High expression of tumor necrosis factor α receptors in peripheral blood mononuclear cells of obese type 2 diabetic women

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ABSTRACT. The tumor necrosis factor system plays an important role in the pathogenesis of obesity and type 2 diabetes (DM), by a complex and only partially understood mechanism. In this study we analyze the mRNA expression levels of TNFα and its receptors (TNFR1 and TNFR2), in peripheral blood mononuclear cells (PBMC) from eleven, non-morbid, obese and 14, obese, type 2 DM women, by real-time quantitative PCR. We show an increase in the TNFR2 to TNFR1 ratio (mTNFR2/mTNFR1) in type 2 DM (r = 0.63; p = 0.021, after adjusting for age). Likewise, a positive correlation between mTNFR2/mTNFR1 and glucose was observed (r = 0.5; p = 0.029) in the whole group. We performed an oral glucose tolerance test with 75 g of glucose in obese, non-diabetic women in order to evaluate the effect of an acute glucose increase on the tumor necrosis factor system at 60 min and 120 min. We show that except for a positive association of mTNFR1 with body mass index at 60 min and of mTNFR2 with plasmatic triglycerids levels, no other significant differences were elicited by acute glucose in obese, non-diabetic women. These findings are in agreement with a functional role for the TNF system in obese women in obesity-linked, type 2 diabetes.

Keywords: mTNFR1, mTNFR2 and PBMC

INTRODUCTION

Insulin resistance is a major problem frequently observed in obesity and obesity-linked, type 2 diabetes. The pathogenic mechanisms responsible for the development of type 2 diabetes, are only partially understood. In particular, how obesity can bring about resistance to insulin, eventually leading to diabetes, remains quite poorly understood. Recently, the cytokine tumor necrosis factor (TNFα) has been shown to be involved as a modulator of glucose metabolism. A direct role for TNFα in the induction of insulin resistance, and the down-regulation of insulin receptor signaling has been reported in cultured adipocytes and skeletal muscle [1, 2]. Indeed, elevated plasma TNFα levels have been observed in obese and DM2 patients, with a reported over-expression in adipose and skeletal muscle tissues [3, 4].

TNFα is a well-known transcriptional regulator, which mediates the expression of many immune and inflammatory response genes in many cell types, including monocytes, macrophages, endothelial cells, fibroblasts, etc.[5-7]. The biological effects of TNFα are mediated through two cell surface receptors, TNFR1 and TNFR2 [8, 9] which can be independently regulated. TNFR1 is expressed on a broad spectrum of tissues, while expression of TNFR2 is restricted to tissues of endothelial or hematopoietic origin. Once the TNFα is bound to its receptors, a soluble (sTNFR) is released, and remains in serum for longer periods of time, resulting in a prolongation of the TNFα effects [10]. However, the regulatory mechanisms for the shedding of TNFR1 and TNFR2 are only partially understood.

We have previously shown that plasma sTNFR2 levels and the shedding of both receptors are linked to insulin resistance in healthy, and type 2 diabetic subjects [11, 12], confirming that the sTNFR2-to-sTNFR1 ratio correlates well with TNFα action and insulin sensitivity [12, 13]. Although, it is known that acute and chronic hyperglycemia can induce an increase in the synthesis and secretion of these pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMC) *in vitro* [14, 15], only in one work has the effect of hyperglycemia been evaluated *in vivo*, in healthy and intolerant subjects, and this showed that TNFα and IL6 levels increased in plasma during the glucose infusion [16]. There is no information for situations of chronic hyperglycemia such as in type 2 diabetes.

In order to verify whether the TNF system is chronically overexpressed in PBMC from obese, type 2 diabetic subjects, we set up a highly sensitive and quantitative real time PCR assay of TNFα, TNFR1 and TNFR2 mRNA. To examine the relationship between post-prandial, acute glucose excursion and PBMC cytokine expression *in vivo*, we

performed an oral glucose tolerance test in healthy, obese women, investigating the variations in the expression of TNFα mRNA and its receptors.

