What Is the Contribution of Differences in Three Measures of Tumor Necrosis Factor-Alpha Activity to Insulin Resistance in Healthy Volunteers?

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To address the potential role that tumor necrosis factor-alpha (TNF-α) might play in modulation of insulin resistance in healthy, nondiabetic individuals, we compared plasma TNF-α and soluble TNF-α receptor 2 (sTNF-R2) concentrations, as well as TNF-α polymorphisms, in 94 healthy individuals, stratified into insulin-resistant (IR) and insulin-sensitive (IS) groups based on their plasma insulin concentrations 120 minutes after oral glucose on 2 occasions (1993 and 2000). The IR group (n = 50; 29 men and 21 women) was in the upper quartile and the IS group (n = 44; 24 men and 20 women) in the lowest quartile of the distribution of post-glucose challenge insulin concentrations in a large unselected population (>50 *v* <23 μU/mL). The IR group had significantly higher values for body mass index, waist-to-hip girth, fasting and post-glucose challenge insulin concentrations, and fasting triglyceride concentrations, and lower high-density lipoprotein cholesterol concentrations as compared to the IS group. Despite the fact that they were relatively more obese, and insulin-resistant, plasma concentrations of TNF-α were similar in the IR (1.6 ± 0.6 pg/mL) and IS (1.7 ± 0.6 pg/mL) groups, as were the concentrations (5.4 ± 1.4 *v* 5.8 ± 2.0 pg/mL) of sTNF-R2. Furthermore, TNF-α polymorphisms (detected by polymerase chain reaction [PCR]) were similar in the 2 groups, with essentially identical allelic frequencies of the 238 (10.3% *v* 9.4%) and 308 polymorphisms (17.9% *v* 18.7%). In conclusion, plasma TNF-α and sTNF-R2 concentrations, as well as TNF-α gene polymorphisms, were not different in healthy volunteers stratified into IR and IS groups on the basis of their plasma insulin response to an oral glucose challenge. Given these data, it does not appear that differences in TNF-α activity contribute to the marked variations in insulin action that occur in healthy individuals.

EVIDENCE THAT tumor necrosis factor-alpha (TNF-α) is produced by adipose tissue, and that its expression is increased in adipose tissue of obese, insulin-resistant rodents and humans, led to the hypothesis that increased concentrations of TNF-α played an important role in obesity-induced insulin resistance in humans.[1-3] Additional support for the postulated relationship between TNF-α and obesity-induced insulin resistance was derived from studies showing that TNF-α secretion was increased in tissue explants from obese individuals, and that there was excellent correlation between fasting plasma insulin concentrations and adipose tissue TNF-α mRNA expression.[4] It was subsequently shown that TNF-α was detected in skeletal muscle from both rats and humans, and that it was expressed at a higher level in muscle tissue and cultured muscle cells from insulin-resistant individuals.[5] Furthermore, plasma levels of TNF-α have been shown to be elevated in patients with type 2 diabetes, as well as in a population at increased risk to develop type 2 diabetes.[6-8]

As compelling as the initial evidence that differences in TNF-α concentrations might play a role in obesity-induced insulin resistance seemed to be, results of more recent studies attempting to evaluate this conclusion have not been totally successful. For example, not all studies in humans have found a relationship between insulin sensitivity and plasma TNF-α concentrations in healthy volunteers,[9] and administration of antibodies or antagonists to TNF-α have not improved insulin sensitivity in insulin-resistant individuals.[10,11] In addition to the importance of increases in TNF-α concentrations, reports have also been published suggesting that polymorphisms of the TNF-α gene might also modulate insulin-mediated glucose disposal. Specifically, reports have been published demonstrating that a biallelic polymorphism in the TNF-α promoter region −308 and −238 might contribute to TNF-α–related insulin resistance.[12-16] However, as with measurements of TNF-α concentrations, not all studies have been able to demonstrate a significant relationship between TNF-α polymorphism and estimates of insulin resistance.[17-22]

Finally, reports have also been published suggesting that increases in the plasma concentration of the TNF-α receptor 2 (sTNF-R2) are also elevated in insulin-resistant individuals.[23-25] However, similar to the situation with plasma TNF-α concentrations and TNF-α polymorphisms, not all studies have been able to demonstrate a relationship between insulin resistance or associated abnormalities, and sTNF-R2 concentrations.[26]

