Shedding of TNF-α receptors, blood pressure, and insulin sensitivity in type 2 diabetes mellitus

José-Manuel Fernandez-Real,1,2 Beogna Lainez,1,3 Joan Vendrell,2 Mercedes Rigla,4 Antoni Castro,5 Georgina Peñarroja,5 Montserrat Broch,2 Antonio Pérez,4 Cristobal Richart,2 Pablo Engel,3 and Wifredo Ricart1,2

1Unitat de Diabetologia, Endocrinologia i Nutricio, 2Servei de Medicina Interna, University Hospital of Girona, 17001 Girona; 3Servei de Medicina Interna, University Hospital of Tarragona; Institut d’Estudis Avançats, Tarragona; 4Immunology Unit, Department of Cellular Biology and Pathology, Institut de Investigació Biomèdica August Pi Sunyer, Medical School, University of Barcelona, Barcelona; and 5Servei d’Endocrinologia, Hospital de Sant Pau, Barcelona, Spain

Received 3 October 2001; accepted in final form 7 November 2001

Hypertension is a major cause of morbidity and mortality in diabetic patients (5). The mechanism by which hypertension occurs is most likely multifactorial, based on the chronic metabolic disturbance. Immunopathogenic mechanisms are increasingly recognized to be involved in the pathogenesis of hypertensive disease in which alterations in both humoral and cellular immunity have been described (15, 18). Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that, in addition to its role in the immune response and cancer, is implicated in the phenotypic expression of insulin resistance (17). The complete absence of TNF-α results in a significant improvement in insulin sensitivity in mice with dietary, hypothalamic, or genetic obesity (reviewed in Ref. 17). In humans, recent studies suggest that the TNF-α gene locus is involved in insulin resistance-associated hypertension (27). A positive correlation between serum TNF-α concentration and both systolic blood pressure (SBP) and insulin resistance has been found in subjects with a wide range of adiposity (38). Upregulation of TNF-α secretion has been observed in peripheral blood monocytes from hypertensive patients (12). The available information on direct TNF-α effects also suggests that it is involved in the pathophysiology of hypertension. TNF-α stimulates the production of endothelin-1 (19) and angiotensinogen (7). In the spontaneously hypertensive rat model, TNF-α synthesis and secretion are increased in response to lipopolysaccharide (LPS) stimulation compared with those in nonhypertensive control rats. LPS increased fat angiotensinogen mRNA in the former but not in the latter (24). TNF-α is also thought to cause endothelial dysfunction linked to insulin resistance (37).

TNF-α binds to two TNF-α receptors, TNFR1 and TNFR2. Each receptor is expressed by most cells and can be regulated independently. After binding to its receptors, a proteolytic cleavage of the extracellular parts elicits the soluble forms, named (2, 22) sTNFR1 (55 kDa) and sTNFR2 (75 kDa). The soluble TNFRs...
remain elevated for longer periods of time after the administration of TNF-α and are thought to measure previous TNF-α effects (2). sTNFRs were constantly found in the circulation of patients with sepsis, as a surrogate of the inflammatory state even when TNF activity was undetectable (34). Measurements of the sTNFRs’ concentrations in healthy individuals at different time lapses showed that their concentrations in the same subject were quite stable, at least during a period of 1 yr (3).

Isolated limb perfusion with TNF-α to cancer patients led simultaneously to increased plasma sTNFR1 and sTNFR2 concentrations. Different mechanisms were responsible for shedding of TNFRI and TNFRII (4). The TNFR1 stimulation with excess TNF-α seemed to transactivate preferential TNFR2 shedding (17).

We have previously shown that plasma sTNFR2 concentration was associated with insulin resistance in nondiabetic subjects (16). We tested the hypothesis that the sTNFR2-to-sTNFR1 ratio (R2/R1), as a correlate of TNF-α action and insulin sensitivity, was associated with blood pressure in a sample of 368 individuals. We found that R2/R1 correlated positively with SBP and diastolic blood pressure (DBP; r = 0.144 and 0.14, P = 0.005 and 0.007). The R2/R1 was significantly greater in type 2 diabetic subjects (DM-2, n = 66) than in type 1 diabetic patients (DM-1, n = 46) and was greater than in control nondiabetic subjects (n = 258; 2.65 ± 1.05 vs. 2.24 ± 0.7 vs. 1.76 ± 0.38, ANOVA, P < 0.00001). In fact, the R2/R1 seems to be a good correlate of TNF-α action in subjects with insulin resistance (11). Given these findings, we hypothesized altered TNFRI and TNFRII shedding in insulin resistance-associated hypertension.