MATERIALS AND METHODS

Subjects

The study group consisted of 14 women diagnosed with type 2 diabetes (DM2) (aged 59.9 ± 6.3) and 12, non-diabetic women matched for BMI. All subjects were of Caucasian origin and had not suffered from any infection within the previous month, cancer or any other wasting disease, and their BMI was lower than 40 kg/m^2. The 13.3% of type 2 diabetic patients were treated by diet only, 40% with oral hypoglycaemic agents and 46.7% with insulin therapy. None of the control subjects were receiving medication or had evidence of any metabolic disease, other than obesity. All subjects reported that their body weight had been stable for at least three months prior to the study.

The study was approved by the ethics committee of the Joan XXIII University Hospital, Tarragona, and all subjects provided written informed consent prior to participation in the study.

Anthropometric measurements

Height and weight were measured with the patient standing in light clothes and without shoes. BMI was calculated as body weight divided by height squared (kg/m^2). Waist-hip ratio was assessed. Body composition was determined using a bioelectrical impedance analyzer (TANITA Analyzer, LTD, USA), the precision of this test in determining body fat (BF) is within ± 3%. Blood pressure was measured at rest, with a mercury sphygmomanometer, with subjects in a sitting position. The mean of three measurements was used. All examinations were done by the same physician (JV). Obesity was classified according to the World Health Organization criteria [17].

Analytical methods

Blood samples were drawn from each subject before breakfast, between 8.00-9.00 a.m., and after overnight rest in bed in order to determine glucose, creatinine, cholesterol, HDLc and LDLc, triglycerides, "C-reactive protein" (CRP) and sTNFRI, sTNFRII.

An oral glucose tolerance test (OGTT) was performed according to WHO recommendations [18] in all healthy, control participants. After a 12-h, overnight fast, 75 g of glucose was ingested, and blood samples were collected through a venous catheter from the antecubital vein at 0 min, 60 min and 120 min to measure serum glucose. All healthy subjects showed normal glucose tolerance.

Serum glucose was measured using a glucose oxidase method employing an Hitachi autoanalyzer (L-9100; Hitachi Scientific Instruments, Inc., Japan). Serum creatinine was determined using a routine laboratory method. Serum insulin was measured using a specific immunoradiometric assay (Medgenix Diagnostics, Fleurus, Belgium), in which proinsulin did not cross-react. The intra- and inter-assay coefficients of variation were 6% and 7%, respectively. Insulin resistance was estimated in obese, non-diabetic women using the homeostasis model assessment for insulin resistance (HOMA), using the following formula: insulin (U/ml) × glucose (mmol/l/22.5). Total serum cholesterol was measured using the cholesterol esterase/cholesterol oxidase/peroxidase reaction. HDL cholesterol was quantified after precipitation with polyethylene glycol at room temperature. Serum triglycerides were measured by means of the glycerol-phosphatase-oxidase and peroxidase reaction. The sTNFRI and sTNFRII were determined by solid phase enzymoimmunoassay with amplified reactivity (Bio Source Europe S.A. B-62220, Fleurus, Belgium). The limit of detection was 50 pg/ml for sTNFRI and 0.1 ng/ml for sTNFRII, and the intra- and inter-assays coefficients of variation were 7% and 9% respectively. The sTNFRI assay does not cross-react with sTNFRII and *vice versa*. All samples were assayed in duplicate. HbA1c was measured using a chromatographic method (Glico Hb Quick Column Procedure, Helena laboratories, Beaumont, TX, USA).

Isolation and preparation of cells

Peripheral blood mononuclear cells (PBMC) were obtained from obese women with DM2 after fasting, and from healthy, obese women during the OGTT at 0 min, 60 min, and 120 min. PBMC were isolated from fresh, heparinized venous blood by centrifugation on a Ficoll-Hypaque gradient (SEROMED; Biochrom KG, Berlin, Germany), washed twice with phosphate-buffered saline (PBS) and immediately stored on liquid nitrogen. A patient suffering from rheumatoid arthritis was included in the basal study as a control for an activated TNFα system in PBMC.

