistance (IR) is unclear, although it appears that both genetic and environmental factors contribute to its development (3–5).

Recent research suggests that tumor necrosis factor-α (TNFα), an inflammatory cytokine produced mainly by monocytes and macrophages, may play an important role in obesity-associated IR and diabetes (6). *In vitro* studies have shown that TNFα reduces transcriptional activity of the GLUT4 gene (7) and increases IR by inhibition of insulin receptor tyrosine kinase activity in muscle and fat tissue (8, 9). The latter process possibly occurs via stimulation of the p55 TNF receptor (TNFR) and sphingomyelinase activity (10–12). In humans, adipose tissue TNFα messenger ribonucleic acid (mRNA) levels are correlated positively with percent body fat and body mass index (BMI), and inversely with lipoprotein lipase activity (13, 14). The elevated TNFα mRNA levels in obese subjects are decreased by weight loss, which supports a putative role in the induction of IR (14).

Several attempts have been made to neutralize TNFα *in vivo*. Hotamisligil *et al.* (8, 15) administered a recombinant soluble TNFα receptor-IgG chimeric protein to Zucker fatty rats and reported improvements in insulin sensitivity as well as insulin, glucose, and fatty acid levels. In contrast, Ofei and co-workers (16) used recombinant TNFα-neutralizing antibody and reported no effect on IR in obese subjects with noninsulin-dependent diabetes mellitus. A recently published report demonstrated that targeted disruption of the TNFα gene in mice resulted in reduced body adiposity and triglyceride levels in −/− nonobese animals, but the absence of TNFα was "not sufficient to substantially protect against insulin resistance" (17). These apparently contradictory findings have led to controversy about the role of TNFα in the IR syndrome.

There have been few studies of TNFα in the clinical setting, and none that we are aware of in the context of population-

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based epidemiological studies. It may be particularly informative to compare various populations with differing rates of diabetes and obesity. We studied the relationship between TNFα and the anthropometric and physiological variables associated with IR and diabetes in an isolated Native Canadian population with very high rates of type 2 DM.

Subjects and Methods

Study population

The community of Sandy Lake, Ontario, Canada, accessible only by air for most of the year, comprises approximately 1600 people and is located roughly 2000 km northwest of Toronto. Historically, the inhabitants of this area lived in small nomadic groups and were physically active; their diet was high in protein from wild meat and fish, with seasonal supplementation with berries and roots. Their lifestyle has changed dramatically over the past several decades, with a marked decrease in physical activity and an alteration in diet to one characterized by excess consumption of saturated fat and processed foods (18). This population is consequently undergoing an epidemiological transition (19), with a marked increase in morbidity related to chronic diseases, such as obesity and type 2 DM (20–22).

The methodology of the Sandy Lake Health and Diabetes Project (SLHDP) has been described in detail in previous publications (23–25). From July 1993 to March 1995, 728 of 1018 (72%) eligible residents of Sandy Lake, aged 10–79 yr, volunteered to participate in a cross-sectional survey to determine the prevalence of type 2 DM and its associated risk factors. Signed informed consent was obtained from all participants, and the study was approved by the Sandy Lake First Nation Band Council and University of Toronto human subjects review committee. The present analysis is based on a stratified random sample (n = 80) of the SLHDP study population. Twenty individuals (10 males and 10 females) were randomly selected from each of four glucose tolerance status (GTS) categories: type 2 DM, impaired glucose tolerance (IGT), obese (BMI, ≥24) with normal glucose tolerance (NGT), and nonobese (BMI, <24) NGT.

Metabolic and biochemical testing

Volunteers provided fasting blood samples for glucose, insulin, lipids, leptin, and TNFα after an 8- to 12-h overnight fast. A 75-g oral glucose tolerance test was administered, and a second blood sample for glucose determination was drawn after 120 min. Individuals were excluded from the oral glucose tolerance test if they had physician-diagnosed diabetes and 1) were currently receiving treatment with insulin or oral hypoglycemic agents or 2) had a fasting blood glucose exceeding 11.1 mmol/L. Diabetes and IGT were diagnosed according to established criteria (26).

Fasting plasma insulin concentrations were determined by RIA. IR was estimated using the homeostasis model assessment (HOMA) approach of Matthews and colleagues (27). Plasma concentrations of total triglyceride were measured using procedures described in the *Lipid Research Clinics Manual of Operations* (28). Glucose level was determined using standard laboratory procedures.

