Plasma Levels of the Soluble Fraction of Tumor Necrosis Factor Receptor 2 and Insulin Resistance

José-Manuel Fernández-Real, Montserrat Broch, Wifredo Ricart, Roser Casamitjana, Cristina Gutierrez, Joan Vendrell, and Cristobal Richart

Recent studies have shown that the tumor necrosis factor (TNF) system is implicated in the insulin resistance of human obesity. Plasma concentrations of the soluble fraction of the TNF receptors 1 and 2 (sTNFR1 and sTNFR2) are thought to reflect the degree of activation of the TNF system. The purpose of this study was to explore whether this activation, as measured by the levels of circulating sTNFR1 and sTNFR2, is associated with insulin resistance. A total of 19 men (mean age 36.2 ± 1.9; BMI 28.8 ± 1.2, range 22.2–35.7) and 17 premenopausal women (age 34.9 ± 1.4; BMI 28.1 ± 0.8, range 19–37.9) were studied. Men showed higher levels of plasma sTNFR1 and sTNFR2 than women. However, obese men showed increased levels of sTNFR2 but similar levels of sTNFR1 in comparison with obese women. In fact, sTNFR2 levels correlated with BMI (r = 0.50, P = 0.002), fat-free mass (FFM) (r = 0.61, P < 0.0001), and waist-to-hip ratio (WHR) (r = 0.39, P = 0.02), but not with fat mass or percent fat mass. sTNFR2 levels correlated with basal glucose levels (r = 0.45, P = 0.007), area under the curve (AUC) for glucose during an oral glucose tolerance test (r = 0.42, P = 0.013), and with the quotient AUC glucose/log AUC insulin (r = 0.41, P = 0.015). sTNFR2 also correlated negatively with insulin sensitivity (S_I), evaluated using the frequently sampled intravenous glucose tolerance test with minimal model analysis (r = –0.38, P = 0.02). Plasma sTNFR1 levels were not associated with any of these variables. Because WHR influenced both S_I and sTNFR2 levels, we constructed a multiple linear regression to predict S_I, with WHR and sTNFR2 as independent variables. In this model, both WHR (P = 0.0078) and sTNFR2 levels (P = 0.025) contributed to 47% of the variance in S_I. In parallel with higher FFM, lean and obese men showed a lower S_I (2.9 ± 0.9 vs. 5.2 ± 1.3 $min^{-1} \cdot mU \cdot l^{-1}$, P = 0.001; and 1.15 ± 1.1 vs. 1.8 ± 0.8 $\cdot min^{-1} \cdot mU \cdot l^{-1}$, P = 0.035, respectively) and higher sTNFR2 levels in comparison with lean and obese women, respectively. After controlling for FFM, the correlation between S_I and sTNFR2 levels disappeared, indicating that FFM was significantly influencing these associations. In summary, plasma sTNFR2 levels, but not sTNFR1, were proportional to BMI, WHR, FFM (a well-known confounder in the evaluation of insulin sensitivity), basal and postload glucose levels, and insulin resistance. These findings support TNF-α as a system regulating insulin action in human obesity. ***Diabetes*** **47:1757–1762, 1998**

In the last years, it has been demonstrated that tumor necrosis factor (TNF)-α has important effects on whole-body lipid and glucose metabolism (1,2). 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 (3,4) and muscle (5) tissues. TNF-α actions in obesity appear to occur via an autocrine-paracrine mechanism in these latter tissues (4,5). Neither the mechanisms that control this restricted activity nor the receptor systems that are involved in TNF-α signaling are clearly understood (6,7). TNF signals through at least two known cell-surface receptors (6,7) (TNFRs), TNFR1 (p60) and TNFR2 (p80), that are present in virtually all cells of higher mammals, including adipocytes. It appears that TNFR1 can signal for virtually all known activities of TNF, including apoptosis, differentiation, and proliferation. TNFR2 seems to signal metabolic actions (8,9).