RNA isolation and cDNA synthesis

Total RNA from PBMC was extracted using two, phenolic acid guanidinium thiocyanate extraction steps, followed by a chloroform-isoamyl alcohol step (29:1), and precipitation with 2-propanol [19]. Total RNA extracted from cells was quantified, and 1 μg of PBMC-derived total RNA was used for cDNA synthesis. The mRNA expression coding for TNFα, TNF-RI, TNF-RII and β-actin was determined by RT-PCR, using mRNA specific primers. To reduce the RNA secondary structure, and to maximise the stringency of the cDNA synthesis, each RNA sample was preheated to 70 °C for 3 min. Subsequent cDNA synthesis was carried out for 60 min at 42 °C in a 20 μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 37.5 mM KCl, 5 mM $MgCl_2$, 1 mM each of deoxynucleoside triphosphate, Rnasin (50 U), 20 U AMV reverse transcriptase (Roche Diagnostics, Mannheim, Germany), and 1.0 μM of the corresponding polarity (antisense) primer. Prior to the amplification of the cDNA by the LightCycler, cDNA samples were heated to 95 °C for 5 min and then treated with 100 μg of RNase per ml.

Primer design and normalization

The primer pairs for TNFα, TNF-RI and TNF-RII were chosen with the assistance of the Oligo 4.0 computer program (National Biosciences, Inc., Plymouth, MN, USA). Cytokine primer pairs were spanning exon-exon conjunctions and were therefore mRNA/cDNA-specific and non-reactive with genomic DNA. The primer sequences used in this study are listed in *Table 1*.

To minimize variability in the results caused by differences in the RT efficiency or RNA integrity among the samples,

Table 1
Sequences, amplicon sizes and exon localizations of primers

Primers	Sequence (5' →3')	Exon	Size cDNA (bp)
TNF-α forward	CAGAGGGAAGAGTTCCCCAG	2	325
TNF-α reverse	CCTTGGTCTGGTAGGAGACG	4	
TNFR1 forward	GTGCTGTTGCCCCTGGTCAT	7	162
TNFR1 reverse	GCTTAGTAGTAGTTCCTTCA	9	
TNFR2 forward	AAACTCAAGCCTGCACTC	4	208
TNFR2 reverse	GGATGAAGTCGTGTTGGAGA	5	
β-actin forward	AGCGGGAAATCGTGCGTG	4	311
β-actin reverse	CAGGGTACATGGTGGTGCC	6	

Primers were chosen on different exons in order to avoid possible genomic DNA amplification. The cDNA amplicon was much shorter than the genomic DNA amplicon (TNFα: > 780 bp, TNFR1: 628 bp, TNFR2: 761 bp, β-actin: 422bp).

the target cytokine transcripts were normalized to an internal housekeeping gene (β-actin). The specific primers for β-actin mRNA are shown in *Table 1* [20]. The normalized values of cytokine transcript copies in each sample were reported as the ratio of the target cytokine transcript copy number to the β-actin transcript copy number.

Real time PCR quantification

Quantification of the mRNA coding for TNFα, TNF-RI, TNF-RII and β-actin was performed using LightCycler technology (Roche Diagnostics). The reactions were performed in a volume of 20 µl of a mixture containing 0.5 µM of each oligonucleotide primer and 2 µl of DNA LC-FastStart DNA Master SYBR green I (Roche Molecular Biochemicals) containing Taq DNA polymerase, reaction buffer, dNTP mix and the double stranded DNA (dsDNA)-specific fluorescent dye SYBR green I. The final concentration of $MgCl_2$ was adjusted to 4.5 mM. LightCycler mastermix (18 µl) was put into the LightCycler glass capillaries and 2 µl cDNA (TNFα, TNF-RI, TNF-RII and β-actin reverse-transcribed total RNA) was added as a PCR template. To improve SYBR Green I quantification, a high temperature, fluorescence measurement point was included in the amplification protocol performed. The following LightCycler protocol was used: denaturation program (95 °C for 10 min), 45 cycles of amplification and quantification with four segments of: denaturation at 95 °C for 2 min, anneling at 60 °C for 3 min, elongation at 72 °C for 13 min and a fluorescence acquisition at 88 °C for 2 min for the TNFα cDNA; 95 °C for 3 min, 56 °C for 10 min, 72 °C for 10 min and 82 °C for 2 min for the TNFR1 cDNA; 95 °C for 5 min, 56 °C for 5 min, 72 °C for 10 min and 82 °C for 2 min for the TNFR2 cDNA; 95 °C for 3 min, 60 °C for 5 min, 72 °C for 12 min and 82 °C for 2 min for the β-Actin cDNA, followed by a melting curve program (60-99 °C, with a heating rate of 0.1 °C/s and continuous fluorescence measurements), and finally a cooling program down to 40 °C.

