Lowered Tumor Necrosis Factor Receptors, but Not Increased Insulin Sensitivity, with Infliximab

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Abstract

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Objectives: To verify whether Infliximab could modify insulin sensitivity and *TNF-α* and *GLUT4* mRNA expression in muscle and adipose tissue of morbidly obese subjects. Soluble TNF receptors I and II (TNFR-I and TNFR-II) were also assayed.

Research Methods and Procedures: Six obese subjects were investigated before and 2 weeks after a single intravenous administration of 5 mg/kg Infliximab; insulin sensitivity was evaluated by euglycemic hyperinsulinemic clamp, and *TNF-α* and *GLUT4* mRNA expression were assessed by reverse-transcriptase polymerase chain reaction on muscle and adipose tissue. TNF-α, TNFR-I, and TNFR-II were determined using the ELISA technique.

Results: Infliximab infusion did not affect fasting plasma insulin or fasting plasma glucose levels; whole body glucose uptake did not change significantly. *TNF-α* and *GLUT4* mRNA did not show any significant change in muscle or adipose tissue. Serum TNF-α was undetectable before and after treatment, whereas TNFR-I and TNFR-II concentrations significantly decreased ($p < 0.01$).

Discussion: An explanation for the absence of effect of Infliximab on insulin resistance in morbidly obese subjects may be the paracrine way of action of this cytokine. Because Infliximab is predominantly distributed within the vascular compartment, its effectiveness in penetrating muscle and adipose tissue is potentially low. The significant decrease of TNFR-I and TNFR-II might be ascribed to a targeted effect of Infliximab on the immune system.

Key words: Infliximab, insulin sensitivity, euglycemic hyperinsulinemic clamp, tumor necrosis factor α receptors

Introduction

Recently, the role of tumor necrosis factor α (TNF-α)[1] has been stressed in the development of insulin resistance in animals and humans (1–4). In fact, the complete lack of TNF-α function through targeted mutations in the *TNF-α* gene or both of its receptors results in a significant improvement of insulin sensitivity in dietary, chemical, and genetic models of rodent obesity (5). The molecular mechanism underlying this phenomenon is represented by the action of TNF-α on the early steps of the insulin-signaling cascade, leading to an impaired insulin-stimulated glucose transport (6). In fact, TNF-α seems to reduce the phosphorylation of insulin receptor substrate 1 (IRS 1), with subsequent impairment of the insulin uptake by its receptor and, thus, a frank reduction of insulin-mediated glucose uptake.

TNF-α is expressed not only in adipose tissue but also in human muscle, which is the major site of insulin-mediated glucose uptake. The degree of *TNF-α* expression is higher in muscle tissue and cultured muscle cells from insulin-resistant and diabetic subjects than normal subjects (7).

Infliximab is a chimeric IgG human monoclonal antibody that links with high affinity and specificity to both soluble and transmembrane forms of TNF-α. This drug inhibits the

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[1] Nonstandard abbreviations: TNF-α, tumor necrosis factor α; IRS 1, insulin receptor substrate 1; TNFR-I, tumor necrosis factor receptor I; TNFR-II, tumor necosis factor receptor II; TBW, total body water; EHC, euglycemic hyperinsulinemic clamp; RT-PCR, reverse-transcriptase polymerase chain reaction; FFM, fat-free mass; FM, fat mass.

binding of TNF-α with its receptors in vitro through the formation of stable complexes and, therefore, leads to the loss of the biological activity of this cytokine (8).

The effect of the neutralization of TNF-α with anti-TNF antibodies has been studied in animal models. Hotamisligil et al. (9,10) showed that this neutralization results in an increase in insulin-stimulated autophosphorylation of both the insulin receptor and IRS 1 in muscle and adipose tissue, causing a significant increase in the peripheral glucose uptake in response to insulin.

To our knowledge, only two studies in the literature concern the use of anti-TNF-α antibodies in obese insulin-resistant subjects. The first shows that the administration of the anti-TNF-α antibody CDP751 does not affect insulin sensitivity in obese type 2 diabetic subjects (11). Similarly, negative results have been reported when a recombinant TNF-α receptor:Fc fusion protein was administered to obese insulin-resistant patients (12).