Cytokines circulate bound to a number of binding proteins that appear to alter their clearance rates and/or enhance their biological activity. Both TNFRs exist also in soluble forms (10–13), apparently derived by proteolytic cleavage from the cell-surface forms (14). These soluble TNFRs (sTNFRs) can compete with the cell-surface receptors and thus block TNF activity, safeguarding against its potentially harmful effects. However, sTNFRs affect TNF function also by stabilizing its activity (15), most likely by preventing dissociation of the homotrimeric TNF molecules (16) to inactive monomers. It has been suggested that sTNFR1 and sTNFR2 represent a buffer system that prolongs the biologic effects of TNF-α by forming a "slow release reservoir" and impeding spontaneous denaturation of the cytokine (15).

Limited information is currently available concerning the effects of the two TNFRs in energy metabolism and glucose homeostasis. In a recent study, obese women expressed approximately twofold more TNFR2 mRNA in fat tissue and approximately sixfold more sTNFR2 in circulation relative

From the Department of Endocrinology (J.M.F.-R., W.R.), University Hospital of Girona "Dr. Josep Trueta," Girona; the Department of Endocrinology (M.B., C.G., J.V., C.R.), University Hospital of Tarragona Joan XXIII, Tarragona; and the Hormonal Laboratory (R.C.), University Hospital Clínic, Barcelona, Spain.

Address correspondence and reprint requests to J.M. Fernández-Real, MD, Department of Endocrinology, Hospital de Girona, Ctra. França s/n, 17007 Girona, Spain. E-mail: hosptrueta@comgir.com.

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ASF, abdominal skinfold thickness; AUC, area under the curve; BSF, biceps skinfold thickness; CV, coefficient of variation; FFM, fat-free mass; FSIGTT, frequently sampled intravenous glucose tolerance test; IGT, impaired glucose tolerance; MAMA, mid-arm muscle area; MAMC, mid-arm muscle circumference; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; S_G, glucose effectiveness; S_I, insulin sensitivity index; SSF, subscapular skinfold thickness; sTNFR, soluble tumor necrosis factor receptor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TSF, triceps skinfold thickness; WHR, waist-to-hip ratio.

to lean control subjects (8). Adipose tissue expression of TNFR2 strongly correlated with BMI and the level of insulinemia. In contrast, no correlation was observed between any of the metabolic variables that were measured (glucose, insulin, lipids, BMI) and TNFR1 expression (8). More specifically, TNFR1 expression and protein levels were similar in lean and obese subjects. sTNFR1 levels correlated with leptin levels independently of BMI in another study (17). In these latter studies, the relationships among sTNFR1, sTNFR2, and glucose or insulin resistance were inferred from isolated plasma levels of glucose and insulin.

TNF-α is a strong candidate for abnormalities in glucose metabolism, but circulating TNF-α levels are usually not informative. This is probably due to difficulties in measuring TNF in plasma, where it normally is in a very low concentration, in the range of picograms per milliliter. Compared with circulating TNF-α, sTNFR1 and sTNFR2 levels remain elevated for longer periods of time and are of more value for monitoring inflammatory responses (15). Furthermore, sTNFRs are significantly elevated in plasma, are very stable proteins, and are quite easy to measure.

The purpose of this study was to explore whether activation of the TNF-α system, measured through the levels of circulating sTNFR1 and sTNFR2, is associated with insulin resistance.

RESEARCH DESIGN AND METHODS

Subjects. We studied 36 subjects: 19 men (mean age 36.2 ± 1.9; BMI 28.8 ± 1.2, range 22.2–35.7) and 17 premenopausal women (mean age 34.9 ± 1.4; BMI 28.1 ± 0.8, range 19–37.9). Characteristics of the subjects are summarized in Table 1. Inclusion criteria for the subjects were *1*) a BMI (weight in kilograms divided by the square of height in meters) >30 and <40 kg/m^2 for obese and <27 (for men) or <25 kg/m^2 (for women) for lean subjects, *2*) the absence of any systemic disease, and *3*) the absence of any infections. 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 and normolipemic (data not shown). Because the BMI only provides a crude measurement of body fatness, some men and women with BMI values <25 kg/m^2 had relatively higher levels of body fat, as assessed by bioelectric impedance, and thus the two subgroups considerably overlapped for body fatness indexes. Metabolic and hormonal data on some of the subjects were reported in two previous publications (18,19). The protocol was approved by the hospital ethics committee, and informed consent was obtained from each subject.