All PCR reactions for cytokine mRNA quantification were performed in duplicate by the same blinded (for clinical data) researcher.

Calibration curves

To prepare the standard curve, PCR products encoding TNFα, TNF-RI, TNF-RII and β-actin were cloned in the pCRII-TOPO (TOPO TA cloning kit; Invitrogen, Carlsbad, CA, USA) and sequenced in both directions using the ABI PRISM 310 Genetic Analyzer. The plasmid DNA template was rendered linear with Eco RI and transcribed with T7 and SP6 RNA polymerases (Riboprobe Transcription Systems; Promega, Madison, WI, USA), producing RNA strands. The concentration of the synthesized RNA was measured by spectrophotometry and stored as a stock solution. Serial logarithmic dilutions of each plasmid were prepared to generate standard curves.

Statistical analysis

Statistical comparisons were performed using the Statistical Package for Social Sciences (SPSS) version 10.0, Chicago IL, USA. Descriptive results of continuous variables are expressed as mean ± SD. Before statistical analysis, normal distribution and homogeneity of the variables were tested. Comparison of variables between groups of subjects was performed using Student's test. Relationships between variables were determined by Pearson's correlation coefficient and stepwise multivariate linear regression analysis with forward selection. Levels of statistical significance were set at $p < 0.05$.

RESULTS

Clinical and metabolic characteristics of the subjects included in the study

At the end of the study, results from 11, healthy and 14, type 2 diabetic women were suitable for analysis. One healthy woman was excluded due to the detection in serum of a CRP protein above the upper normal value. For the remaining women, CRP levels were within the normal ranges of our laboratory. The main clinical and metabolic characteristics are shown in *Table 2*. Healthy, obese women were slightly younger than the DM2 group (52.0 ± 2.9 versus 59.9 ± 6.38; $p = 0.001$ respectively). BMI and blood pressure were similar in both groups. Glucose and HbA1c were significantly higher in DM2 patients, and the lipid parameters showed high plasma levels of total cholesterol and HDLc in the healthy women's group *(Table 2)*. The mean age at diagnosis in the DM2 group was 50.3 ± 7.9 years and the time of evolution of diabetes at the moment of inclusion into the study was 10.1 ± 6.9 years. All of the DM2 patients had normal serum creatinine levels.

Primer specificity and sequence analysis

To determine the specificity of the real-time RT-PCR assay developed in this study, the RT-PCR products were sepa-

Table 2
Anthropometric and clinical characteristics of the studied groups

	Obese women (n = 11)	Type 2 diabetic women (n = 14)
Age (years)	52.0 ± 2.9	59.9 ± 6.3**
BMI (kg m^{-2})	32.1 ± 4.8	31.6 ± 3.8
SBP (mm Hg)	126.4 ± 6.1	135.7 ± 24.4
DBP (mm Hg)	80.0 ± 6.6	75.3 ± 7.7
Total cholesterol (mmol/L)	6.0 ± 0.6*	5.1 ± 0.8
HDL-cholesterol (mmol/L)	1.6 ± 0.2*	1.2 ± 0.2
Triglycerides (mmol/L)	1.1 ± 0.5	1.3 ± 0.6
HbA^{1c} (%)	4.8 ± 0.1	7.5 ± 1.6**
Plasma glucose (mg/dL)	91.8 ± 10.8	181.8 ± 45.1**
sTNFR1(ng/ml)	1.8 ± 0.1	2.0 ± 0.4
sTNFR2 (ng/ml)	5.1 ± 0.8	6.92 ± 1.7*
sTNFR2/sTNFR1 (ratio)	2.8 ± 0.4	3.4 ± 0.7*