The aim of this study was to verify whether intravenous administration of Infliximab has the ability to improve the insulin-mediated glucose uptake and the *TNF-α* and *GLUT4* mRNA expression in muscle and adipose tissue of morbidly obese insulin-resistant subjects. Serum soluble TNF receptors I and II (TNFR-I and TNFR-II) serum concentrations were also assayed because their variations represent an index of TNF-α concentration change.

Research Methods and Procedures

Subjects

Six obese white subjects (three men and three women; age, 25.5 $\pm$ 11.2 years; BMI, 46.9 $\pm$ 6.4 kg/m^2) were enrolled in this study. All subjects gave their written consent, and the experimental protocol was approved by the Ethical Committee of the Catholic University of Rome. The nature and purpose of the study were carefully explained to all participants before obtaining their written consent to participate.

Subjects had maintained a stable body weight for at least 2 months before the beginning of the protocol. They were not on regular medication and did not show known complications of obesity, such as established hypertension, diabetes, impaired glucose tolerance, or dyslipidemia. None showed signs of allergic, autoimmune, or chronic infectious diseases.

All subjects were investigated before and 2 weeks after a single intravenous administration of Infliximab at the dose of 5 mg/kg. Muscle and adipose tissue biopsies were performed twice to measure *TNF-α* and *GLUT4* mRNA.

Body Composition

Body weight was measured to the nearest 0.1 kg using a beam scale, and height was measured to the nearest 0.5 cm using a stadiometer (Holatin; Crosswell, Wales, United Kingdom). Total body water (TBW) was determined using 0.19 becquerels of tritiated water in 5 mL of saline solution administered as an intravenous bolus injection (13). Blood samples were drawn before and 3 hours after the injected dose. The disintegrations/min were counted in duplicate in 0.5 mL of plasma using a beta-scintillation counter (Model 1600TR; Canberra-Packard, Meriden, CT). Corrections were made (5%) for nonaqueous hydrogen exchange (14), and water density at body temperature was assumed to be 0.99371 kg/L. TBW (kilograms) was computed as ^3H_2O dilution space (liters) $\times$ 0.95 $\times$ 0.99371. The within-person day-by-day coefficient of variation reported for this method is 1.5% (15).

Euglycemic-Hyperinsulinemic Clamp procedure

Peripheral insulin sensitivity was evaluated by the euglycemic-hyperinsulinemic clamp (EHC) procedure (16). After insertion of a cannula in a dorsal hand vein for sampling arterialized venous blood and another one in the antecubital fossa of the contralateral arm for infusions, the subjects rested in a supine position for at least 1 hour. The subjects were placed with one hand in a heated air box set at 60 °C to obtain arterialized blood samples. Whole-body glucose uptake (M value) in micromoles per kilograms of fat-free mass per minute was determined during a primed-constant infusion of insulin (at the rate of 7 pmol/min per kilogram). The fasting plasma glucose concentration was maintained throughout the insulin infusion by means of a variable glucose infusion and blood glucose determinations every 5 minutes. Whole-body peripheral glucose use was calculated during the last 40-minute period of the steady-state insulin infusion. At the time of the clamp studies, subjects were on a weight-maintaining diet consisting of at least 250 grams of carbohydrate/d for at least 1 week before each study.

Blood Chemistry

Plasma glucose levels were measured by a glucose-oxidase method (Beckman, Fullerton, CA). Serum immunoreactive insulin was assayed using a microparticle enzyme immunoassay (Abbott, Pasadena, CA).

Muscle and Subcutaneous Adipose Tissue Biopsies

While patients were under local anesthesia, muscle biopsies were obtained from the middle region (15 cm above the patella) of the vastus lateralis portion of the quadriceps femoris muscle by the percutaneous needle-biopsy technique using 14-gauge, 20-cm needles (Gallini Medical Devices, Brussels, Belgium). Adipose tissue biopsies were obtained with the same technique and needle from the subcutaneous area of the same region before and 2 weeks after Infliximab treatment. Tissue samples were immediately placed in liquid nitrogen and stored at -80 °C for further analysis.

