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ARTICLE

Associations between insulin resistance and TNF-α in plasma, skeletal muscle and adipose tissue in humans with and without type 2 diabetes

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Abstract
Aims/hypothesis Clear evidence exists that TNF-α inhibits insulin signalling and thereby glucose uptake in myocytes and adipocytes. However, conflicting results exist with regard to the role of TNF-α in type 2 diabetes.
Methods We obtained blood and biopsy samples from skeletal muscle and subcutaneous adipose tissue in patients with type 2 diabetes (n=96) and healthy controls matched for age, sex and BMI (n=103).
Results Patients with type 2 diabetes had higher plasma levels of fasting insulin ($p<0.0001$) and glucose ($p<0.0001$) compared with controls, but there was no difference between groups with regard to fat mass. Plasma levels of TNF-α ($p=0.0009$) and soluble TNF receptor 2 (sTNFR2; $p=0.002$) were elevated in diabetic patients. Insulin sensitivity was correlated with quartiles of plasma TNF-α after adjustment for age, sex, obesity, WHR, neutrophils, IL-6 and maximum O_2 uptake ($\dot{V}O_2$/kg) in the diabetes group ($p<0.05$). The *TNF* mRNA content of adipose or muscle tissue did not differ between the groups, whereas muscle TNF-α protein content, evaluated by western blotting, was higher in type 2 diabetic patients. Immunohistochemistry revealed more TNF-α protein in type 2 than in type 1 muscle fibres.
Conclusions/interpretation After adjustment for multiple confounders, plasma TNF-α is associated with insulin resistance. This supports the idea that TNF-α plays a significant role in the pathogenesis of chronic insulin resistance in humans. However, findings on the TNF-α protein levels in plasma and skeletal muscle indicate that measurement of *TNF* mRNA content in adipose or muscle tissue provides no information with regard to the degree of insulin resistance.

Keywords Cytokines · Insulin resistance · Insulin sensitivity · Low-grade inflammation · Metabolic syndrome

Abbreviations

CRP	C-reactive protein
HOMA2-IR	Homeostasis model assessment of insulin resistance, version 2
proTNF-α	membrane-bound form of TNF-α
sTNFR2	Soluble TNF receptor 2
$\dot{V}O_2$	maximum O_2 uptake

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Introduction

Low-grade inflammation is associated with the metabolic syndrome and type 2 diabetes [1, 2]. Several studies have demonstrated association between circulating TNF-α, obesity and type 2 diabetes [3–5], whereas other studies have failed to do so [6, 7]. A direct effect of TNF-α on insulin sensitivity in skeletal muscle has been demonstrated in vitro [8], in vivo in animals [9] and in vivo in humans [10]. TNF-α inhibits the insulin signalling cascade at several pivotal regulatory proteins, such as the insulin receptor substrate (IRS) and Akt substrate 160 in human skeletal muscle in vitro [11] and in vivo [10]. These two studies indicate that elevated TNF-α is not secondary to the pathological conditions associated with insulin resistance, but that TNF-α plays a direct pathogenic role in glucose metabolism.

Impaired insulin sensitivity in skeletal muscle is a major feature of type 2 diabetes [12]. An increased amount of TNF-α protein in skeletal muscle has been found in patients with insulin resistance [13] and chronic obstructive pulmonary disease [14]. Regular exercise improves insulin sensitivity [15] and the amount of TNF-α protein is reduced by resistance exercise in frail elderly patients [16].

Obesity is strongly associated with reduced insulin sensitivity [17], and increased expression of *TNF* mRNA in human adipose tissue from obese patients has been demonstrated [18]; however, this is not a consistent finding [19]. Furthermore, it has been suggested that obese patients have a higher level of membrane-bound TNF-α than lean subjects [20], whereas release of TNF-α from human adipose tissue into the circulation has yet to be demonstrated in vivo [21]. Interestingly, it may not be the adipocytes per se, but the surrounding stromal cells—including macrophages—that produce most of the TNF-α in the adipose tissue [22].

Shedding of TNF receptor 2 (TNFR2) from plasma membranes seems changed in type 2 diabetes [23] and soluble TNFR2 (sTNFR2) has been demonstrated to correlate negatively with insulin sensitivity [23]. Furthermore, upon activation of TNFR2 the receptor is cleaved and shed into the circulation, where it can be measured as sTNFR2 [24]. The amount of sTNFR2 in the circulation could therefore be interpreted as the degree of activation of TNF-α signalling in the tissues, making sTNFR2 an interesting parameter for TNF-α activation in the tissues.

The aim of the present study was to test the hypothesis that elevated levels of TNF-α in plasma, muscle and adipose tissue and plasma levels of sTNFR2 are associated with insulin resistance independently of obesity. Given that type 2 diabetes and obesity often coexist and that several other factors might influence the levels of TNF-α, we studied the levels of TNF-α using a cross-sectional case-control design in which patients with type 2 diabetes and healthy controls were closely matched, not only according to age and sex but also according to BMI.