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein.
Data are expressed as mean ± SD. Student's T-test * $p < 0.05$ ** $p < 0.001$

rated using high resolution gel electrophoresis and resulted in a single product with the desired length *(Table 3)*. The specificity of the RT-PCR products was additionally documented using melting curve analysis by LightCycler software 3.5 (Roche). On the other hand, sequencing of the amplification products from the RNAs transcribed *in vitro* from plasmid pCRII-TOPO always gave the expected nucleotide sequence previously known, confirming that no nucleotide misincorporation due to Taq polymerase activity had occurred during the PCR procedures.

Real-time RT-PCR assay validation

All real-time assays performed were product-specific, and effective, PCR amplification kinetics were shown by high PCR efficiency *(Table 3)*. Assay sensitivities were confirmed by detection limits down to a few molecules. PCR efficiency (E) was calculated from the given slope of the calibration curve in the LightCycler software, according to the equation: $E = 10^{[-1/slope]}$ [21]. Test linearity was given between 10^2 and 10^{11} molecules, demonstrating linear quantification over a wide quantification range. To confirm accuracy and reproducibility of real-time PCR, the intra- and inter-assay variations were determined *(Table 3)*. The advantage of a high temperature fluorescence acquisition in the fourth segment during the amplification program results in reliable and sensitive mRNA quantification with high linearity (Pearson correlation coefficient: $r > 0.95$), up to nine orders of magnitude.

TNF system in the whole study group

The soluble fractions of TNFR2 and the ratio between sTNFR2 and sTNFR1 were at a significantly higher level in the DM2 group than in the healthy women *(Table 3)*. sTNFR1 levels showed no differences. The mononuclear mRNA expression in the basal state for both soluble receptors, showed no statistical differences, however, the ratio of TNFR2 mRNA (mTNFR2) and TNFR1 mRNA (mTNFR1) appears to be significantly higher in DM2 women than in the healthy group (2.58 ± 1.94 versus 1.01 ± 1.30; $p = 0.025$ respectively), even after adjusting for age ($r = 0.63$, $p = 0.021$) *(Figure 1)*. Considering all subjects, we observed a significant, positive correlation between the sTNFR2/sTNFR1 ratio and age and glucose levels ($r = 0.45$; $p = 0.027$ and $r = 0.51$; $p = 0.013$ respectively). Likewise, a positive correlation between the mTNFR2/mTNFR1 expression and blood glucose levels was observed ($r = 0.5$; $p = 0.029$) (Figure 2A). In a multiple, linear regression analysis, controlling for age and glucose levels, the sTNFR2/sTNFR1 ratio was only positively correlated with glucose (B = 0.096 IC 0.013-0.18, $p = 0.025$). Likewise the mTNFR2/mTNFR1 ratio remained significantly associated with glucose levels (B = 0.42 IC 0.016-0.84, $p = 0.043$).

If we analyze the population affected by DM2 separately, the basal expression of the mTNFR2/mTNFR1 ratio was only positively correlated with the BMI ($r = 0.57$; $p = 0.04$) *(Figure 2B)*. In the group of healthy, obese women, a negative correlation between sTNFR2 and the mTNFR2/mTNFR1 ratio ($r = -0.83$; $p = 0.021$) was observed. No significant correlation between the basal receptor expression and fat mass and fat-free mass was noted.

The TNF-α mRNA content in PBMC was below the detection limit of the technique in all study samples, barring the patient affected by rheumatoid arthritis.