Semiquantitative RT-PCR

Frozen muscle biopsies were pulverized in liquid nitrogen, and total cellular RNA was obtained using guani-

dinium thiocyanate-phenol-chloroform extraction. The amount of RNA was quantified spectrophotometrically, and its purity was assessed by the ratio 260:280.

The Perkin Elmer Gene Amp RNA PCR kit was used for all the reverse-transcriptase polymerase chain reaction (RT-PCR) reactions, which were performed in the Gene Amp PCR System 9600 (Perkin Elmer Cetus, San Diego, CA). After removal of contaminating chromosomal DNA with DNase I treatment, 200 ng were reverse transcribed with 2.5 units of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI) at 42 °C for 30 minutes. Two micrograms of cDNA products were used in each PCR reaction. The PCR reactions were carried out using different cycling parameters for each set of primers. The following primers were used: for *GLUT4*, 5′-CCCCCTCAGCAGCGAGTGA-3′ and 5′-GCACCGCCAGCACATTGTTG-3′; and for *TNF-α*, 5′-TGTTGCCCCTGGTCATTTTC-3′ and 5′-CCGCAAAGTTGGGACAGTCA-3′. The target cDNAs were coamplified with myoglobulin as an internal control using the following set of primers at a concentration of 0.24 μM: 5′-ACCCCCACTGAAAAAGATGA-3′ and 5′-ATCTTCAAACCTCCATGATG-3′.

The number of cycles and reaction conditions were chosen to ensure that none of the target cDNA reached a plateau and that the two pairs of primers would not compete with each other.

GLUT4 was amplified with 1 U of AmpliTaq Gold DNA polymerase and 4 mM $MgCl_2$ using primers at a final concentration of 0.06 μM. A first incubation of 10 minutes at 95 °C, 45 seconds at 65 °C, and 1 minute at 72 °C was followed by 26 cycles of 45 seconds at 95 °C, 45 seconds at 65 °C, and 1 minute at 72 °C. *TNF-α* was amplified in 1 U of AmpliTaq Gold DNA polymerase and 5 mM $MgCl_2$, using a final concentration of primers of 0.12 μM. A first incubation of 10 minutes at 95 °C, 45 seconds at 65 °C, and 1 minute at 72 °C was followed by 30 cycles of 45 seconds at 95 °C, 45 seconds at 65 °C, and 1 minute at 72 °C. The myoglobulin was coamplified with target cDNA using the primers at a final concentration of 0.24 μM.

PCR products were electrophoresed on a 2% agarose gel; images on the ethidium bromide-stained gels were acquired by a Cohu charge-coupled device camera and quantified with Phoretix 1D (Phoretix International, Newcastle on Tyne, United Kingdom).

ELISA

The determination of circulating TNF-α and soluble TNFR-I and TNFR-II was performed using the quantitative sandwich enzyme immunoassay technique according to the manufacturer's indications (Quantikine; R&D Systems, Minneapolis, MN).

Statistical Analysis

Statistical analysis was performed using SPSS 10.1 for Windows (SPSS Inc., Chicago, IL). Intragroup comparison was done using a Wilcoxon test. Spearman correlation coefficients and regression analyses were used to examine association of M values with soluble TNFR levels. Results were considered significant at the $p \leq 0.05$ level.

Table 1. Characteristics of the patients

	Pretreatment	Post-treatment
Weight (kg)	132.54 ± 28.30	131 ± 29.08
BMI (kg/m^2)	46.80 ± 6.42	46.74 ± 6.55
Percent body fat	49.33 ± 5.81	49.16 ± 5.81
Fasting glucose (mM)	4.76 ± 0.34	4.69 ± 0.28
Insulin (pM/l)	159.18 ± 83.52	155.58 ± 91.50
M values (μmol/kg$_{FFM}$ per minute)	21.6 ± 9.48	20.64 ± 8.34
TNFR-I (pg/mL)	1470.6 ± 224.9	1215.4 ± 203.1*
TNFR-II (pg/mL)	3388.6 ± 886	2512.9 ± 590.4*

* $p < 0.001$.