The objectives of this study were to evaluate whether TNFRI and TNFRII shedding was associated with insulin action and with vascular dysfunction, as a surrogate complication of insulin resistance, in DM-2 patients and to test whether shedding of TNF-α receptors changes after lowering of blood pressure.

**MATERIALS AND METHODS**

**Patients: Control Subjects**

Two hundred fifty-eight subjects (109 women) were evaluated between July 1 and September 15, 1997 as a part of an ongoing epidemiological study dealing with the influence of inflammatory factors on body composition. None of the subjects was taking any medication or had any evidence of metabolic disease other than obesity. All subjects were of Caucasian origin and reported that their body weight had been stable for at least 3 mo before the study. Inclusion criteria were as follows: 1) body mass index (BMI, weight in kilograms divided by the square of height in meters) < 40 kg/m², 2) absence of any systemic disease, 3) absence of any infections in the previous month, and 4) SBP < 165 mmHg. Resting blood pressure was measured with a mercury sphygmomanometer after subjects had been in a sitting position for a minimum of 15 min and was read three times in the right arm by the same investigator. The mean of three measurements was used for this study. Liver and renal diseases were specifically excluded by biochemical workup.

**Inclusion and Exclusion Criteria for Diabetic Patients**

Forty-six (21 women) consecutive DM-1 patients and 66 (20 women) DM-2 patients were prospectively recruited from diabetes outpatient clinics on the basis of the following criteria: 1) aged 40–70 yr, 2) current BMI < 40 kg/m², 3) stable metabolic control in the previous 6 mo, and 4) no history of ketoadiosis. Exclusion criteria included the following: 1) clinically significant hepatic, neurological, endocrinological, or other major systemic disease, including malignancy; 2) history of drug or alcohol abuse, defined as > 80 g/day in men and > 40 g/day in women, or serum transaminase activity over two times the upper limit of normal; 3) SBP > 165 mmHg, previous antihypertensive therapy for uncontrolled hypertension and elevated serum creatinine concentration; 4) acute major cardiovascular event in the previous 6 mo; 5) acute illnesses and current evidence of acute or chronic inflammatory or infective diseases; and 6) mental illness rendering the subjects unable to understand the nature, scope, and possible consequences of the study. DM-1 patients were defined according to World Health Organization criteria (36) and, except for history of ketoadiosis, fulfilled the preceding criteria. All patients underwent a full medical history, including age, duration of diabetes, BMI, eating habits, smoking habits, blood pressure, total cholesterol, and a full examination to screen for diabetic complications. The experimental protocol was approved by the Ethics Committee of the Girona University Hospital. Informed written consent was obtained after the purpose, nature, and potential risks were explained to the subjects.

**Definition of Chronic Diabetic Complications**

The clinical diagnosis of diabetic retinopathy was based on the examination of the ocular fundus after dilatation of the pupils by experienced ophthalmologists. Simplex retinopathy was defined as one or more microaneurysms or hemorrhages. Diabetic macroangiopathy complications were diagnosed according to clinical findings, Doppler sonography, and angiopathy. Persistent microalbuminuria was defined as an albumin excretion rate of 30–300 mg/day.

**Measurements**

Each subject was studied in the research laboratory in the postabsorptive state. The room was quiet, lights were dimmed, and temperature was controlled at 23°C. Alcohol, caffeine, and all medications, including sulfonylurea, metformin, and insulin, were withheld within 12 h of the different tests.

**Study of insulin sensitivity.** After the intravenous injection of regular insulin, glucose levels were determined every minute during 15 min. Insulin sensitivity was indicated by the first-order rate constant for the disappearance rate of glucose (KITT) estimated from the slope of the regression line of the logarithm of blood glucose against time during the first 3–15 min.