Expression pattern of mTNFRs in healthy women during the OGTT

A positive correlation was observed between the basal expression of mTNFR1 and mTNFR2, and their expression at 60 min after glucose ingestion ($r = 0.89$; $p = 0.001$ and $r = 0.80$; $p = 0.017$ respectively). No significant increase in the mRNA expression levels was seen at 60 min and 120 min during the OGTT for either recep-

Table 3
Characteristics and validation parameters of real-time PCR

	TNFR1	TNFR2	TNFα	β-actin
Product length (bp)	123	251	325	311
PCR efficiency	1.84	1.79	2.2	2.1
Detection limit (m)	42	41	40	40
Quantification limit (m)	420	410	400	40
Quantification range (m)	420-4 × 10^{11}	410-4 × 10^{11}	400-4 × 10^{11}	40-4 × 10^{11}
Test linearity (r)	0.99	0.97	0.95	0.99
Intra-assay variation (%)	2% (n = 3)	0.7% (n = 3)	ND	2% (n = 3)
Inter-assay variation (%)	14% (n = 3)	3.5% (n = 3)	ND	2.5% (n = 3)

TNFR1: tumor necrosis factor receptor 1. TNFR2: tumor necrosis factor receptor 2. TNFα: tumor necrosis factor alpha.
(bp) base pairs
(m) molecules

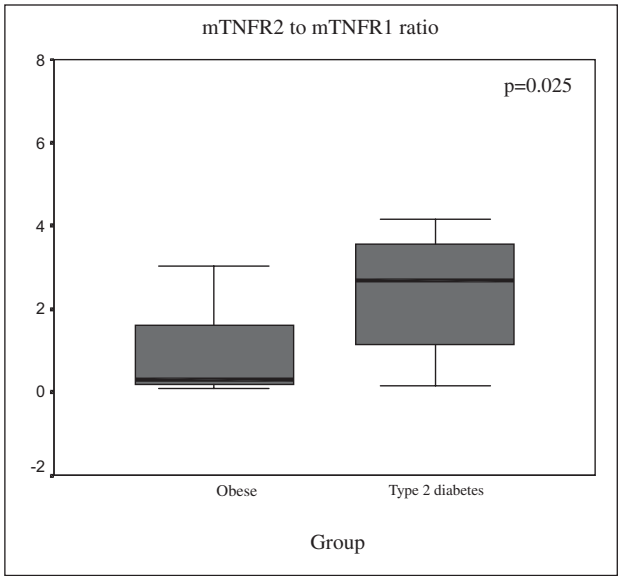

Figure 1
Basal expression of TNFR2 mRNA to TNFR1 mRNA ratio in healthy, obese (n = 11) and type 2 diabetic women (n = 14), all the samples were performed in duplicate.

tor. In order to predict the 60 min response of mRNA of both receptors after the OGTT, multiple linear regression analysis was performed. For the mTNFR1 60 min response, a positive association with basal levels ($p < 0.001$) and with BMI ($p = 0.014$) was observed. When 60 min mTNFR2 was considered as a dependent variable, a significant, positive association with basal mTNFR2 levels ($p < 0.001$) and with the plasma trygliceride levels ($p < 0.001$) was observed.

TNFα mRNA was not detected during the OGTT in healthy women.