Results

The anthropometric characteristics of the patients are summarized in Table 1. Body weight remained constant during the study period in all subjects (±1 kg). No variation was observed in body composition [fat-free mass (FFM): 57.16 ± 5.82% vs. 57.33 ± 5.81%; fat mass (FM): 42.83 ± 5.81% vs. 42.66 ± 5.81%].

Neither fasting plasma insulin concentration nor fasting plasma glucose levels changed after treatment (159.18 ± 83.52 vs. 155.58 ± 91.50 pM, p = not significant; 4.76 ± 0.34 vs. 4.69 ± 0.28 mM, p = not significant); whole body glucose uptake was not significantly affected by Infliximab infusion (21.6 ± 9.48 vs. 20.64 ± 8.34 μmol/kg$_{FFM}$/min, p = not significant).

The levels of *GLUT4* mRNA, expressed as a ratio to the internal control β_2-microglobulin mRNA [relative amounts (RA)], in muscle and adipose tissues showed no significant changes (muscle: 4.80 ± 0.38 vs. 5.38 ± 2.69 RA; adipose tissue: 1.347 ± 0.671 vs. 1.148 ± 1.055 RA; Figure 1). Similarly, no significant variation of *TNF-α* mRNA levels was observed in either tissue (muscle: 1.50 ± 0.6 vs. 1.7 ± 0.8 RA; adipose tissue: 0.495 ± 0.236 vs. 0.487 ± 0.179 RA; Figure 2).

Serum TNF-α was undetectable before and after Infliximab administration.

Soluble receptor TNFR-I concentrations in serum significantly decreased after treatment (1470.6 ± 224.9 vs. 1215.4 ± 203.1 pg/mL, $p < 0.01$), as did TNFR-II levels (3388.6 ± 886.8 vs. 2512.9 ± 590.4 pg/mL, $p < 0.01$).