The TNFα concentration was measured using the quantitative sandwich enzyme immunoassay technique (R & D Systems, Minneapolis, MN), which has an interassay coefficient of variation of 7.5–10.4% and a lower limit of detection of 0.5 pg/mL. Measurements were made using specimens that had been stored at −70 C for approximately 3 yr. Although we have no direct information on the stability of TNFα over time at this temperature, it has been suggested that other cytokines show no loss of activity after storage at various temperatures (up to 56 C) for 1 yr (29). In addition, Thavasu and colleagues demonstrated that there was "no effect on TNFα levels after six repeat freeze/thaw cycles" (samples frozen at −40 C) (30).

Assessment of anthropometry, blood pressure (BP), and medication use

Anthropometric measurements were performed without shoes and with the volunteer wearing either undergarments and a hospital gown or light athletic clothing. Each measurement was performed twice, and the average was used in the analysis. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Weight was measured to the nearest 0.1 kg using a hospital balance beam scale. BMI was defined as weight (kilograms)/height (meters)^2. The waist was measured to the nearest 0.5 cm at the minimal circumference between the umbilicus and xiphoid process; the hips were measured to the nearest 0.5 cm at maximum extension of the buttocks. The waist/hip ratio (WHR) was calculated as the ratio of these two circumferences.

The percent body fat was estimated by bioelectrical impedance analysis using the Tanita TBF-201 Body Fat Analyzer (Tanita Corp., Tokyo, Japan). We have documented high reproducibility of percent fat estimates using this machine (intraclass correlation coefficient = 0.99) (31) in a sample from this population. The instrument has been validated against dual energy x-ray absorptiometry in a number of populations (32–34).

BP was measured in the right arm with the volunteer seated and the arm bared. Systolic BP was recorded to the nearest 2 mm Hg at the appearance of the first Korotkoff sound (phase I), and diastolic BP was recorded to the nearest 2 mm Hg at the appearance of the fifth Korotkoff sound (phase V). Two measurements were performed using a hand-held aneriod sphygmomanometer, and the average of the two was used in the analysis.

Current use of medications for diabetes and hypertension was determined from patient medical files and pharmaceutical log books.

Statistical analysis

All statistical analyses were conducted using SAS version 6.09 in the VMS environment (35). Data are presented as means, SDs, medians, and ranges. Relationships between continuous variables were assessed using Spearman correlation coefficients. Distributions of continuous variables were tested for normality, and, if appropriate, the natural log transformations of skewed variables were used in subsequent analyses. *t* tests were employed to assess differences between groups, and analysis of covariance was used to test for differences between groups while adjusting for other factors.

Multiple linear regression models were constructed to examine factors that were associated with variations in TNFα concentration. The log transformation of serum TNFα was used as the dependent variable. Independent variables were included in the initial models according to the strength of their univariate relationships with serum TNFα and their biological importance based on the scientific evidence available at the time the analysis was conducted. Terms for interaction effects between gender and other independent variables were initially added to the full regression model as a global test for interactions. None of these interactions was statistically significant (all $P > 0.05$; data not shown), and thus subsequent modelling was performed with the genders pooled. In addition to the full regression model approach, a backward stepwise elimination procedure was employed to assist in the construction of models that best predicted TNFα concentration in this sample (36).

Results

The mean TNFα concentration was 5.6 pg/mL (SD = 2.18) and ranged from 2.0–12.9 pg/mL. There were no differences between males and females in terms of age, TNFα, fasting insulin, glucose (fasting and 2 h post-challenge), fasting triglycerides, and waist circumference (Table 1). The percent body fat and leptin levels were significantly higher among females ($P < 0.001$), whereas males had significantly elevated WHRs and BP levels (both systolic and diastolic) relative to females ($P < 0.05$; Table 1).