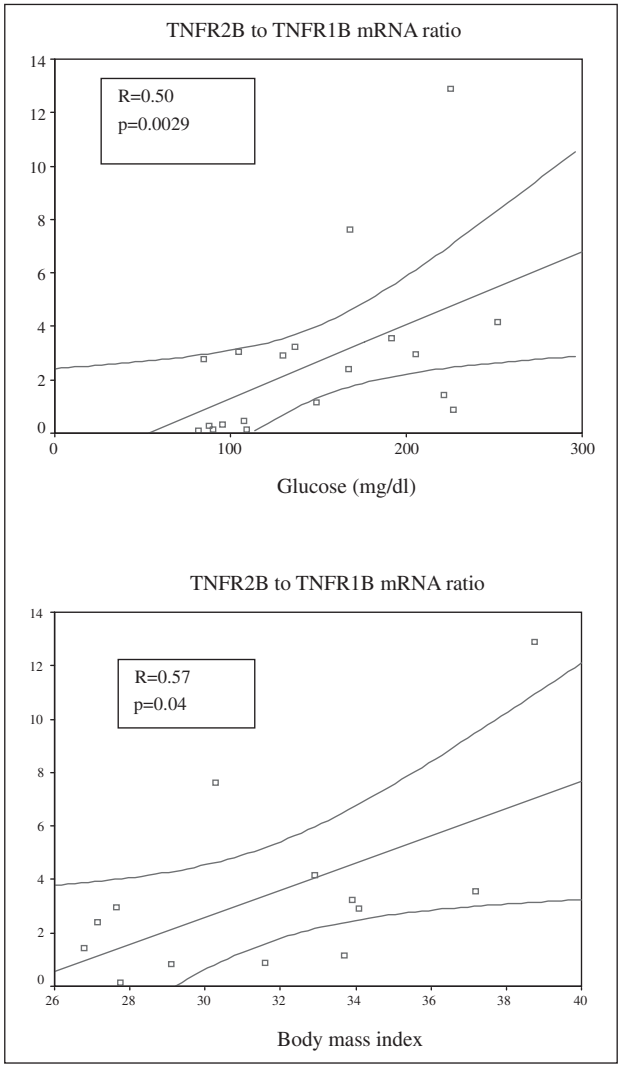

Figure 2
A: Correlation between the TNFR2 to TNFR1 mRNA ratio and glucose in healthy, obese women (n = 25). B: Correlation between the TNFR2 to TNFR1 mRNA ratio and body mass index in diabetic women (n = 14).

DISCUSSION

The aim of this study was to assess the in vivo state of the TNFα system in PBMC from healthy, obese and type 2 diabetic women. The data obtained provide evidence, for the first time, of an increased activation of TNFR mRNA in obese, type 2 DM patients.

Abnormalities in immune system function and in inflammatory mediators have been claimed to be responsible for obesity and type 2 diabetes. In fact, a recent hypothesis suggests a common inflammatory process in the pathogenesis of insulin-resistance disorders, with the direct participation of the TNF-system [22]. The majority of models of experimental obesity and insulin resistance seem to produce significantly higher levels of TNFα mRNA and protein compared to lean counterparts [23-26]. The impairment of pro-inflammatory cytokines has been proposed as an important pathogenic mechanism linking obesity and type 2 diabetes [4, 27, 28]. Elevated circulating levels of TNFα protein and its soluble receptors have been clearly demonstrated in obesity and/or insulin resistance syndromes, mainly in women [29]. In our population, sTNFR2 was significantly greater in diabetic women *(Table 3)*, in agreement with previous observations [29], suggesting greater synthesis and secretion of this protein in muscle and adipose tissue in the context of a higher insulin resistance, as is observed in type 2 diabetes [4, 27]. However, it should be noted that the plasma levels of this soluble fraction may arise from different sources, including the complex equilibrium between the synthesis and the shedding of both soluble TNFα receptors. Besides the well known mechanisms involved in TNFα receptor shedding, our group has recently demonstrated that insulin resistance is associated with decreased TNFR1 shedding in monocytes from type 2 diabetic patients, contributing to increased serum levels of sTNFR2 and an increased sTNFR2/sTNFR1 ratio [12], as observed in the diabetic women in our study. Interestingly, the basal ratio of mTNFR2/mTNFR1 expression was clearly increased in DM2 patients compared to non-diabetic, obese women *(Figure 1)*, suggesting a higher proportion of mTNFR2 synthesis in PBMC of diabetic patients, which may contribute to the increase in the sTNFR2 plasma levels observed in DM2 subjects. The negative correlation observed in the group of healthy, obese women between sTNFR2 and the mTNFR2/mTNFR1 ratio ($r = -0.83$; $p = 0.021$), supports previous observations in which TNFR1 stimula-

tion was capable of preferentially transactivating TNFR2 shedding in the PBMC [30].