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), mid-arm muscle circumference (MAMC), and mid-arm muscle area (MAMA). TSF, BSF, SSF, and ASF were measured with a skinfold caliper (Holtain, Cambridge, U.K.). MAMC and MAMA were calculated as previously described (20). 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 (20). The percentage of body fat and fat-free mass (FFM) were measured by bioelectric impedance analysis (Holtain BC Analyser).

Study protocol. An oral glucose tolerance test (OGTT) was performed according to the recommendations of the National Diabetes Data Group (21). 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 (AUCs) during the OGTT were determined by the trapezoidal method.

For the frequently sampled intravenous glucose tolerance test (FSIGTT), 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. 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. The serum glucose level during the FSIGTT was measured in duplicate by the glucose oxidase method with a Beckman Glucose Analyzer 2 (Brea, CA). The coefficient of variation (CV) 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 intra-assay CV was 5.2% at a concentration of 10 mU/l and 3.4% at 130 mU/l. The interassay CVs were 6.9 and 4.5% at 14 and 89 mU/l, respectively.

The Medgenix sTNF-R1 EASIA and sTNF-R2 EASIA (BioSource Europe, Fleunes, Belgium) are solid-phase enzyme-amplified sensitivity immunoassays performed on microtiter plate. The minimum detectable concentration was estimated to be 0.1 ng/ml and was defined as the sTNFR1 or sTNFR2 concentration corresponding to the average of 20 replicates of the zero standard + 2 SDs. The intra-assay and interassay CVs were <7 and <9%. sTNFR1 EASIA does not cross-react with sTNF-R2. TNF-α does not interfere with the assay.

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 times course of glucose and insulin concentrations. The glucose disappearance model, by accounting for the effect of insulin and glucose on glucose disappearance, provides the parameters S_I (10^{-4} per minute per microunit per milliliter) or the insulin sensitivity index, a measure of the effect of insulin concentrations above the basal level to enhance glucose disappearance, and S_G (per minute) or glucose effectiveness, 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 (22).

Statistical analyses. Descriptive results of continuous variables are expressed as means ± SD. Non–Gaussian-distributed variables were $\log_{10}$ transformed to achieve normality. This applied to insulin sensitivity and sTNFR2. Relationships between variables were sought by Pearson's correlation coefficient and stepwise multivariate linear regression analysis with forward selection. The regression coefficient generated by this analysis indicates the slope of the association between the dependent variable and the specified independent variable, after adjusting for other independent variables in the model. The SE represents the variability in this association, and the significance is reflected by the P value. The model R^2 indicates the percent of the variance in the dependent variable that is accounted for by the independent variables included in the model. Comparison of variables across lean and obese men and women was performed by one-way analysis of variance using Fisher's test for multiple comparisons. Levels of statistical significance were set at $P < 0.05$. All these analyses were performed with the BMDP statistical package (BMDP Statistical Software, Cork, Ireland).

RESULTS

Tables 1 and 2 summarize the characteristics of the study subjects at the time of entry into the study. Among the 36 subjects, 4 men and 3 women (all obese) had impaired glucose tolerance (IGT), whereas 29 had normal glucose tolerance (NGT), as judged by the OGTT. No significant differences were found in sTNFR1 or sTNFR2 levels between obese subjects with NGT or IGT. Exclusion of these participants from the subsequent analyses resulted in little change, so they were retained in the analyses to increase statistical power.