There were moderate, statistically significant correlations between log-normal (ln) TNFα and fasting insulin, HOMA IR, waist circumference, fasting triglyceride, and systolic BP; in all cases, coefficients for females were higher than those for males (Table 2 and Figs. 1 and 2). The associations between TNFα and BMI, WHR, fasting glucose, and 2-h PC glucose were of borderline significance, with higher sex-specific co-

TABLE 1. Metabolic and anthropometric characteristics of a sample of participants from the Sandy Lake Health and Diabetes Project, by gender (n = 80)

Variable	Males (n = 40)			Females (n = 40)			*P* value
	Mean	SD	Range	Mean	SD	Range	
Age (yr)	36.81	15.04	13.49–75.32	34.90	14.52	11.20–63.20	0.564
TNFα	5.58	2.03	3.28–12.79	5.55	2.34	1.96–12.91	0.666
BMI (kg/m^2)	26.77	5.36	16.56–36.47	27.44	5.84	17.17–38.04	0.657
% Body fat (%)	26.82	8.88	4.10–40.90	42.69	10.82	18.30–57.80	<0.001
Waist (cm)	96.99	13.55	72.50–124.75	92.80	12.16	69.00–115.00	0.149
Waist hip ratio	0.95	0.07	0.82–1.09	0.88	0.06	0.75–1.01	<0.001
Fasting glucose (mmol/L)	6.76	2.93	4.60–17.90	7.01	3.87	3.70–21.30	0.743
2-h glucose (mmol/L)	8.27	6.11	1.70–29.60	8.35	5.59	2.7–32.1	0.952
Fasting insulin (pmol/L)	108.70	63.67	30.00–372.00	143.18	87.78	25.00–354.00	0.106
HOMA IR	4.78	3.76	1.00–18.60	6.26	4.58	0.77–19.19	0.198
Triglyceride (mmol/L)	1.78	1.02	0.63–5.04	1.66	0.95	0.63–4.77	0.661
Fasting leptin (ng/L)	7.70	5.76	1.10–27.40	19.71	11.05	3.30–45.30	<0.001
Systolic BP	124.89	13.78	101.0–180.0	115.91	16.59	83.5–170.0	0.005
Diastolic BP	70.61	13.09	42.0–96.0	65.14	9.77	49.0–90.0	0.037
	n	%		n	%		
Diabetes (%)	10	25		10	25		
IGT (%)	10	25		10	25		

Values are means (SD) unless otherwise indicated.

TABLE 2. Spearman correlation coefficients between TNFα and selected metabolic and anthropometric variables among a sample of participants in the Sandy Lake Health and Diabetes Project (n = 80)

	Age	BMI	% Fat	Waist	WHR	Insulin	IR	Triglycerides	Leptin
Both sexes	0.14	0.19	0.12	0.24	0.22	0.32	0.34	0.23	0.17
P	0.2140	0.0887	0.2714	0.0328	0.0486	0.0043	0.0019	0.0366	0.1280
Males	0.13	0.10	0.10	0.19	0.36	0.16	0.26	0.13	0.09
P	0.4413	0.5214	0.5486	0.2408	0.0240	0.3378	0.1087	0.4299	0.5613
Females	0.15	0.24	0.13	0.29	0.15	0.47	0.38	0.38	0.33
P	0.3416	0.1322	0.4223	0.0671	0.3656	0.0021	0.0151	0.0142	0.0394

	Fasting glucose	2-h glucose	Systolic BP	Diastolic BP
Both sexes	0.21	0.21	0.25	0.18
P	0.0600	0.0744	0.0225	0.1095
Males	0.25	0.30	0.03	0.10
P	0.1257	0.0671	0.8713	0.5501
Females	0.18	0.12	0.46	0.28
P	0.2600	0.4865	0.0030	0.0750

BP, Blood pressure.

efficients among males for the latter three variables (Table 2). The relationships between TNFα and age, percent body fat, diastolic BP, and leptin were generally weak, although the positive correlation with leptin among females was notable (Table 2). When individuals with diabetes were excluded from the correlation analysis, coefficients were stronger for all variables among women and weaker for all variables among men (data not shown). With the sexes pooled, correlations were slightly stronger for all factors except fasting and 2-h PC glucose (data not shown).

Individuals with NGT had lower TNFα concentrations than those with IGT or type 2 DM. These differences were statistically significant, both unadjusted as well as adjusted for age and sex. When these comparisons were further controlled for log HOMA IR, however, differences were no longer significant (both $P > 0.25$; Fig. 3).

We used multiple linear regression to examine factors that were associated with variation in ln TNFα concentration. Independent variables that were considered included age, WHR, percent body fat, and diastolic BP; the log transformations of triglyceride concentration, HOMA IR, leptin concentration, and systolic BP; and indicator variables for gender, IGT, diabetes, and use of hypertension medication. Waist circumference and BMI were not included in the same multivariate model due to very strong colinearity between these two variables.