Methods

Study design A cross-sectional case–control design was employed. Participants (n=199) were recruited by advertising in a local newspaper and information on the diagnosis of type 2 diabetes was based on oral information from each subject. Participants received oral and written information about the experimental procedures before giving their written informed consent. The study was approved by the Ethics Committee of the Copenhagen and Frederiksberg Communities (KF 01-141/04). To verify the diagnosis, the WHO diagnostic criteria for type 2 diabetes were used, and the participants were thus divided into 103 healthy controls and 96 patients with type 2 diabetes. Participants were screened to isolate metabolic conditions other than type 2 diabetes that are known to influence body composition and the immune system. Exclusion criteria were treatment with insulin, recent or ongoing infection, a history of malignant disease, and known dementia. Participants reported to the laboratory between 08:00 and 10:00 hours after an overnight fast. They did not take any medication in the 24 h preceding the examination, and those with type 2 diabetes did not take their oral glucose-lowering medication for 1 week preceding the examination. A general health examination was performed. Sphygmomanometric measurement of brachial blood pressure was performed while the participants were resting in the supine position. Blood samples were drawn from an antecubital vein. An OGTT was performed on the same day.

OGTT Blood samples were drawn before and 1 and 2 h after the participant drank 500 ml of water containing 75 g of dissolved glucose. The WHO diagnostic criteria were applied. Participants found to have IGT were excluded from the study.

Plasma samples Blood samples were drawn into glass tubes containing EDTA, which were immediately spun at 3,500×g for 15 min at 4°C. Plasma was isolated and stored at −20°C until analysed. Plasma concentrations of TNF-α, sTNFR2 and IL-6 were measured by ELISA (R&D Systems, Minneapolis, MN, USA). Samples were analysed in duplicate and mean concentrations were calculated. In plasma, levels of cholesterol (HDL and LDL), triacylglycerol, C-reactive protein (CRP), glucose and insulin were measured using routine laboratory methods. Based on the fasting plasma concentrations of glucose and insulin, the level of insulin resistance was calculated using the homeo-

stasis model assessment of insulin resistance, version 2 (HOMA2-IR) (software available at http://www.dtu.ox.ac.uk/, last accessed in August 2007) [25].

Tissue samples Biopsies were obtained from skeletal muscle and adipose tissue using a Bergström biopsy needle [26]. The biopsies were immediately frozen in liquid nitrogen and stored at −80°C until analysed. From the muscle biopsies, mRNA was obtained from 94 controls and 83 diabetic patients and from the adipose tissue biopsies mRNA was obtained from 84 controls and 83 diabetic patients.

RNA isolation, reverse transcription and real-time PCR Total RNA was extracted from ~40 mg muscle or adipose tissue using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. In summary, muscle tissue was homogenised in 1 ml Trizol Reagent for 15 s using a Qiagen Tissuelyser (Qiagen Nordic, Copenhagen, Denmark). Chloroform was added and the phases were separated by centrifugation. The aqueous phase with the RNA was transferred to a fresh tube and the RNA precipitated by adding isopropanol and left at −20°C for 1 h. After another centrifugation, the RNA pellet was washed in 75% ethanol and finally dissolved in 50 μl diethylpyrocarbonate-treated water.

The RNA concentration was determined spectrophotometrically and 2 μg total RNA was reversed-transcribed in a total volume of 100 μl using the Taqman Reverse Transcription Kit (Applied Biosystems, Branchburg, NJ, USA) and random hexamers as primers.

Real-time PCR was performed using an ABI 7900 Sequence Detection System (Applied Biosystems). The mRNAs for *TNF* and the endogenous control, β-actin, were amplified using predeveloped assays (Applied Biosystems). The PCR conditions followed the procedure recommended by the manufacturer, with 10 μl reaction volume and each sample run in triplicate for 50 cycles. The mRNA content of both the target and the endogenous control gene was calculated from the cycle threshold values by using a standard curve constructed from a serial dilution of aliquots of cDNA pooled from all the samples.

Muscle TNF-α protein quantification Muscle TNF-α protein was measured in a subpopulation consisting of 16 controls (eight of normal weight and eight obese) and 16 diabetic patients (eight of normal weight and eight obese). They were matched according to age and sex.

Muscle lysate Muscle tissue was freeze-dried and dissected free of visual blood, fat and connective tissues. Muscle lysate was then prepared by mixing the muscle tissue with a modified RIPA cell lysis buffer (50 mmol/l Tris-HCl, pH 7,4, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l EDTA, 0.25% sodium deoxycholate, 1% Triton X) containing 1 μg/ml pepstatin A, 1 mmol/l sodium orthovanadate, 1 mmol/l sodium fluoride, and a complete protease inhibitor cocktail (Roche, Basel, Switzerland) followed by homogenisation in precooled racks using a Tissuelyser (Qiagen, Valencia, CA, USA) for 1 min at 30 Hz followed by 15 min incubation on ice. Homogenisation and incubation on ice were repeated two or three times to obtain the required degree of homogenisation. Homogenates were then rotated end over end for 1 h at 4°C and centrifuged at 16,000×*g* at 4°C for 1 h. The supernatant protein concentrations were determined using the Bio-Rad DC kit (Bio-Rad, Hercules, CA, USA) using BSA as standard. All determinations were done in triplicate.