Influence of sex and body composition. Overall, compared with women, men showed increased levels of plasma sTNFR1 (1.65 ± 0.22 vs. 1.44 ± 0.26 ng/ml, $P = 0.019$) and sTNFR2 (3.3 ± 0.5 vs. 2.66 ± 0.47 ng/ml, $P = 0.001$). Lean men showed increased levels of sTNFR1 and sTNFR2 in comparison with lean women (Table 2). Obese men showed increased levels of sTNFR2 but similar levels of sTNFR1 compared with obese women (Table 2).

Obese men and women showed levels of sTNFR1 similar to that of lean men and women, respectively (NS). In contrast, sTNFR2 was higher in obese men and women than in lean control men and women ($P = 0.023$ and 0.045, respectively). In fact, sTNFR2 correlated with BMI ($r = 0.50$, $P = 0.002$), FFM

TABLE 1
Anthropometric and clinical characteristics of the study subjects

Characteristics	Lean men	Lean women	Obese men	Obese women
n	9	7	10	10
Age (years)	34.5 ± 6	33.7 ± 6.3	38.5 ± 4.7	35 ± 8.1
BMI (kg/m^2)	24.4 ± 1.7	19.8 ± 1.6	32.7 ± 2	32.3 ± 2.7
Waist (cm)	88 ± 4.6	77.7 ± 11.4	105.9 ± 7.8	107.1 ± 9.7
WHR	0.98 ± 0.04	0.85 ± 0.05	0.99 ± 0.06	0.98 ± 0.01
Fat mass (kg)	13.28 ± 7.8	11.15 ± 3.2	30.56 ± 8.9	33.42 ± 6.2
Body fat (%)	17.51 ± 9.4	19.95 ± 3.7	30.16 ± 6.2	39.29 ± 5.6
FFM (kg)	61.94 ± 9.1	43.9 ± 4.7	69.9 ± 7.7	51.2 ± 4.8
TSF (%)	108.2 ± 35.5	90.2 ± 21.6	178.1 ± 68.3	153.2 ± 25.4
SSF (%)	112.3 ± 17	73.04 ± 21	219 ± 59.7	213.9 ± 20.9
ASF (%)	109.5 ± 28.8	71.2 ± 25	176.4 ± 35	172.1 ± 35.2
MAMC (%)	102.7 ± 6.4	100.19 ± 5.9	112.6 ± 10.3	119.9 ± 10
MAMA (%)	106.2 ± 13.4	100.6 ± 12.2	127.8 ± 23.5	144.7 ± 23.3

Data are means ± SD.

(r = 0.61, P < 0.0001; Fig. 1), and WHR (r = 0.39, P = 0.02) but not with fat mass or percent fat mass. sTNFR2 also correlated positively with MAMC and MAMA (both r = 0.61, P < 0.0001; Fig. 1), two indicators of the muscle compartment. In a multiple linear regression in a stepwise manner to predict sTNFR2, with FFM and sex as independent variables, only FFM predicted sTNFR2 (P = 0.04), contributing to 39% of the variance in sTNFR2.

Insulin sensitivity. sTNFR2 levels correlated with basal glucose levels (r = 0.45, P = 0.007) and with AUC glucose during the OGTT (r = 0.42, P = 0.013). This latter relationship improved when subjects with IGT were excluded from the analyses (r = 0.75, P < 0.0001; Fig. 2). An association was observed between sTNFR2 levels and the quotient AUC glucose/log AUC insulin (r = 0.41, P = 0.015). sTNFR2 also correlated negatively with S_I (r = –0.38, P = 0.02; Fig. 3). Because WHR influenced both S_I and sTNFR2 levels, we constructed a multiple linear regression to predict S_I, with WHR and sTNFR2 as independent variables. In this model, both WHR (P = 0.0078) and sTNFR2 (P = 0.025) levels contributed to 47% of the variance in S_I.

In parallel with higher FFM, lean and obese men showed a lower S_I (P = 0.001 and 0.035, respectively) and higher sTNFR2 levels in comparison with lean and obese women, respectively (Table 2). After controlling for FFM, the correlation between S_I and sTNFR2 disappeared, indicating that FFM was significantly influencing these associations.