**Brachial artery vascular reactivity.** High-resolution external ultrasound (128XP/10 mainframe with a 7.5-MHz linear array transducer; Toshiba SSH-140A) was used to measure changes in brachial artery diameter in response to 400 μg of sublingual nitroglycerin (NTG), an endothelium-independent, direct smooth muscle dilator, as described by Celemajer et al. (9). The lumen diameter of the artery was defined as the distance between the leading edge of the echo of the near wall-lumen interface to the leading edge of the far wall-lumen interface echo. All scans were ECG-controlled for the R wave. All images were recorded on a super-VHS videotape.
Endothelial-independent vasodilation is induced after sublingual administration of a 400-μg metered, l-dose of NTG (Solinotrin spray; Almirall Prodesfarma, Barcelona, Spain) and is expressed as the percentage of change in the arterial diameter 3 min later. A scan was recorded from 2 min after NTG administration during 70 s. All images registered on super-VHS tape were analyzed afterward by two independent observers blinded to the randomization of the subject and the stage of the experiment. Each observer analyzed the arterial diameter for four cardiac cycles for each condition, and these measurements were averaged. Previous to the initiation of the study in diabetic subjects, validation of this technique was performed through the evaluation of reproducibility inter- and intraindividual in 22 healthy subjects (12 men and 10 women, mean age 30.1 yr [95% confidence interval (CI) 27.1, 33.2], BMI 22.6 kg/m² (CI 21.3, 23.8)). Measurements were performed by two observers (A and B). The intraobserver correlation coefficient of fixed effects between observers A and B was 0.90. The coefficient of variation (CV) between means obtained by observers A and B was 9%. The CV obtained by a same observer was 3%. The reproducibility (CI 95%) was 0.27 mm (observer A). With observer B the CV was 4%, with a reproducibility (CI 95%) of 0.39 mm. The study of the variability of the means by the same observer in five consecutive days showed a CV of 6% (observer A) and 2% (observer B). The NTG-induced vasoconstriction correlated with basal artery diameter (r = 0.67; P = 0.025).

**Analytical methods.** Serum creatinine was determined by a routine laboratory method. HbA1c was measured by high-performance liquid chromatography with the use of a fully automated glycosylated hemoglobin analyzer system (Hitachi L-9100; reference level 4.6–5.43%)

**Plasma sTNFR1 and sTNFR2 levels.** Plasma samples were obtained at baseline and were stored at −30°C until assay. Plasma sTNFR1 and sTNFR2 levels were analyzed by commercially available solid-phase enzyme-amplified sensitivity immunoassays (EASIA) MEDGENIX sTNFR1 and sTNFR2 EASIA (BioSource Europe, Fleunnes, Belgium). The intra- and interassay CV were <7 and 9%, respectively. sTNFR1 EASIA does not cross-react with sTNFR2. TNF-α does not interfere with the assay.

**Measurement of TNFR1 expression levels in monocytes by flow cytometry.** Blood cells sedimented through Ficoll-Hypaque gradients were washed with RPMI 1640 medium, and monocytes [identified by staining with anti-CD14 monoclonal antibodies (Becton-Dickinson)] were analyzed for CD120a (TNFR1) expression by flow cytometry using biotinylated anti-CD120a monoclonal antibody (Caltag Laboratories, Burlingame, CA) and avidin-phycoerythrin (Southern Biotechnology Associated). Cells (4 × 10⁶) were incubated with conjugated antibodies or isotype controls for 30 min at 4°C, washed twice with PBS containing 0.5% BSA, and incubated with avidin-phycoerythrin for 30 min at 4°C. The cells were washed again, resuspended in 500 μl of PBS, and analyzed on a FACScan Calibur flow cytometer (Becton-Dickinson).

**Study of shedding.** Blood cells sedimented through Ficoll-Hypaque gradients were washed, resuspended at 2 × 10⁶ cells/ml, and incubated at 37°C for 15 or 30 min in RPMI 1640 medium containing 1% FCS with or without phorbol 12-myristate 13-acetate (PMA, 10 ng/ml; Sigma). Blood cells sedimented through Ficoll-Hypaque gradients were washed, resuspended at 2 × 10⁶ cells/ml, and incubated at 37°C for 15 or 30 min in RPMI 1640 medium containing 1% FCS with or without PMA. Monocytes were analyzed for CD120a and CD120b expression before and after PMA activation for 15 and 30 min (Fig. 1). L-selectin expression was measured as an internal control. We calculated the percentage of induced shedding (SHED) by

\[
\%\text{SHED}_{R1_{15}} = 100 \left( 1 - \frac{\text{PMA}_{15R1} - \text{NC}_{15R1}}{\text{t}_{15R1} - \text{NC}_{t_{15R1}}} \right)
\]