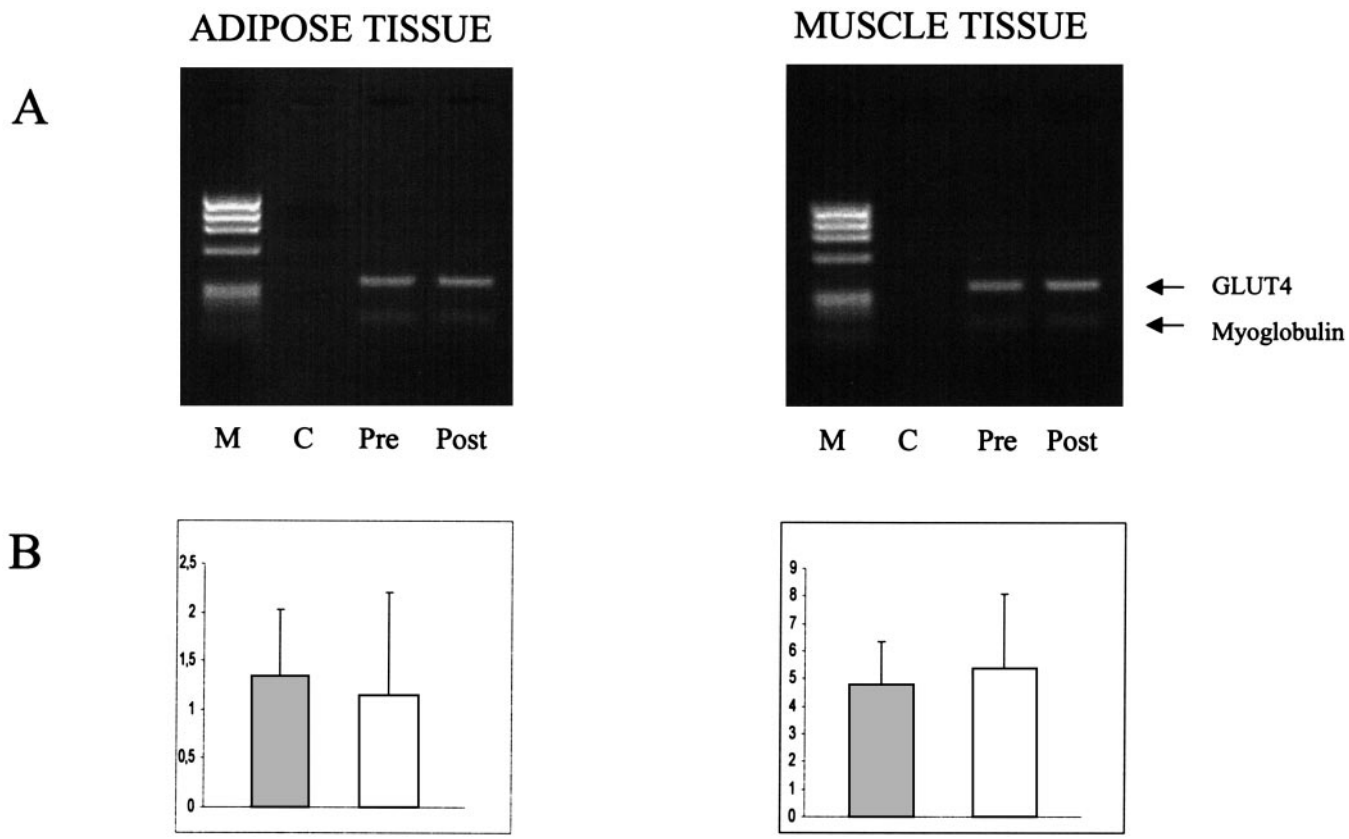

Figure 1: GLUT4 mRNA expression before and after Infliximab administration in skeletal muscle and adipose tissue biopsies. (A) Representative agarose gel showing RT-PCR analysis of *GLUT4* and myoglobulin mRNA content in muscle and adipose tissue of one subject before and after Infliximab administration (M, marker; C, negative control). (B) Densitometric analysis of the ratio *GLUT4*: myoglobulin mRNA abundance before (dark bar) and after (light bar) treatment. Bars represent the mean ± SD of six subjects.

Discussion

The major result of this study was that the acute infusion of Infliximab did not improve insulin-mediated glucose uptake in morbidly obese subjects. This drug did not affect *TNF-α* and *GLUT4* gene expression in skeletal muscle and adipose tissue, although the levels of soluble TNFR-I and TNFR-II were significantly reduced.

Concerning the use of specific anti-TNF-α antibodies in insulin-resistant subjects, Ofei et al. (11) showed that, in obese type 2 diabetic subjects, TNF-α neutralization over a period of 4 weeks with its specific antibody CDP751, which was administered in a single dose of 5 mg/kg_{bw}, did not affect insulin sensitivity, measured by insulin-tolerance test. Using EHC, Paquot et al. (12) did not find changes in insulin sensitivity in obese insulin-resistant patients after a single intravenous dose of a recombinant TNF-α receptor:Fc fusion protein, which consists of the soluble TNF receptor (p55) linked to the Fc portion of human IgG1.

Infliximab is the commercial form of the experimental drug used by Ofei et al. (11). Because the patients examined were obese and had type 2 diabetes, and because insulin sensitivity was not assessed by EHC, which is commonly considered the gold standard for evaluating whole-body glucose disposal, we tested the efficacy of Infliximab in obese insulin-resistant subjects with normal glucose tolerance. In fact, should the efficacy of Infliximab have been shown in terms of improvement of insulin sensitivity, the cost/benefit ratio might have been in favor of the use of this drug instead of, for example, thiazolidinediones, because three administrations per year should be sufficient.

Our data showed that a single intravenous infusion of Infliximab in morbidly obese subjects at the dose of 5 mg/kg_{bw} was ineffective on the degree of insulin resistance. Accordingly, *TNF-α* and *GLUT4* mRNA expression in muscle and subcutaneous adipose tissue taken by biopsies was unchanged after treatment.