Unfortunately, there are no obvious explanations to account for the lack of unanimity as to the relationship between insulin resistance and the various estimates of TNF-α that have been evaluated. However, it should be noted that the majority of published studies have addressed this relationship in population-based studies, and relied upon statistical approaches to define associations between the TNF-α measurements and estimates of insulin resistance in narrowly defined groups. The current study was initiated to use a totally different approach to evaluate the relationship between insulin resistance and changes in TNF-α metabolism, in which we compared these

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variables in 2 groups of healthy volunteers, stratified into insulin-resistant (IR) and insulin-sensitive (IS) subgroups on the basis of 2 measurements of the plasma insulin response to oral glucose made several years apart. The results to be presented indicate that there are no differences in plasma concentrations of either TNF-α or sTNF-R2, or in polymorphisms of the TNF-α gene, that could account for the vast differences in insulin action between the 2 experimental groups.

MATERIALS AND METHODS

The experimental population consisted of 94 healthy individuals, stratified into an IR and IS group on the basis of their plasma insulin concentrations 2 hours after a 75-g oral glucose challenge as determined on 2 occasions (1993 and 2000). These individuals were selected from a larger group of 400 apparently healthy volunteers initially studied in 1993. The IS group (24 men and 20 women) and the IR group (29 men and 21 women) were in the lower quartile (IS) and upper quartile (IR) in terms of their plasma insulin concentration 2 hours after the oral glucose challenge when measured both in 1993 and 2000.

In both 1993 and 2000, plasma glucose and insulin concentrations in response to the 75-g oral glucose challenge were determined after the volunteers had been instructed to consume 300 g of carbohydrate/d for 3 days before the study. In addition, blood was obtained after an overnight fast in 2000 for determination of plasma lipid and lipoprotein concentrations, as well as for measurement of plasma TNF-α concentrations and analysis of genomic TNF-α locus polymorphisms. Plasma glucose, insulin, lipid, and lipoprotein concentrations were quantified as described previously.[27] TNF-α plasma concentrations and sTNF-R2 concentrations were measured with commercially available enzyme-linked immunosorbent assays: Quantikine HS (R&D Systems, Minneapolis, MN) for TNF-α and Biosource Europe SA (Milan, Italy) for sTNF-R2.

Genomic DNA was extracted from the whole blood preserved in EDTA by using the phenol-chloroform method. TNF 2 and TNF A alleles were detected by using mutagenic primers containing a single base-pair mismatch adjacent to the polymorphic site to introduce a restriction site into the wild-type nucleotide sequence after amplification.[16,28] We used the following 4 primers (M Medical Florence, Italy), with the mismatch position underlined: F308 (5′-GGG ACA CAC AAG CAT CAA GG-3′), R308 (5′AAT AGG TTT TGA GGG CCA TG-3′), F238 (5′-ATC TGG AGG AAG CGG TAG TG-3′), and R238 (5′ AGA AGA CCC CCC TCC GAA CC-3′) The fragment containing the TNF 2 polymorphism was amplified by using F308 and R308 primers, and TNF A was amplified with F238 and R238.

DNA samples were amplified in 50 μL ammonia reaction buffer (Bioline, London, UK) containing 200 μmol/L deoxynucleoside triphosphate, 10 μmol/L each primer, 2 μL DNA sample, and 2 U Taq polymerase (Bioline). A period of 5 minutes at 95°C was conducted to achieve DNA denaturation, followed by 33 cycles at 94°C for 4 minutes, 59°C for 1 minute, and 70°C for 45 seconds. At the end, a final period of 5 minutes at 70°C was conducted.

The polymerase chain reaction (PCR) products were digested at 37°C with *Nco*l to detect the TNF 2 alleles and with *Msp*l to detect the TNF A alleles and than subjected to 4% agarose gel electrophoresis Each PCR batch included a "blank" to which no DNA had been added to ensure that no contamination of samples had occurred. None of the blank reactions yielded any visible product after gel electrophoresis.

Data are expressed as the mean ± SE, and Student's unpaired *t* test was used to evaluate the statistical significance of differences between the 2 groups.

Table 1. Clinical Characteristics of the Experimental Groups

Variable	IS (n = 44)	IR (n = 50)	*P*
Age (yr)	57 ± 7	59 ± 7	NS
Gender (M/F)	24/20	29/21	NS
BMI (kg/m^2)	25.6 ± 3.8	29.6 ± 4.4	<.001
Waist (cm)	90 ± 10	100 ± 11	<.001
Smokers (n)	10	7	NS

Abbreviation: NS, not significant.