\[
\%\text{SHED}_{R1_{30}} = 100 \left( 1 - \frac{\text{PMA}_{30R1} - \text{NC}_{PMA_{30R1}}}{\text{t}_{30R1} - \text{NC}_{t_{30R1}}} \right)
\]

\[
\%\text{SHED}_{R2_{15}} = 100 \left( 1 - \frac{\text{PMA}_{15R2} - \text{NC}_{15R2}}{\text{t}_{15R2} - \text{NC}_{t_{15R2}}} \right)
\]

\[
\%\text{SHED}_{R2_{30}} = 100 \left( 1 - \frac{\text{PMA}_{30R2} - \text{NC}_{PMA_{30R2}}}{\text{t}_{30R2} - \text{NC}_{t_{30R2}}} \right)
\]

\[
\%\text{SHED LSEL}_{30} = 100 \left( 1 - \frac{\text{PMA}_{30LSEL} - \text{NC}_{PMA_{30LSEL}}}{\text{t}_{30LSEL} - \text{NC}_{t_{30LSEL}}} \right)
\]

where PMAt15R1, PMAt30R1, PMAt15R2, and PMAt30R2 are the mean fluorescence intensities of TNFR1 and TNFR2 in monocytes incubated at 37°C with PMA for 15 and 30 min; NC PMAt15R1, NC PMAt15R2, NC PMAt30R1, and NC PMAt30R2 are the mean fluorescence intensities of negative controls for these conditions; t15R1 and t30R1, t15R2, and t30R2 are the mean fluorescence intensities of TNFR1 or TNFR2 in monocytes incubated at 37°C without PMA for 15 and 30 min; NC t15R1, NC t15R2, NC t30R1, and NC t30R2 are the mean fluorescence intensities of negative controls for these conditions; and LSEL and *t30LSEL are the mean fluorescence intensities of L-selectin incubated without PMA at 0 and 30 min and PMAt30LSEL with PMA for 30 min.

**Serum nitrate/nitrite levels.** Serum nitrate/nitrite levels were measured with the Nitrile Oxide Colorimetric Assay Kit (Calbiochem-Novabiochem, San Diego, CA), based on the Griess method. Amounts of nitrite in the plasma were estimated by a standard curve obtained from enzymatic conversion of KNO₂ to nitrite. The total (pro and active) matrix metalloproteinase-9 (MMP-9) in serum samples was measured using a commercially available enzyme-linked immunosorbent assay kit [QuantiKine Human MMP-9 (total) Immunoassay; R&D Systems, Minneapolis, MN] according to the manufacturer’s instructions.
Exercise Test: Study Protocol

Twenty diabetic patients (11 with DM-1 and 9 with DM-2) were allocated to a training program lasting 3 mo at the same fitness center in all cases. The protocol and blood pressure changes in these patients have been published previously (33). In brief, patients were instructed to attend the fitness center at least 3 times/wk and did not modify their usual daily activities. An individualized aerobic exercise program was designed according to the patients’ characteristics and degree of physical fitness, which was determined by means of a treadmill exercise test carried out at the beginning of the study and repeated after 3 mo of training. All exercise sessions were supervised by a coach specifically trained by the investigators for that purpose. Each session included 10 min of warm-up, 30–40 min of aerobic activity, and 10 min of cool down. The exercise consisted mainly of walking or running on a treadmill, cycling, or a combination of both. Initially, subjects exercised at an intensity corresponding to 60–65% maximal O2 uptake (VO2max; 1–2 wk) and 65–75% VO2max thereafter to improve their aerobic capacity. All patients had been instructed in diabetes care (diet, blood glucose monitoring, insulin administration, self-adjustment, etc.) and presented stable body weight and glycemic control.

Patients were seen in the outpatient unit every 4 wk to reinforce their program compliance and were instructed on insulin dose modifications. Aerobic capacity, anthropometric parameters, ambulatory blood pressure, glycemic profile, and laboratory analyses were evaluated before and after the planned exercise program. Twenty-four-hour ambulatory blood pressure was registered using a Spacelab 90207 device (Spacelab, Redmond, WA) both before and after the exercise program. During the estimated waking hours (0700–2300), blood pressure was recorded every 20 min and every 30 min during the overnight period.

Statistical Methods

We used a χ² test for comparisons of proportions and unpaired or paired t-tests for comparisons of quantitative variables. Before statistical analysis, normal distribution and homogeneity of the variances were tested. Parameters that did not fulfill these tests were log transformed. The relations between variables were analyzed by simple correlation, partial correlation, and multiple regression in a stepwise manner. Levels of statistical significance were set at P < 0.05.