Backward stepwise elimination was used to assist in the construction of a regression model that best predicted the TNFα concentration in this sample. Using an inclusion criterion of $P = 0.05$, log HOMA IR and log systolic BP were determined to be significant independent factors by this procedure (model F = 11.45; $P < 0.0001$; model $r^2 = 0.23$; log HOMA IR: $b = 0.16$, $t = 3.23$, $P = 0.0018$; log systolic BP: $b = 1.03$, $t = 3.44$, $P = 0.0009$). The full linear regression model is presented in Table 3. Log HOMA IR and log systolic BP were independently related to log TNFα; none of the other factors in the model was significantly associated in the presence of other variables (model $r^2 = 0.28$).

Discussion

The role of TNFα in the pathogenesis of IR in humans is controversial. In this study of an isolated Native Canadian

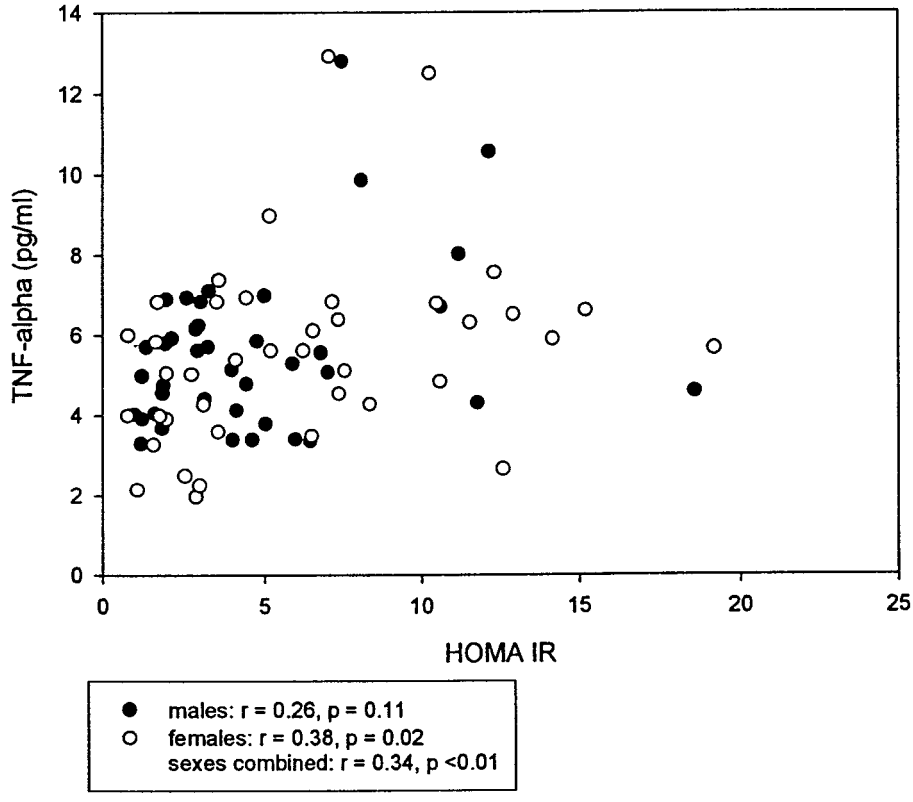

FIG. 1 Correlations between TNFα and HOMA IR in men (●) and women (○).