Western blotting Twenty-five micrograms of muscle protein lysate per lane was boiled in Laemmli buffer and separated on 12% Bis-Tris gels (Invitrogen, Taastrup, Denmark) and transferred to PVDF (polyvinylidene difluoride) membranes (Hybond-P; GE Healthcare, Little Chalfont, UK). Membranes were then blocked for 1 h at room temperature in blocking buffer [Tris-buffered saline with 0.1% Tween 20 and 5% Top-Block (Sigma-Aldrich, St Louis, MO, USA)]. The membranes were then incubated overnight at 4°C in a blocking buffer containing a primary antibody against human TNF-α (catalogue no. 3707; Cell Signaling Technology, Danvers, MA, USA) at 1:1,000 dilution. The membranes were then washed three times for 5 min in a wash buffer (Tris-buffered saline with 0.1% Tween 20) and incubated for 1 h at room temperature with secondary antibody (goat anti-rabbit horseradish peroxidase, P0448; Dako, Glostrup, Denmark) at 1:15,000 dilution in blocking buffer, followed by three 5-min washes in wash buffer. The protein bands were detected using Supersignal West Femto (Pierce, Rockford, IL, USA) and quantified using a CCD image sensor (ChemiDoc XRS; Bio-Rad) and software (Quantity One; Bio-Rad). Following detection, the membranes were stripped in strip buffer (25 mmol/l glycine, 1% SDS, pH 2) for 1–2 h and then blocked in blocking buffer for 1 h at room temperature. In order to normalise for equal protein, the membranes were reprobed overnight at 4°C with anti-β-actin (A3853; Sigma) at 1:5,000 dilution in blocking buffer, followed by washing and incubation for 1 h with secondary antibody (anti-mouse horseradish peroxidase, P0260; Dako) at 1:2,000 dilution in blocking buffer, followed by three 5-min washes in a wash buffer. The protein bands were detected using enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, Bucks, UK) and quantified using a CCD image sensor (ChemiDoc XRS) and software (Quantity One). The content of TNF-α protein (cleaved form or membrane-bound) was expressed as arbitrary units relative to β-actin protein content.

Muscle TNF-α histology Muscle samples from eight healthy controls and eight diabetic patients were evaluated for TNF-α by immunohistochemistry. The subjects were matched according to sex, age and obesity. For identification of muscle fibres, frozen biopsies of the vastus muscles were cut on a cryostat at −20°C in 6 μm consecutive transverse sections. All sections were immediately collected on glass slides.

Histology We used rouine ATPase histochemistry performed after preincubation at pH 4.57 and pH 10.30.

Immunohistochemistry For the immunostaining procedure, we applied enzymatic epitope retrieval, for which the sections were preincubated overnight in a Tris–EGTA buffer (1.211 g Tris, 0.95 g EGTA, 1 l distilled water) at 60°C and afterwards in 1.5% H_2O_2 in Tris-buffered saline (TBS)/Nonidet (TBS 0.05 mol/l Tris, pH 7.4, 0.15 mol/l NaCl; with 0.01% Nonidet P-40) (code N-6507; Sigma-Aldrich) for 15 min at room temperature (20°C) to quench endogenous peroxidase. Subsequently, sections were incubated in 10% goat serum (code 04009-1B; In Vitro, Fredensborg, Denmark) for 30 min at room temperature in order to block potential, non-specific binding of the primary and secondary antibodies. Afterwards, sections were incubated overnight at 4°C with polyclonal rabbit anti-TNF-α diluted 1:100 (catalogue no. AMC3012; Biosource, Solingen, Germany).

Primary antibodies were detected using biotinylated anti-rabbit immunoglobulin G diluted 1:400 (catalogue no. B3275; Sigma-Aldrich) followed by streptavidin–biotin–peroxidase complex (StreptABComplex/horseradish peroxidase; DakoCytomation, Glostrup, Denmark) for 30 min at room temperature. Afterwards, staining was enhanced using biotinylated tyramide and streptavidin–peroxidase complex (catalogue no. NEL700A; NEN Life Science Products, Boston, MA, USA) prepared according to the manufacturer's recommendations. The immunoreaction was visualized using 0.015% H_2O_2 in 3,3-diaminobenzidine tetrahydrochloride/TBS for 10 min at room temperature. Sections were always processed and stained simultaneously and under the same laboratory conditions.

Negative control sections were incubated without the primary or secondary antibody or in the blocking serum. Results were considered only if these controls were negative.