RESULTS

The clinical characteristics of the IS and IR groups are listed in Table 1. The ages, gender distribution, and smoking habits of the 2 groups were not different. However, the IR group was significantly ($P < .001$) more obese, as estimated by either body mass index or waist circumference.

Fig 1 compares the plasma insulin and glucose of the 2 groups when determined in 1993 and 2000. By selection, plasma insulin concentrations were significantly ($P < .001$) higher when measured on both occasions in the IR group. It can also be seen in Fig 1 that plasma glucose concentrations, both in fasting and 2 hours post-oral glucose, were higher ($P < .001$) in the IR group in both 1993 and 2000. It should also be noted that both the fasting and 2-hour values for plasma glucose and insulin concentrations were similar in 1993 and 2000 in the IS group. In contrast, the 2-hour values in the IR group were higher for both insulin (119 ± 15 *v* 92 ± 6 uU/mL) and glucose (139 ± 4 *v* 123 ± 3 mg/dL) in 2000.

Table 2 compares several hemodynamic and metabolic characteristics of the 2 groups when determined in 2000. These results show that heart rate ($P < .05$) and both systolic and diastolic blood pressure ($P < .001$) were higher in the IR group. In addition, the IR group had significantly higher plasma triglyceride and lower high-density lipoprotein cholesterol (HDL-C) concentrations ($P < .001$). Concentrations of total plasma cholesterol and low-density lipoprotein cholesterol (LDL-C) were also elevated in the IR group, but only the LDL-C increase reached the conventional level of statistical significance.

Plasma TNF-α and sTNF-R2 concentrations and the allelic frequency of the 238 and 308 TNF-α gene polymorphisms are shown in Table 3. It is apparent from these comparisons that values for these experimental variables were essentially identical in the IR and IS groups.

DISCUSSION

To our knowledge this study is the only one in which the relationship between insulin action and 3 estimates of TNF-α activity—TNF-α concentrations, concentrations of sTNF-R2, and TNF-α gene polymorphisms—have been evaluated in 2 relatively large groups of healthy volunteers, stratified on the basis of their being either insulin-sensitive or insulin-resistant. Before discussing the implications of our findings, it seems important to review the criteria for selecting the 2 groups, as well as the magnitude of metabolic differences between them. The 94 participants in this study were healthy volunteers, without apparent disease, and evaluated on 2 occasions within a period of 7 years. Categorization of an individual as being

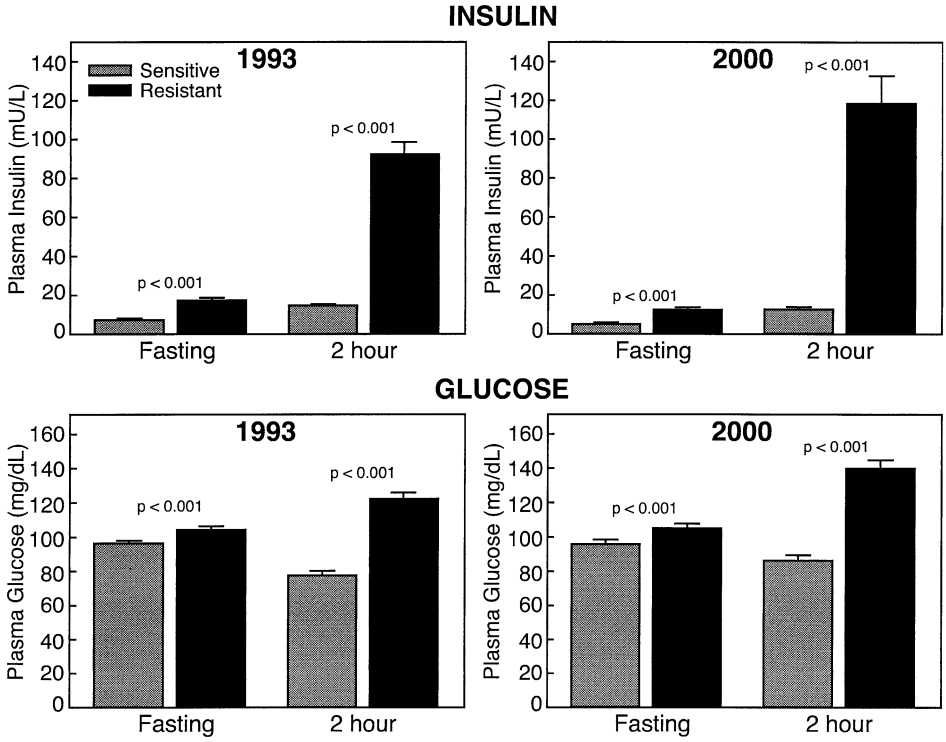

Fig 1. Plasma insulin and glucose concentrations measured before (fasting) and 2 hours after a 75-g oral glucose challenge in 1993 and 2000. Sensitive = subjects whose insulin levels were in the lower quartile on both occasions, whereas resistant = subjects whose insulin levels were in the upper quartile on both occasions.