RESULTS

General characteristics of the study subjects are shown in Table 1 (means ± SD). Age, years of evolution of diabetes, glycosylated hemoglobin, or renal function did not influence the R2/R1 in the diabetic groups. In contrast, a significant negative association between R2/R1 and serum creatinine was observed in the control group (r = −0.17, P = 0.01). R2/R1 was significantly higher in DM-2 than in the other groups (Table 1). The differences in R2/R1 paralleled differences in mean SBP and DBP among groups (Table 1). These differences in R2/R1 were mainly because of decreased circulating sTNFR1 levels in DM-2 compared with age- and BMI-matched control subjects (1.60 ± 0.8 vs. 2.14 ± 0.5 ng/ml, P = 0.001), whereas sTNFR1 levels were similar in DM-1 patients to those present in age- and BMI-matched control subjects (2.10 ± 0.6 vs. 1.97 ± 0.5 ng/ml, P = 0.2). The type of treatment of diabetes or associated drugs was not associated with R2/R1 differences.

Table 1. Anthropometrical and clinical variables in the study subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Group</th>
<th>Type 2 Diabetes</th>
<th>Type 1 Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>258</td>
<td>66</td>
<td>46</td>
</tr>
<tr>
<td>Men/women</td>
<td>149/109</td>
<td>46/20</td>
<td>25/21</td>
</tr>
<tr>
<td>Age, yr</td>
<td>38.8 ± 10.3</td>
<td>57.8 ± 9.5</td>
<td>31.3 ± 10.2</td>
</tr>
<tr>
<td>Range, yr</td>
<td>17–68.5</td>
<td>31–70</td>
<td>18–48</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.8 ± 4.2</td>
<td>28.6 ± 4.1Ⅹ</td>
<td>23.6 ± 4.4</td>
</tr>
<tr>
<td>Range, kg/m²</td>
<td>16.3–39.3</td>
<td>20.8–40</td>
<td>19.6–30.8</td>
</tr>
<tr>
<td>Years of diabetes</td>
<td>8.5 ± 8.9</td>
<td>13.2 ± 9.5</td>
<td>3.2 ± 9.5</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>121 ± 13.5</td>
<td>135.4 ± 14.7Ⅹ</td>
<td>129.7 ± 15.3</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>69.8 ± 9.4</td>
<td>81.4 ± 9.1Ⅹ</td>
<td>74.3 ± 10.2Ⅹ†</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.97 ± 0.14</td>
<td>0.97 ± 0.16</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>HbA1c</td>
<td>7.08 ± 1.3</td>
<td>7.39 ± 1.9</td>
<td>7.39 ± 1.9</td>
</tr>
<tr>
<td>sTNFR1 ratio</td>
<td>4.4–8.8</td>
<td>4.3–8.7</td>
<td>4.3–8.7</td>
</tr>
<tr>
<td>sTNFR2 ratio</td>
<td>2.03 ± 0.5</td>
<td>1.75 ± 0.73Ⅹ</td>
<td>2.13 ± 0.65</td>
</tr>
<tr>
<td>R2/R1 ratio</td>
<td>3.5 ± 0.91</td>
<td>4.16 ± 1.4Ⅹ</td>
<td>4.72 ± 2.03</td>
</tr>
<tr>
<td>Pharmacological treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin, %</td>
<td>41</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Oral hypoglycemic drugs, %</td>
<td>36</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Diet only, %</td>
<td>35</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Diabetic complications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous clinical macroangiopathy, %</td>
<td>23</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Diabetic retinopathy, %</td>
<td>35</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Microalbuminuria, %</td>
<td>27</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

† P < 0.00001 compared with the type 1 diabetes and control groups (Ⅹ) and compared with the control group (†).

Values are means ± SE; n, no. of subjects. sTNFR1 and sTNFR2, soluble tumor necrosis factor-α (TNF-α) receptors 1 and 2, respectively; R2/R1 ratio, ratio of sTNFR2 to sTNFR1.