To determine the degree of false-positive staining due to endogenous biotin, we pretreated sections sequentially with Cruz Block Avidin/Biotin blocking kit (catalogue no. sc-24967; Santa Cruz Biotechnology, Santa Cruz, CA, USA) before immunohistochemistry was performed. This approach revealed that muscular endogenous biotin is unlikely to induce a false-positive signal by binding to the streptavidin included in the immunohistochemistry process. In order to assess the specificity of the primary antibodies, we preabsorbed these with their corresponding human antigen for 2 at 20°C. We used human TNF-α (catalogue no. sc-1350P; Santa Cruz Biotechnology). Results were considered only when this preabsorbtion resulted in negative immunostaining. For the simultaneous examination and recording of staining we used a Zeiss Axio Imager D1 microscope with an AxioCam MRc5 camera.

Statistics Data are generally presented as mean±SEM. If the data were not normally distributed, logarithmic transformation was applied and the data are presented as geometric mean and geometric SEM. For comparisons between groups (controls and diabetic patients) the Student's *t* test was used for continuous variables and the χ^2 test for categorical variables. Levels of TNF-α, sTNFR2 in plasma and *TNF* mRNA in muscle and adipose tissue were correlated with various clinical and paraclinical parameters based on the Spearman correlation and were divided into quartiles. Multiple regression analysis was done using a general linear model. For evaluation of the protein content in muscle, two-way ANOVA was performed and the Student's *t* test with Bonferroni correction was used as a post hoc test. The residuals obtained from the regression and ANOVA models were further evaluated and the model was only accepted if the residuals were normally distributed. All analyses were performed using SAS software version 9.1 (SAS Institute, Cary, NC, USA). Significance was accepted at $p<0.05$.

Results

Clinical and laboratory variables The cohort consisted of 96 patients with type 2 diabetes and 103 healthy controls. Patient characteristics appear in Table 1. There were no differences with regard to sex and obesity, but the control group had a slightly lower mean age than the diabetes group ($p<0.05$). Fasting glucose and insulin, HbA_{1c} and HOMA2-IR were elevated in the diabetic group. The total percentage fat mass, determined by dual-energy X-ray absorptiometry scanning, did not differ between groups, but WHR and triacylglycerol were higher in the diabetes group compared with the controls. HDL-cholesterol was lower in the group of diabetic patients; however, LDL-cholesterol was also lower in the diabetic group when compared with the controls. Markers of increased inflammation (neutrophil number, CRP, TNF-α, sTNFR2 and IL-6) were elevated in the diabetes group. However, no difference was observed for orosomucoid.

Surprisingly, there were no significant differences between the two groups with regard to *TNF*-α mRNA content in muscle or adipose tissue (Table 1).

Table 1 Clinical and laboratory variables in healthy controls and patients with type 2 diabetes

	Healthy controls		Type 2 diabetes (n=96)		
	Mean	95% CI	Mean	95% CI	p value
Clinical					
Age (years)	52.9	50.5–55.3	58.2	56.1–60.4	0.0014
Sex (female/male)	33/70	–	24/72	–	NS (χ^2 test)
WHR	0.95	0.93–0.97	0.98	0.96–1.00	0.015
Total fat mass (%)	30.2	27.2–33.2	29.8	27.6–32.0	NS
BMI (kg/m^2)	30.06	28.8–31.3	30.9	29.7–32.0	NS
Diastolic BP (mmHg)	88.6	86.5–90.7	90.5	88.4–92.5	NS
Systolic BP (mmHg)	142.1	138.3–145.8	151.7	147.1–156.2	0.0014
HDL-cholesterol (mmol/l)	1.46	1.38–1.54	1.26	1.18–1.34	0.0009
LDL-cholesterol (mmol/l)	3.50	3.31–3.69	2.94	2.74–3.14	<0.0001
Fasting plasma glucose (mmol/l)	5.14	5.04–5.24	9.06	8.38–9.79	<0.0001
Fasting insulin (pmol/l)	45.82	40.01–52.48	71.96	61.62–84.04	<0.0001
HOMA2-IR	0.86	0.75–0.99	1.64	1.40–1.93	<0.0001
HbA_{1c} (%)	5.52	5.47–5.58	7.14	6.85–7.44	<0.0001
Triacylglycerol (mmol/l)	1.17	1.05–1.31	1.56	1.35–1.81	0.002
$\dot{V}O_2$/kg (l/kg)	31.0	28.6–33.5	24.4	22.75–26.00	<0.0001
Inflammation					
Neutrophils (10^9/l)	3.14	2.93–3.38	3.81	3.56–4.09	0.0002
Plasma CRP (mg/l)	2.26	1.89–2.69	2.93	2.45–3.51	0.039
Plasma orosomucoid (g/l)	0.80	0.77–0.83	0.76	0.72–0.80	NS
Plasma TNF-α (ng/l)	2.40	2.29–2.50	2.72	2.56–2.88	0.0009
sTNFR2 (ng/l)	2.28	2.2.6–2.30	2.32	2.30–2.34	0.002
IL-6 (ng/l)	1.27	1.08–1.48	1.63	1.41–1.90	0.022
Tissue *TNF* mRNA					
Muscle *TNF* mRNA (arbitrary units)	4.13	3.67–4.64	4.38	3.84–4.99	NS
Adipose *TNF* mRNA (arbitrary units)	2.37	2.06–2.74	2.28	2.08–2.50	NS