A novel and interesting finding is, however, that serum concentrations of soluble TNFR-I and TNFR-II were significantly decreased.

Previous studies have shown that anti-TNF-α antibody levels remain elevated over a period of 4 weeks (11). Infliximab has a prolonged half-life (9.5 days) and is predominantly distributed within the vascular compartment. Therefore, its effectiveness in penetrating inside muscle and

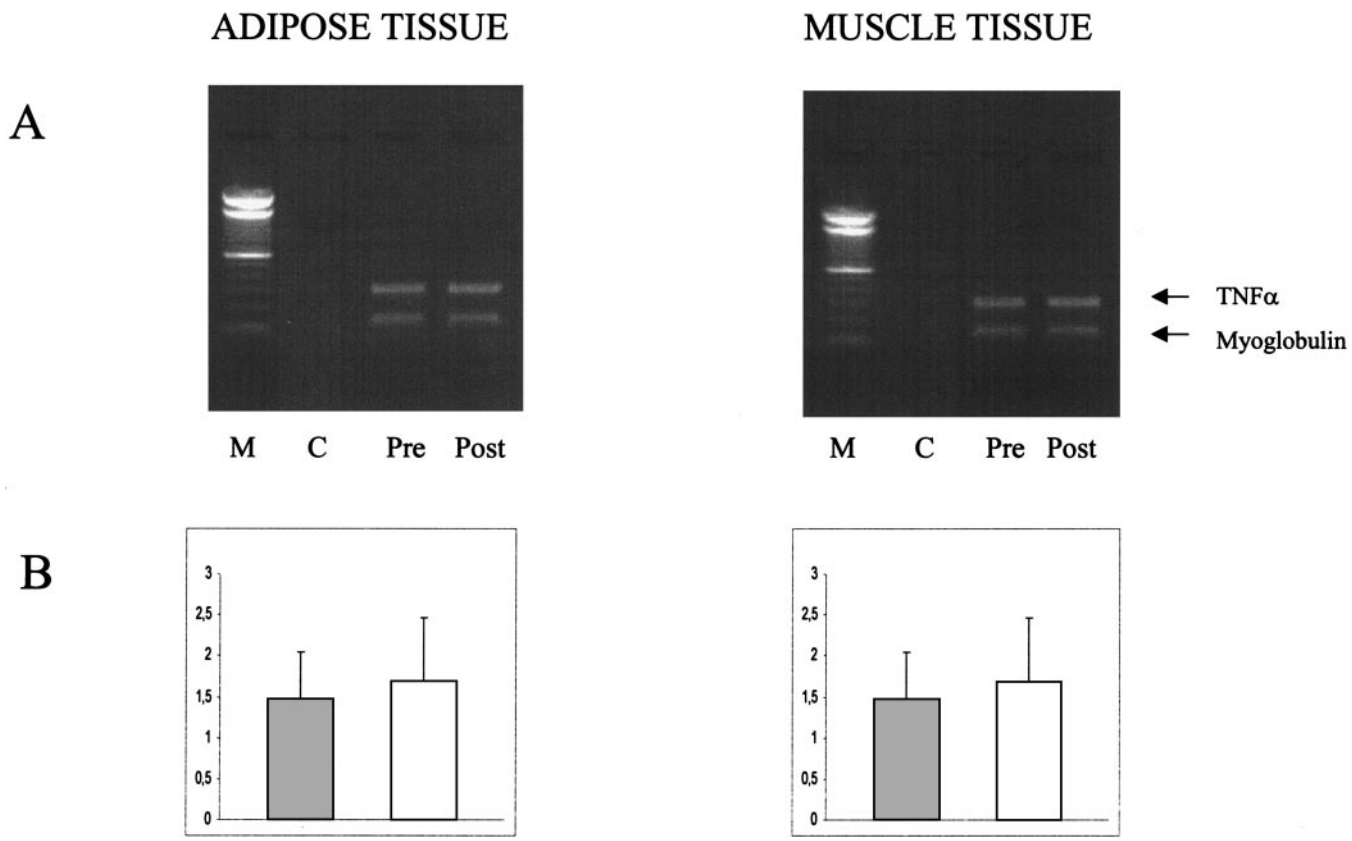

Figure 2: TNF-α mRNA expression before and after Infliximab administration in skeletal muscle and adipose tissue biopsies. (A) Representative agarose gel showing RT-PCR analysis of *TNF-α* and myoglobulin mRNA content in muscle and adipose tissue of one subject before and after Infliximab administration (M, marker; C, negative control). (B) Densitometric analysis of the ratio *TNF-α*:myoglobulin mRNA abundance before (dark bar) and after (light bar) treatment. Bars represent the mean ± SD of six subjects.

adipose tissue should be potentially low. Infliximab acts in the vascular compartment, with the induction of proinflammatory cytokines and acute-phase proteins (17,18). It has been demonstrated that serum concentrations of both soluble TNFRs are elevated in obesity and other insulin-resistant states (19,20).

Variations in the concentration of plasma soluble TNFRs after weight loss and exercise training have also been demonstrated (21,22). TNFR-II is better correlated to measures of insulin sensitivity (23).