DISCUSSION

TNF-α is a candidate mediator of insulin resistance in obesity, as it is overexpressed in the adipose and muscle tissues of rodents and humans and because it blocks the action of insulin in cultured cells and whole animals (3–5,23,24). Weight loss caused by dietary treatment of obesity results in a significant decrease in the amount of TNF-α expression in adipose and muscle tissues (3–5). The induction of insulin resistance is mediated through its ability to produce serine phosphorylation of insulin receptor substrate 1, decreasing the tyrosine kinase activity of the insulin receptor (25). In a recent study, obese mice with a targeted null mutation in the gene encoding TNF-α were spared from obesity-induced deficiencies in insulin receptor signaling in fat and muscle tissues (26).

The correlation between basal and postload serum glucose levels and sTNFR2 may suggest that both glucose and sTNFR2 might be regulated by the same obesity-related signal. In fact, increased expression of TNF-α strongly correlates with the level of hyperinsulinemia (4) and the glucose disposal rate during the euglycemic clamp technique (5). Because TNF-α is a strong inducer of TNFR2 expression in adipocytes (8) and other cell types (27), the negative correlation between insulin sensitivity and sTNFR2 might be attributed to increased TNF-α action in situations of insulin resistance. Here we describe, for the first time, to our knowledge, a relationship between insulin action and plasma sTNFR2 concentration.

TABLE 2
Biochemical variables of the study subjects

	Lean men	Lean women	P value	Obese men	Obese women	P value
Fasting glucose (mmol/l)	5.31 ± 0.48	4.58 ± 0.33	NS	5.77 ± 0.53	5.43 ± 1.25	NS
Fasting insulin (mU/l)	8.27 ± 2.5	5.7 ± 1.5	NS	15.9 ± 8.8	12.1 ± 5.2	NS
Glucose AUC (mmol/l)	7.31 ± 0.6	5.95 ± 1.83	NS	10.6 ± 2.7	8.8 ± 3.6	NS
Insulin AUC (mU/l)	69.1 ± 27.9	49.3 ± 25.3	NS	114.1 ± 50	82.6 ± 51.4	NS
S_I (min^{-1} · mU · l^{-1})	2.98 ± 0.9	5.26 ± 1.36	0.001	1.15 ± 1.1	1.8 ± 0.8	0.035
sTNFR1 (ng/ml)	1.68 ± 0.12	1.38 ± 0.31	0.034	1.62 ± 0.28	1.48 ± 0.22	NS
sTNFR2 (ng/ml)	3.07 ± 0.45	2.38 ± 0.39	0.014	3.5 ± 0.5	2.79 ± 0.46	0.003

Data are means ± SD. S_I was derived from the frequently sampled intravenous glucose tolerance test with minimal model analysis.