AJP-Endocrinol Metab • VOL 282 • APRIL 2002 • www.ajpendo.org
Insulin Sensitivity, Vascular Dysfunction, and Circulating sTNFR1 and sTNFR2 Concentrations

In a subsample of 19 consecutive unselected DM-2 patients, we evaluated insulin sensitivity in relation to TNFR1 and TNFR2 shedding. These patients were not significantly different in age, years of diabetes, or usual treatment compared with the remaining group of DM-2 patients. Insulin sensitivity was negatively associated with serum sTNFR2 levels \((r = -0.66, P = 0.002)\), as previously found in nondiabetic subjects (16). Insulin sensitivity appeared to be associated with serum sTNFR1 \((r = -0.49, P = 0.03)\), but the latter association disappeared after one subject with sTNFR1 >3.5 ng/ml was excluded.

The mean ± SD vasodilatory response to glyceryl trinitrate was 14 ± 7% (range 2.3–27.6%). This response was positively associated with serum sTNFR1 concentration \((r = 0.62, P = 0.01, n = 16; \text{Fig. } 2)\), indicating that the lower the sTNFR1, the lower the vasodilation. Serum nitrate/nitrite concentrations were used as a surrogate of NO action, i.e., the higher the levels, the higher the resistance to NO action. Serum nitrate/nitrite concentrations correlated positively with DBP \((r = 0.58, P = 0.02)\).

Insulin Sensitivity and In Vitro Shedding of TNFR1 and TNFR2

The TNFR1 density in peripheral blood monocytes was similar in DM-2 patients compared with five nondiabetic subjects \((\text{mean } ± \text{ SE, } 47.8 ± 22 \text{ vs. } 38.7 ± 8.5, P = \text{ not significant})\). After PMA, shedding of TNFR1 was significantly decreased in DM-2 compared with control subjects \((35.6 ± 9.8 \text{ vs. } 65.1 ± 19.2, P = 0.04)\). In vitro TNFR1 shedding was positively associated with insulin sensitivity in DM-2 patients \((r = 0.54, P = 0.04, n = 14; \text{Fig. } 3)\). Conversely, TNFR2 shedding was negatively associated with insulin sensitivity \((r = -0.54, P = 0.03)\). The shedding of L-selectin, used as control, showed no significant association with insulin sensitivity.

sTNFR1 and sTNFR2 Changes After Lowering of Blood Pressure

In a sample of 20 eligible patients, previously reported (33), we evaluated whether changes in blood pressure run in parallel with variations in R2/R1. The main features of these patients were as follows: 11 type 1 diabetic patients (6 men, 5 women; mean age 26.4 ± 6.5; range: 19–42 yr) with a mean diabetes duration of 6 ± 5 (0.7–16.2) yr and 9 type 2 diabetic patients (7 men, 2 postmenopausal women; mean age: 54 ± 5.2; range: 48–64 yr) with a mean diabetes duration of 8.2 ± 5 (2.1–19.8) yr. All were treated with diet plus insulin except two type 2 diabetic patients who were on diet therapy alone. Ten patients smoked, and six were regular alcohol consumers (<200 g/wk). Two patients had background retinopathy and two microalbuminuria.

At baseline, R2/R1 correlated negatively with \(\dot{V}O_2\text{max} \) in type 2 diabetic patients \((r = -0.77, P = 0.003)\). This association was not statistically significant in type 1 diabetic patients. After the exercise program, we observed a positive association between the decrease in mean blood pressure during the 24-h ambulatory blood pressure recording and the decrease in R2/R1 \((r = 0.46, P = 0.042 \text{ for } \text{SBP}; r = 0.47, P = 0.03 \text{ for } \text{DBP})\). When the analysis was performed separately in DM-1 and DM-2 groups, this association remained significant only in the DM-2 group \((r = 0.71, P = 0.032; \text{Fig. } 4)\).

DISCUSSION

Abnormalities in immune system function and inflammatory mediators have been claimed to be responsible for the onset of hypertension (15, 18) and type 2
Type 2 diabetes mellitus and insulin resistance have been hypothesized as chronic inflammatory disorders (17, 31). Here we report that insulin resistance was associated with increased serum sTNFR2 concentration and decreased TNFR1 shedding in type 2 diabetic patients. We also found that altered shedding of TNF-α receptors is linked to vascular dysfunction and blood pressure. After blood pressure is decreased, circulating sTNFR1 increases and sTNFR2 decreases, leading to a lower R2/R1. We propose that the latter is mediated through increased and decreased shedding, respectively, of TNFR1 and TNFR2.