either IR or IS was based on their plasma insulin response to a 75-g oral glucose challenge, a measurement shown to be highly correlated to specific determinations of insulin-mediated glucose disposal.[29] A volunteer was considered to be IR if their plasma insulin concentration was in the upper quartile of approximately 800 healthy volunteers when measured on both occasions. In a similar manner, a volunteer who was in the lowest quartile on both occasions would be classified as being IS.

It is apparent from the results in Tables 1 and 2 that the IR group displayed all of the manifestations previously shown to be associated with the insulin resistance/compensatory hyperinsulinemia in nondiabetic subjects.[30] Furthermore, the IR group was more obese, with higher values for both body mass index and abdominal girth. Consequently, it appears that we have been successful in creating 2 groups of healthy volunteers, who differ significantly in terms of their degree of insulin sensitivity. As such, we are able to test the hypothesis that the IR group would have higher plasma concentrations of TNF-α and/or sTNF-R2, or would differ in terms of the distribution of TNF-α gene polymorphisms.

Despite our ability to create 2 groups of individuals with strikingly different degrees of insulin action, we were unable to discern any differences in their plasma TNF-α concentrations, sTNF-R2 concentrations, or TNF-α gene polymorphisms. In addition, the IR group had significantly higher values for both body mass index and abdominal circumference. Thus, our results do not permit us to ascribe any causal role for differences in TNF-α activity as being responsible for insulin resistance in our population of healthy volunteers. As discussed earlier, there is no consensus as to the role of elevations of plasma TNF-α and/or sTNF-R2 concentrations, or TNF-α gene polymorphisms, in modulation of insulin sensitivity. Although our findings do not help reconcile the opposing views that have been proposed, the nature of our experimental protocol permits us to draw conclusions that avoid some of the pitfalls of earlier studies. Perhaps of greatest relevance is that our studies were performed in healthy volunteers, drawn from the population at

Table 2. Hemodynamic and Metabolic Characteristics of the Experimental Groups

Variable	IS (n = 44)	IR (n = 50)	*P*
Heart rate (beats/min)	65 ± 7	68 ± 8	<.05
Systolic BP (mm Hg)	121 ± 11	135 ± 15	<.001
Diastolic BP (mm Hg)	77 ± 1	89 ± 16	<.001
Cholesterol (mg/dL)	217 ± 37	234 ± 41	NS
LDL-C (mg/dL)	149 ± 32	167 ± 36	<.05
HDL-C (mg/dL)	50 ± 13	40 ± 9	<.001
TG (mg/dL)	91 ± 38	129 ± 41	<.001

Abbreviations: BP, blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride.

Table 3. TNF-α Plasma Concentrations and Gene Polymorphisms 238-308

Variable	IS (n = 44)	IS (n = 50)	*P*
TNF-α (pg/mL)	1.7 ± 0.6	1.6 ± 0.5	NS
sTNF-R2 (pg/mL)	5.4 ± 1.4	5.8 ± 2.0	NS
TNF-α −238	10.3% (6/58)	9.4% (6/64)	NS
TNF-α −308	17.9% (10/56)	18.7% (12/64)	NS

large, rather than in special groups, eg, patients with type 2 diabetes, obese individuals, or family members of patients with diabetes or fatty liver disease. Furthermore, by stratifying the groups at the outset as being either insulin sensitive or insulin resistant, we could directly compare TNF-α plasma concentration (as cytokine levels as well as soluble receptor concentrations) and gene polymorphisms into extremely disparate groups in terms of the variable of interest. As such, our results provide strong support for the view that substantial differences in insulin-mediated glucose disposal can occur in the population at large, irrespective of any variation in the plasma level of either TNF-α or TNF-R2, or in the distribution of TNF-α gene polymorphisms. Whether or not the same conclusion applies to specialized population groups cannot be addressed by our results.

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