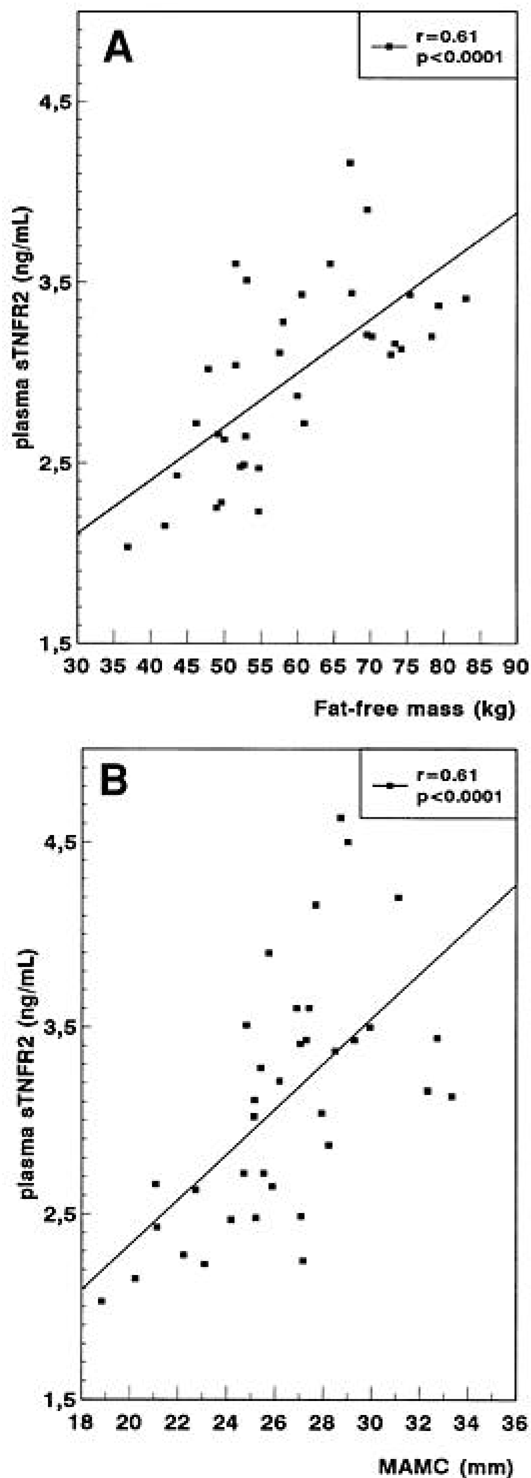

FIG. 1. Linear correlations between circulating sTNFR2 levels and FFM (*A*) and between sTNFR2 and MAMC (*B*).

We found differences in sTNFR2 but not in sTNFR1 levels between obese and lean subjects. These findings are identical to those described by Hotamisligil et al. (8). However, the differences were less marked, probably because in that study, obese women had a significantly higher mean BMI (39 kg/m^2). Adipose tissue TNFR2 expression was also associated with WHR in the latter study (8), and a similar relationship

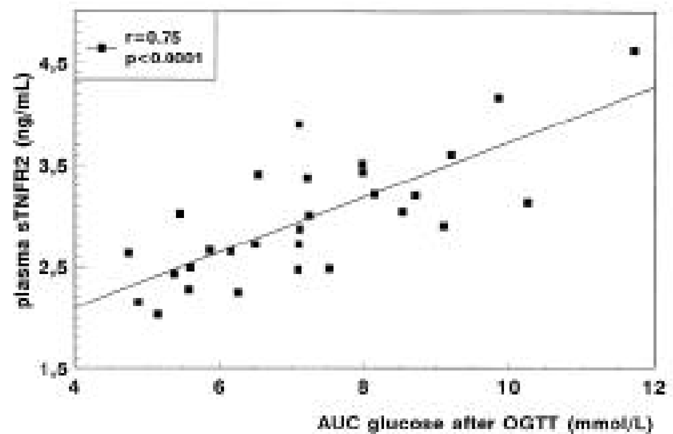

FIG. 2. Linear correlation between circulating sTNFR2 levels and AUC glucose after an OGTT in subjects with NGT.

between sTNFR2 and WHR has been found in this report. This correlation could merely reflect the degree of insulin resistance found in abdominal obesity, but we have demonstrated that both WHR and sTNFR2 independently predict insulin sensitivity in a multiple regression analysis. On the other hand, abdominal obesity is a well-known cardiovascular risk. In this sense, the correlation between sTNFR2 and AUC glucose is in concordance with recent finding in young postinfarction patients, in whom TNF-α correlated with fasting and postload glucose levels (28). Furthermore, circulating sTNFR2 levels were three times greater in patients with ischemic heart disease in a recent study (29).

The sTNFRs are present constitutively in serum at concentrations that increase significantly in both inflammatory and noninflammatory disease states (30). The effect of these proteins may differ, however, depending on their concentration at the site of TNF action, the relation of their concentration to the local concentration of TNF, and the rates at which the sTNFRs and TNF are cleared from the site of TNF action in relation to the decay of TNF activity (15). Thus, the TNFRs may in some situations inhibit the effects of TNF, in others, serve as carriers for TNF, and in some cases they may even augment the effects of TNF by prolonging its function (15).