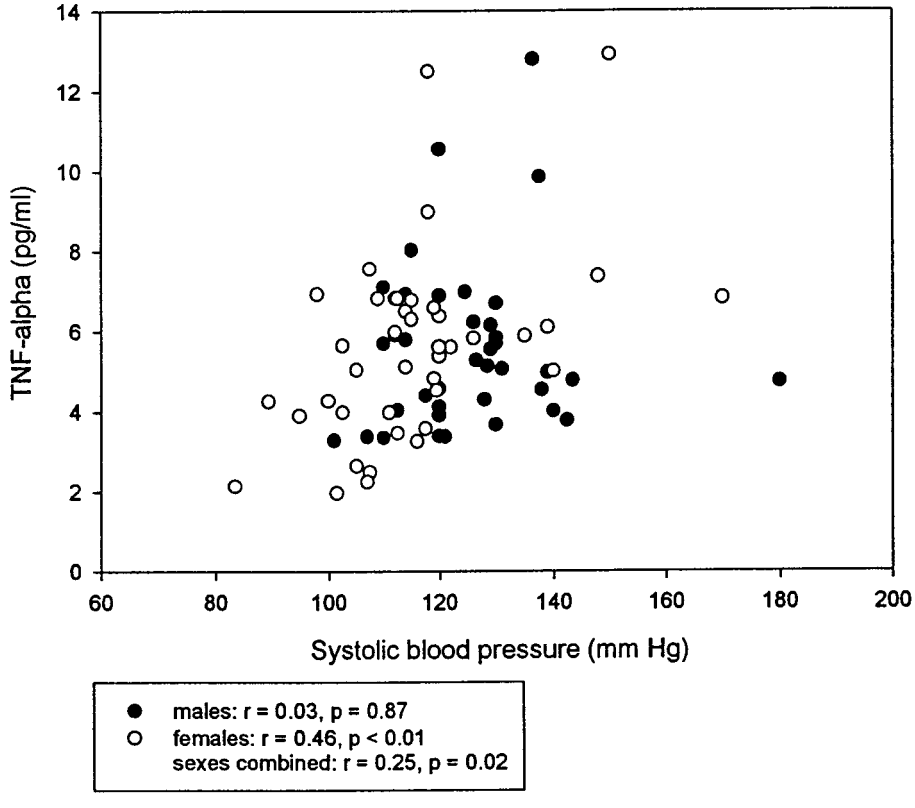

FIG. 2. Correlations between ln TNFα and systolic BP in men (●) and women (○).

community, we found that in subjects with varying degrees of obesity and glucose intolerance there was a significant correlation of circulating TNFα concentrations with fasting insulin as well as with HOMA IR, a measure of IR. TNFα was also significantly correlated with waist circumference, WHR, fasting triglycerides, and systolic blood pressure, parameters

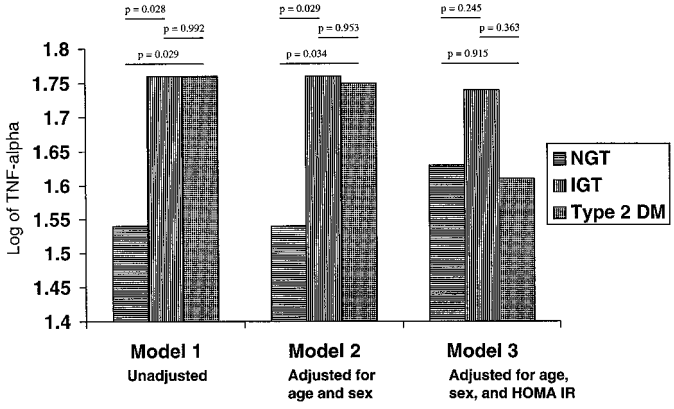

FIG. 3. TNFα concentrations in NGT, IGT, and type 2 DM groups.

TABLE 3. Results of multiple linear regression analysis: independent association of anthropometric and metabolic factors with log TNFα

Variable	Parameter estimate	SE	*t*	*P*
Age	−0.0005	0.003	−0.143	0.886
Gender	0.2456	0.171	1.436	0.156
Log HOMA insulin resistance	0.2211	0.093	2.377	0.020
Log triglyceride concentration	0.0383	0.100	0.383	0.703
% Body fat	−0.0119	0.008	−1.579	0.119
Waist/hip ratio	1.1334	0.890	1.273	0.207
Diabetes	−0.1253	0.124	−1.009	0.317
Log systolic BP	1.1366	0.393	2.893	0.005
Diastolic BP	−0.0008	0.004	−0.191	0.849
Log leptin	0.0306	0.092	0.334	0.740

Model $r^2 = 0.28$.

previously demonstrated to be positively associated with IR. Multiple linear regression analysis along with backward stepwise elimination revealed that only HOMA IR and systolic blood pressure remained significant and independent predictors of TNFα.

Studies in rodents support a role for TNFα as a mediator of IR (8, 15, 37). In human subjects, elevated adipose tissue TNFα mRNA content and fasting insulin are correlated, and both decrease with weight loss (13, 14). Although circulating TNFα concentrations are elevated in obese rodents (15), this has been difficult to substantiate in human subjects (6, 13, 37). This is due in part to the low circulating levels and previous lack of ultrasensitive assays. Furthermore, elevated local production of TNFα in adipose tissue may not always be reflected in peripheral blood, as the cytokine is synthesized and secreted by other tissues, notably monocytes/macrophages (38), and may not always be released into the circulation by the adipose tissue (39). A local autocrine/paracrine action may also explain in part the inability to reverse the IR with anti-TNFα antibody administration in human subjects (16).