Our study showed a significant correlation between TNFR-II and M values (Figure 3), although the average M

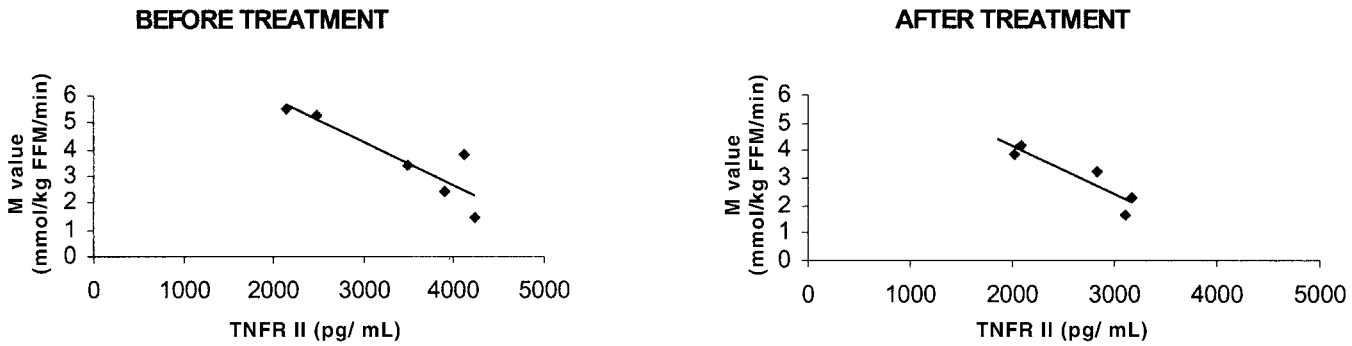

Figure 3: Correlation between insulin sensitivity (M values) and soluble TNFR-II concentration of obese patients before ($y = 0.0016x + 9.0421$; $R^2 = 0.7963$) and after ($y = 0.0018x + 7.6829$; $R^2 = 0.831$) Infliximab administration.

value did not change significantly. It has been hypothesized that, in obese subjects, adipose tissue might be an important source of TNFRs (24). Because, in our patients, body weight and FM remained constant, it is likely that the reduction of serum soluble TNFR-I and TNFR-II levels was independent of the modification of FM. Therefore, a possible explanation of this behavior in our patients could have been a targeted effect of Infliximab on the immune system, rather than its action on muscle and adipose tissue. Moreover, the lack of an effect of Infliximab on insulin sensitivity might depend on the low diffusion of this drug in skeletal muscle and fat tissue, whereas it seems likely that TNF-α acts in a paracrine way in these tissues (25). However, it cannot be excluded that insulin resistance might be mediated through other molecular mechanisms that do not include the TNF-α system. Thus, it is conceivable that a high liposolubility might allow Infliximab to enter the muscle and adipose cells, with a positive effect on insulin sensitivity.

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