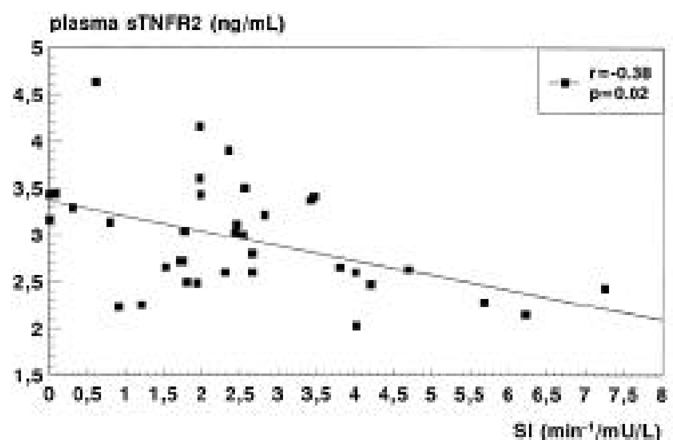

FIG. 3. Linear correlation between sTNFR2 and the S_I derived from the FSIGTT with minimal model analysis.

The increased levels of sTNFR2 in lean and obese men (with significantly increased FFM) in comparison with lean and obese women, and the correlations between sTNFR2 with BMI and FFM itself, suggest that circulating sTNFR2 is produced not only by the adipocyte but also possibly by the muscle. This hypothesis is supported by the correlations between two markers of the muscle compartment (MAMC and MAMA) and sTNFR2. Increased expression of TNF-α has been demonstrated at the muscle tissue (5). Because this latter tissue is the main target for insulin-stimulated glucose disposal (31,32), FFM constitutes, among other factors, a determinant of insulin sensitivity (33). In fact, a good correlation between FFM and insulin sensitivity was observed in our patients ($r = -0.40$, $P = 0.02$). On the other hand, gender-related differences in insulin sensitivity have been well characterized (34–38). Women are more insulin sensible despite higher fat mass than men, and this is thought to be related to differences in FFM metabolism (34–38). Because TNF expression is variably increased with increased adiposity and is decreased with decreased adiposity (3), this variability could be partially explained by differences in FFM. It is tempting to speculate that the association between FFM and sTNFR2 might be attributed to increased production by the muscle of sTNFR2, leading to stabilization of TNF-α homotrimers, thus resulting in insulin resistance at the level of the adipocyte. This latter hypothesis would explain the differences in sTNFR2 in parallel with FFM and insulin sensitivity. Favoring this hypothesis, we have observed increased (two- to threefold) plasma levels of sTNFR1 and sTNFR2 in myopathies in parallel with insulin resistance (J.M.F.-R., J.M. Gomez, J.M. Matos, W.R., J.V., unpublished observations).

On the other hand, increased lean body mass leads to increased insulin sensitivity under many circumstances, such as after endurance or strength training. However, insulin utilizes a phosphatidylinositol-3-kinase–dependent mechanism, whereas the exercise signal is initiated by calcium release from the sarcoplasmic reticulum leading to the activation of other signaling intermediaries (39). Indeed, the hypoglycemic response to insulin is only slightly affected by the level of training (40).

In summary, plasma sTNFR2 but not sTNFR1 levels were proportional to BMI, WHR, FFM (a well-known confounder in the evaluation of insulin sensitivity), basal and postload glucose levels, and insulin resistance. These findings support TNF-α as a system regulating insulin action in human obesity.

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Author Queries (please see Q in margin and underlined text)

Q1: <<Au: Title OK as edited?>

Q2: <<Au: Please supply first names of authors. Thank you.>

Q3: OK to rearrange unit of measure like this?

Q4: What does OD stand for here?

Q5: SD correct in table legends?

Q6: Please spell out the last names of J.M.G. and J.M.M., since they are not authors of the present study.