It has been suggested that TNFα interaction with its cell surface receptor results in proteolytic cleavage of the extracellular binding domain, releasing a substantial amount of soluble receptor (40–44). One study found that circulating levels of soluble TNFR2, the soluble portion of the larger 80-kDa TNFR, but not TNFR1 (55 or 60 kDa), were elevated in obese subjects and correlated with IR (45), whereas another study reported that circulating levels of the smaller p55 TNFR correlated with insulin levels, BMI, and serum leptin concentrations (46). Although it has been proposed that the longer circulating $t_{1/2}$ of the soluble receptors may provide a more accurate reflection of TNFα action (42–44), further studies are required to determine whether this is the case for obesity and IR, and which form of receptor best reflects the cytokine action in adipose tissue.

In another study elevated circulating TNFα levels were found in Caucasian men (but not in women) with type 2 DM compared with levels in nondiabetic men (47). In our study, TNFα levels were also higher in type 2 DM as well as in IGT compared to NGT subjects in both genders. Notably, adjustment for HOMA IR eliminated these differences, supporting the concept that the association of elevated TNFα concentrations with abnormalities in glucose metabolism is related to the IR. The stronger correlation of TNFα with HOMA-IR in women in our study may be due to their greater degree and range of obesity, as adipose tissue is probably the major source of excess TNFα in these subjects.

IR precedes the development of glucose intolerance in the offspring of type 2 DM subjects (1–4). Kellerer *et al.* reported a significant negative correlation between circulating TNFα levels and glucose disposal rate determined by the euglycemic hyperinsulinemic clamp technique in both German and Finnish nondiabetic offspring of type 2 DM parents (48). In that study multiple linear regression analysis revealed a significant correlation of TNFα with the percentage of desirable body weight. These data are consistent with the increased synthesis and secretion of TNFα in adipose tissue of obese subjects, but did not show that TNFα was directly related to the IR. It should be noted that lean subjects with type 2 DM are also insulin resistant (3–5). Thus, the etiology of IR as well as that of type 2 DM appear to be multifactorial and heterogeneous among different populations (2–5). Our study is unique, in that a genetically homogeneous isolated Native Canadian population was examined.

Another approach to substantiate a role for TNFα in human obesity is genetic analysis. Studies of the TNFα gene-coding sequence and promoter region in both Caucasians and Pima Indians did not find any polymorphisms associated with type 2 DM or obesity (49, 50). However, a marker located 10 kb from the TNFα gene was associated with BMI in the Pima study (50). In another study, the *Nco*I restriction site of the TNFα promoter was associated with increased percent body fat, leptin levels, and IR (51). However, circulating TNFα levels were not different. These data do not prove but suggest that the up-regulation of TNFα is, in general, an acquired accompaniment of the obese state.

The explanation of the independent predictive value of systolic BP on TNFα concentration is not known. However, recent studies in the spontaneously hypertensive rat (SHR) model found that TNFα synthesis and secretion are increased in response to lipopolysaccharide stimulation compared with those in nonhypertensive control rats (52, 53); interestingly, this is most marked in adipose tissue (54). Furthermore, TNFα has been reported to stimulate angiotensinogen gene expression in liver (55). These data raise the possibility of TNFα contributing to the elevated BP. On the other hand, Ferreri *et al.* found that TNFα production by the thick as-

cending tubule of the renal medulla was elevated in angiotensin II-dependent hypertensive rats and that neutralization with anti-TNFα antiserum exacerbated the hypertension (56). Thus, the mechanism of the relationship between BP and TNFα and its pathophysiological significance in hypertensive human subjects remains to be determined.

In summary, this study demonstrates that in a genetically homogeneous Native Canadian population there is a significant and independent association between the circulating TNFα concentration and IR. Systolic BP, well documented to be associated with IR and obesity (4), also showed a significant independent association. Furthermore, elevated levels of TNFα in subjects with abnormal glucose tolerance and overt diabetes could be accounted for by their degrees of IR. Thus, our data support a significant contribution of TNFα to the pathogenesis of obesity-related IR and glucose intolerance in this population.

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