NS Not significant

Correlation analyses Correlations were calculated for plasma TNF-α and sTNFR2, muscle and adipose TNF-α mRNA, and various parameters of diabetes. As presented in Table 2, plasma TNF-α correlated with several characteristics of the metabolic syndrome: BMI and insulin levels in both groups, and HDL-cholesterol, fasting glucose and HbA_{1c} in the diabetic group. Associations with these parameters were also observed for sTNFR2, which correlated well (r=0.7) with plasma TNF-α concentration. However, regarding *TNF* mRNA expression in muscle and adipose tissue, no correlation or only weak correlations were found; in regard to muscle, negative correlations were observed with systolic blood pressure (r=−0.2) and triacylglycerol (r=−0.3), whereas muscle *TNF* mRNA correlated positively with maximal oxygen uptake (r=0.3). In adipose tissue, maximal oxygen uptake correlated negatively with *TNF* mRNA content.

Plasma TNF-α concentrations were divided into quartiles and related to HOMA2-IR. A clear association between plasma TNF-α and HOMA2-IR was found in both controls and patients with diabetes (Fig. 1a). This was also reflected in plasma sTNFR2; however, in the diabetes group the association was only borderline significant (Fig. 1b). The mRNA content in muscle or adipose tissue was not associated with insulin resistance as measured by HOMA2-IR (Fig. 1c,d).

In univariate models, plasma TNF-α was associated with several variables, such as obesity and HDL-cholesterol (Table 2). To determine the independent relationship between TNF-α levels and insulin resistance, four models that included successively increasing numbers of confounders were tested for plasma TNF-α, plasma sTNFR2 and muscle and adipose tissue *TNF* mRNA content (Table 3). Only plasma TNF-α in the diabetes group showed a significant relationship, even after adjustment for age, sex, obesity, neutrophil number, IL-6 and $\dot{V}O_2$/kg. No associations were observed for muscle and adipose tissue *TNF* mRNA content.

TNF-α protein in muscle tissue To examine the production of TNF-α in the muscle tissue, a semiquantitative analysis of TNF-α protein (western blotting) was performed in a subpopulation including eight non-obese controls, eight obese controls, eight non-obese diabetic patients and eight obese diabetic patients. Two forms of TNF-α protein were detected, a cleaved form and the membrane-bound form

Table 2 Spearman correlations for plasma TNF-α, plasma sTNFR2 and *TNF-α* mRNA in biopsies of muscle and adipose tissue in healthy controls and patients with type 2 diabetes

	Plasma				Tissue *TNF-α* mRNA			
	TNF-α		STNFR2		Muscle		Adipose tissue	
	Controls	Diabetes	Controls	Diabetes	Controls	Diabetes	Controls	Diabetes
Clinical								
Age (years)	−0.0370	0.1590	−0.1758	0.3040**	0.0775	−0.0205	0.1646	−0.2136
WHR	0.2130*	0.0956	0.2126*	0.1325	−0.0432	0.0596	−0.0917	0.0145
Total fat mass (%)	0.2963**	0.1422	0.4223***	0.1150	−0.1904	−0.2174	0.0526	−0.1021
BMI (kg/m^2)	0.3109**	0.2086*	0.4029***	0.1662	−0.1434	−0.1541	0.0475	−0.0831
Diastolic BP (mmHg)	0.0074	0.1598	0.0494	0.0667	−0.1828	−0.1559	0.0750	−0.0371
Systolic BP (mmHg)	0.0151	0.1064	−0.0226	0.1085	−0.2035*	0.0374	0.0310	−0.0983
HDL-cholesterol (mmol/l)	−0.1819	0.3050**	0.2770**	−0.2077*	0.0631	0.0908	0.0141	−0.0171
LDL-cholesterol (mmol/l)	−0.1193	−0.1681	−0.1946*	−0.3474***	−0.0770	−0.1064	0.0264	−0.1891
Fasting plasma glucose (mmol/l)	−0.0072	0.2196*	0.1396	0.2024*	−0.0871	−0.0107	−0.0108	−0.1491
Fasting insulin (pmol/l)	0.2003*	0.2795**	0.2670**	0.2077*	−0.0899	−0.1437	−0.1194	0.0273
HOMA2-IR	0.1968	0.3088**	0.2693**	0.2136*	−0.0949	−0.0883	−0.1118	0.0022
HbA_{1c} (%)	−0.0066	0.2213*	−0.0548	0.2337*	−0.1192	0.0423	0.0662	−0.0356
triacylglycerol (mmol/l)	0.1642	0.1625	0.0822	0.0972	0.1218	−0.2917**	0.0546	0.0102
$\dot{V}O_2$/kg (l/kg)	−0.2014*	−0.2361*	−0.2673**	−0.2531*	0.1499	0.2593*	−0.2595*	0.0196
Inflammation								
Neutrophils (10^9/l)	0.0170	0.0022	0.1839	0.0890	−0.1643	−0.0489	−0.0892	−0.0508
Plasma CRP (mg/l)	0.1745	0.1790	0.2382*	0.3032**	−0.1653	−0.0277	0.0575	0.0340
Plasma orosomucoid (g/l)	0.0770	−0.0207	0.2364*	0.1220	−0.0696	0.1525	−0.0077	0.0017
Plasma TNF-α (ng/l)	–	–	0.6614***	0.7084***	0.1566	−0.1626	0.0121	−0.0889
sTNFR2 (ng/l)	0.6614***	0.7084***	–	–	0.0310	0.0502	0.0680	0.0053
IL-6 (ng/l)	0.4745***	0.2441*	0.4402***	0.3309**	0.0073	0.0196	0.1893	0.0325
Tissue *TNF* mRNA								
Muscle *TNF* mRNA	0.1566	−0.1626	0.0310	0.0502	–	–	0.1893	0.0844
Adipose *TNF* mRNA	0.0121	−0.0888	0.0753	0.0053	0.1893	0.0844	–	–