The response of cells to TNFα can be regulated by the number of functional receptors on the cell surface, which is the result of the balance between synthesis and shedding. It is tempting to speculate that type 2 diabetic patients may be genetically predisposed to present a higher TNFR2 synthesis and a lower shedding of cell surface TNFR1 than non-diabetic, obese subjects for similar levels of circulating TNFα. We need further, *in vitro* studies analyzing the cytokine mRNA expression in PBMC and the shedding of the cell surface receptors to confirm this hypothesis.

It is known that, in human obesity, the soluble TNFα receptors, mainly sTNFR2, have an increased expression in adipose tissue of obese subjects compared to lean subjects [29, 31]. However, no significant differences in adipose tissue expression levels have been observed between obese individuals with and without type 2 diabetes [31-33]. Interestingly, in our study the basal expression ratio of mTNFR2/mTNFR1 was only positively correlated with BMI in DM2 women and with glucose in the entire obese group. It is tempting to extrapolate the changes observed in PBMC to the adipose tissue, and speculate a predominant role of glucose in receptor expression in the context of obesity, and only when diabetes has been established would BMI have a modulating effect over these receptors.

The effect of *in vivo* hyperglycemia on TNFα and TNFRs receptor expression in humans remains unknown, despite the fact that the OGTT can be considered a reflection of postprandial glucose excursion. In our healthy, obese women, there were no differences in TNFRs receptor mRNA expression in the PBMC during the OGTT. Although there was a striking variation of expression rates in this group, the pattern of response depended on different variables according to the receptor evaluated. Thus, for TNFR1 the 60 min response was positively associated with the basal expression levels and with BMI. Interestingly, when the expression of this receptor was evaluated in subcutaneous adipose tissue from obese individuals, it was found to be positively correlated only with BMI and fat cell size [31]. In contrast, the response observed at 60 min for TNFR2 in our obese women, showed a positive association with TNFR2 basal expression and with tryglyceride plasma levels, suggesting a similar regulation pattern to that previously observed in the basal state in subcutaneous adipose tissue for TNFR2 and TNFα mRNA content in obese women [31].

These findings support the idea that the paracrine/autocrine regulatory mechanisms observed in adipose tissue may work in a similar manner in PBMC, at least in non-diabetic, obese women. The acute increase in glucose levels observed mainly at 60 min after glucose ingestion did not produce a clear increase in TNF receptor expression in PBMC in normotolerant women. These results suggest that an increase in glucose levels as observed during the post-prandial state, does not substantially modify the cytokine expression profile in PBMC in obese, non-diabetic women; however, we cannot exclude an effect of chronic hyperglycemia on the PBMC response after an acute glucose increase, as observed after ingestion in type 2 diabetic patients. In fact, in a recent publication, an acute intravenous glucose administration (about 15 mol/l), produced a transitory rise in TNFα and IL6 plasma levels in healthy and glucose intolerant subjects [16], suggesting a role for the oxidative stress in this response, as was previously proved by *in vitro* experiments [34]. The absence of a clear response of TNFα and its receptor expression after the OGTT in our obese women, may be related to the lower hyperglycemic stimulus obtained and the fact that it is produced in a different way. We believe that these findings require further investigation, in other populations with increased cardiovascular risk, such as patients with glucose intolerance, in order to clarify the pathophysiological role of proinflammatory cytokines during postprandial hyperglycemia in cardiovascular events [35].

In conclusion, this study provides evidence that type 2 diabetes is characterized by proportionally elevated levels of TNFR2 mRNA in PBMC than in PBMC from obese, healthy women. Moreover, these are the first data concerning the *in vivo* response of TNF system expression in PBMC after an oral glucose administration, suggesting a similar regulatory pattern to that observed in adipose tissue [29, 31]. Further studies are required to better understand the complexity of the regulatory balance between TNFα and its receptors in the development of obesity-linked, insulin resistance and type 2 diabetes.

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