*$p<0.05$
**$p<0.01$
***$p<0.001$

(proTNF-α), with molecular masses of 17 and 28 kDa, respectively. The amount of proTNF-α was higher than that of the cleaved form. Recombinant TNF-α protein had a molecular mass similar to the cleaved TNF-α protein (Fig. 2a). Levels of both forms of TNF-α protein were higher in patients with type 2 diabetes than in controls, but there was no significant increase due to obesity (Fig. 2b,c). However, the level of proTNF-α protein was higher in the obese diabetic patients than in the obese controls; the difference between non-obese diabetic patients and non-obese controls was only of borderline significance ($p=0.11$; Fig. 2b).

Since the TNF-α protein content was elevated in skeletal muscle tissue in type 2 diabetic patients, we evaluated the level of TNF-α protein by immunohistochemistry in order to locate it in different fibre types of skeletal muscle. We included eight healthy controls and eight patients with type 2 diabetes, who were matched according to sex, age and BMI. The high level of TNF-α protein was confirmed by immunohistochemistry, which demonstrated homogeneously increased immunostaining within the muscle fibres of diabetic patients. Interestingly, the increased level of TNF-α protein was more pronounced in type 2 than in type 1 fibres, although increased levels were not seen in all type 2 fibres but rather in a subgroup of type 2 fibres of the diabetic patients (Fig. 3).

Discussion

The present study demonstrates that plasma TNF-α is associated with insulin resistance even after adjustment for multiple confounders, such as obesity and other markers of inflammation. Moreover, we found elevated levels of TNF-α protein in skeletal muscle biopsies from patients with type 2 diabetes regardless of the level of obesity.

These data add to previous studies on TNF-α and type 2 diabetes, which have either found a similar association [5,

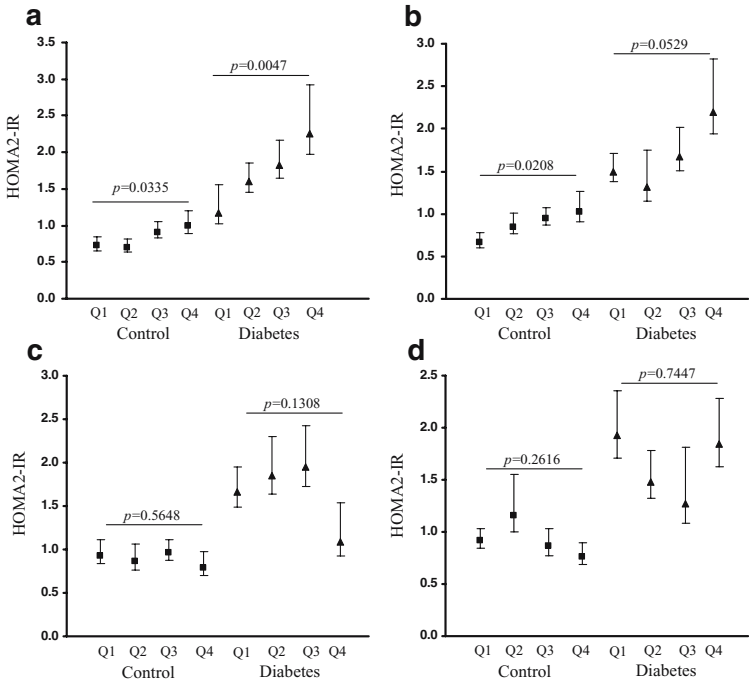

Fig. 1 Insulin resistance as measured by HOMA2-IR in relation to quartiles of plasma TNF-α (**a**), plasma sTNFR2 (**b**), muscle *TNF* mRNA content (**c**) and adipose tissue *TNF* mRNA content (**d**) in the control and diabetes groups. The *p* values indicate significant relations to quartiles

27–29] or no association [6, 7] between TNF-α and insulin resistance. The present study demonstrates that TNF-α is associated with insulin resistance even after adjustment for other inflammatory markers, such as IL-6. Although attempts to improve insulin sensitivity in obese patients with type 2 diabetes by neutralising TNF-α, using anti-TNF-α antibodies [30] or recombinant TNF receptors [31], have been ineffective, the present study lends support to experimental studies suggesting that TNF-α plays a role in chronic insulin resistance. More recently, we obtained direct evidence for a role of TNF-α in insulin resistance. Thus, infusion of TNF-α into healthy humans impaired insulin signalling and whole-body glucose uptake in humans [10].

Diabetic patients have previously been shown to have higher levels of sTNFR2 in the circulation [6], a finding that can be interpreted as higher activity of TNF-α in the tissues, since sTNFR2 is cleaved from the cell membrane upon activation [24]. Accordingly, in the present study plasma TNF-α and sTNFR2 correlated strongly in controls and diabetic patients, with a Spearman correlation coefficient of 0.66 ($p<0.001$) and 0.71 ($p<0.001$), respectively. However, when corrected for confounders, sTNFR2 was not as strongly associated as plasma TNF-α with HOMA2-IR.

There has been much focus on *TNF* mRNA expression in adipose tissue, especially in relation to obesity. Some studies have demonstrated a positive correlation between HOMA2-IR and adipose tissue *TNF* mRNA levels [18, 32],

Table 3 Quartiles of plasma TNF-α, plasma, sTNFR2, muscle *TNF* mRNA and adipose *TNF* mRNA and their relation to insulin sensitivity as measured by HOMA2-IR in five models with different numbers of confounders in healthy controls and patients with type 2 diabetes

	Controls (n=103)				Type 2 diabetes (n=96)			
	Plasma		Tissue *TNF* mRNA		Plasma		Tissue *TNF* mRNA	
	TNF-α	STNFR2	Muscle	Adipose	TNF-α	STNFR2	Muscle	Adipose
Model 1	0.0335	0.0208	(0.5648)	(0.2016)	0.0047	0.0529	(0.1308)	(0.7447)
Model 2	(0.1500)	0.0527	(0.7232)	(0.0681)	0.0027	0.0234	(0.2146)	(0.1986)
Model 3	(0.1177)	0.3444	(0.8639)	0.1663	0.0122	0.0789	(0.4370)	(0.2223)
Model 4	0.2062	0.3095	(0.4712)	(0.0666)	0.0145	0.1022	(0.6645)	(0.2921)
Model 5	0.2122	0.3563	(0.1460)	0.2265	0.0263	0.1153	(0.5348)	(0.3495)

The analysis is based on a general linear model and the *p* values are stated; parentheses indicate that the overall model was not significant.
Model 1: crude model; Model 2: model 1 + age + sex; Model 3: Model 2 + obesity + WHR; Model 4: Model 3 + neutrophils + plasma IL-6; Model 5: Model 4 + $\dot{V}O_2$/kg

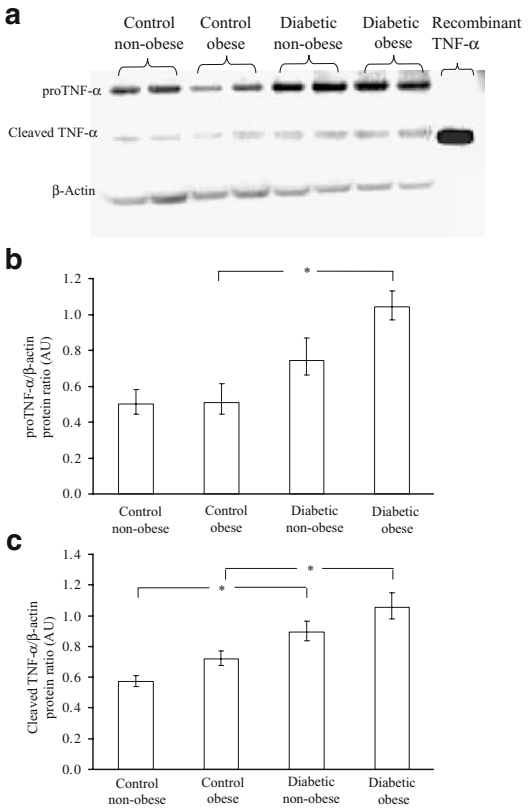

Fig. 2 Western blot analysis of TNF-α protein in the skeletal muscle of healthy controls (eight with normal weight and eight obese) and diabetics (eight with normal weight and eight obese). **a** Representative blots. Recombinant human TNF-α demonstrated a molecular mass similar to that of cleaved TNF-α in muscle tissue. The intensity of the bands was quantified by densitometry. β-Actin was measured as a reference and TNF-α (cleaved and proTNF-α) was expressed as a ratio with respect to β-actin, thereby reducing differences in the total amount of protein and the loading and blotting procedure. **b** Ratio of proTNF-α to β-actin. One-way ANOVA: diabetes $p=0.0002$; obese $p=0.1874$; diabetes × obese p=NS. **c** Ratio of cleaved TNF-α to β-actin. One-way ANOVA: diabetes $p<0.0001$; obese $p<0.0079$; diabetes × obese p=NS. Data are presented as means±SEM. *Significant difference between groups indicated according to ANOVA with post hoc Student's t test. AU, arbitrary units

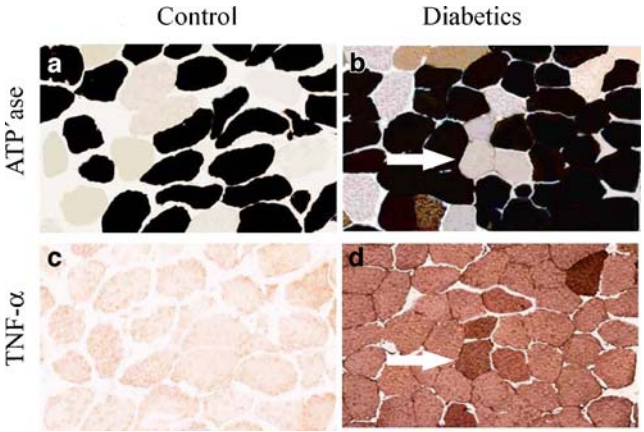

Fig. 3 Representative sections of muscle from a control and a diabetic patient. **a**, **b** Fibre types are distinguished by ATPase stain. **c**, **d** Parallel sections stained for TNF-α. The arrows indicate a type 2 fibre. A subpopulation of type 2 fibres shows increased TNF-α protein levels compared with type 1 fibres; however, this was not observed for all fibres classified as type 2

whereas others have not [33]. These studies [18, 32, 33] are small, with ~20 subjects. A recent study compared type 2 diabetic male patients (n=56) with healthy controls (n=51). *TNF* mRNA was measured in the subcutaneous adipose tissue and there was a higher level only in a subgroup of diabetic patients with a BMI >40 kg/m^2 [34]. In the present study only eight men (three healthy and five with diabetes) had a BMI >40 kg/m^2 and no difference was observed in this subgroup. Visceral fat in particular has been suggested to have a high level of TNF-α [27, 35], which is a possible explanation for the lack of difference in subcutaneous adipose tissue between controls and diabetic patients in the present study. It has not been possible to demonstrate secretion of TNF-α protein from subcutaneous adipose tissue using arteriovenous differences [21, 36], making it unlikely that TNF-α derived from adipose tissue is acting on the skeletal muscle tissue.

Very few studies have attempted to compare the *TNF* mRNA content in skeletal muscle between type 2 diabetics and healthy controls. Saghizadeh et al. [13] compared *TNF* mRNA content from five healthy subjects with five insulin-resistant and five diabetic patients and found an increase in *TNF* mRNA in insulin-resistant and diabetic patients. Furthermore, the amount of *TNF* mRNA correlated negatively with insulin sensitivity. Surprisingly, in the present study (94 controls and 83 diabetic patients), the level of *TNF* mRNA did not differ between the groups and correlation analysis of relevant clinical parameters revealed no association with the metabolic syndrome. These data were somewhat unexpected; however, patients with type 2 diabetes who performed a resistance exercise demonstrated improved insulin sensitivity despite an increase in *TNF* mRNA transcripts [37], suggesting no association of insulin sensitivity with *TNF* mRNA content in skeletal muscle. Taking these results together, the *TNF* mRNA content in muscle has limited clinical significance in relation to insulin resistance.

A higher level of TNF-α protein in skeletal muscle of patients with type 2 diabetes compared with healthy controls has been demonstrated by a modified ELISA in homogenised muscle biopsies [38], and it has been shown that primary muscle cell cultures derived from diabetic patients secrete more TNF-α protein into the medium than primary muscle cell cultures established from healthy controls [13]. In line with these data, in the present study we also observed a higher level of TNF-α protein in muscle from diabetic patients.

Immunohistochemistry demonstrated that TNF-α protein accumulates within muscle fibres. Insulin-resistant and obese patients have a higher proportion of type 2 muscle fibres than

healthy controls [39, 40], which is of interest since the type 2 muscle fibres appeared to contain more TNF-α than type 1 fibres. However, this difference is not reflected in *TNF* mRNA levels, as human soleus and triceps muscles express similar amounts of *TNF* mRNA, despite the fact that the soleus and triceps predominantly consist of type 1 and type 2 muscle fibres, respectively. This is in line with results from studies in which single fibre were dissected out, which found no difference in *TNF* mRNA content between type 1 and 2 fibres from the human vastus lateralis muscle [41]. Taking these results together, it appears that TNF-α levels in muscle are not regulated at the transcriptional level, which would explain the lack of association found when studying the *TNF* mRNA contents of muscle and adipose tissue.

In conclusion, the present study demonstrates that the plasma TNF-α level is associated with insulin resistance even after adjustment for multiple confounders, and that the level of TNF-α protein in muscle is elevated in patients with type 2 diabetes, especially in type 2 muscle fibres. Our findings with regard to TNF-α protein levels in plasma and skeletal muscle indicate that measurement of the *TNF* mRNA content in adipose or muscle tissue provides no information with regard to the degree of insulin resistance.

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