Interestingly, genetic alterations in TNFR1 shedding have been linked to autoinflammatory syndromes (20). Stimulation with PMA, which induces metalloprotease-mediated cleavage of TNFR1, resulted in much less clearance of membrane receptor in patients with the autoinflammatory syndrome than in controls, leading to decreased sTNFR1 levels (20). Thus DM-2 and autoinflammatory syndromes share a low circulating sTNFR1 concentration. Sequencing of the TNFR1 cDNA in some of our DM-2 patients showed that they did not carry this mutation (Fernandez-Real, Vendrell, and Ricart, unpublished results).

Endogenous sTNFR1 is an excellent marker of inflammation (26). In animal models of inflammation and autoimmunity, administration of recombinant sTNFR1 arrests collagen arthritis and protects against lethal endotoxemia (21). In humans, treatment of septic shock patients with p55 TNF fusion protein, but not with p75 TNF fusion protein or antibodies (anti-TNF), reduced the mortality rate (1). Serum sTNFR1 concentrations in DM-1 patients were similar to those present in age- and BMI-matched control subjects, but the sTNFR1 concentration was decreased significantly in DM-2 patients. The circulating density of TNFR1 on the membrane of circulating monocytes and lymphocytes was similar in DM-2 patients and in controls. After PMA stimulation, however, a decreased shedding of TNFR1 was evident in DM-2 subjects, and this shedding was associated with insulin resistance. Decreased shedding was specific for TNFR1 in contrast to TNFR2 and L-selectin. Recent studies have shown that different mechanisms are involved in shedding of both TNF-α receptors as follows: a membrane-bound nonmatrix metalloproteinase is involved in TNFR1 shedding, and a serine proteinase contributes to TNFR2 shedding (14). The TNFR1 stimulation with excess TNF-α seemed to transactivate preferential TNFR2 shedding (4).

We had previously demonstrated that exercise led to decreased blood pressure in DM-2 subjects (33). In fact, physical exercise is a well-established method to enhance insulin sensitivity, making it a useful approach for the treatment of diabetes (6). We evaluated R2/R1 before and after a training program that lasted 3 mo. At baseline, the R2/R1 was significantly associated with VO2 max, an excellent predictor of insulin sensitivity (10, 23), in DM-2 patients. This finding confirms R2/R1 as a good correlate of insulin action. R2/R1 decreased after the training program, and the change in R2/R1 was significantly associated with the decrease in mean blood pressure during a 24-h recording. This association was only observed in DM-2 patients. Thus changes in R2/R1 run in parallel with blood pressure variations in DM-2 and possibly with insulin sensitivity.

In recent studies, insulin resistance has been found to be associated with an impairment in the ability of NO to generate its messenger (cGMP), leading to an increase in nitrate/nitrite (8, 29, 30, 32). This increase could represent an effort to compensate for the defect in cGMP production (30). We found that the vasodila-

Fig. 4. Linear correlation of the association between the change in the sTNFR2-to-sTNFR1 ratio and the change in mean systolic blood pressure during a 24-h ambulatory blood pressure recording in diabetic patients (○ and ●, type 1 and type 2 diabetic patients, respectively).
tion in response to glyceryl trinitrate, a nitric oxide donor, was associated with serum sTNFR1 concentration: the lower the sTNFR1 concentration, the lower the vasodilation. Because NO was recently demonstrated to enhance TNFR1 shedding (25), these findings merit further research.

From our findings, we suggest that the resistance to NO action in DM-2 patients may lead to decreased vasodilatory capacity, decreased TNFR1 shedding, and a subsequent increased R2/R1. The latter would potentiate the effects of TNF-α through an increased plasma half-life (4), finally leading to hypertension. Exercise would enhance NO action (19a), resulting in vasodilation, increased TNFR1 shedding, decreased R2/R1, and lowering of blood pressure. Our scheme is summarized in Fig. 5.

Some authors have found that decreased shedding of TNF-α receptors was mostly related to the development of disease than to elevated secretion of TNF-α itself (28). This suggests that the response of cells to TNF-α can be regulated by the number of remaining functional receptors on the cell surface, and this number will be greater if shedding is reduced. Thus the relatively high levels of unshed, functionally intact TNFR1 remaining on the cell surface may lead to greater responsiveness to TNF-α at physiological concentrations, resulting in increased sensitivity of the TNF-α-TNFR1 axis to TNF-α. Furthermore, sustained upregulation of human TNFR2 production in transgenic mice leads not only to an upregulated level of shed soluble receptors but also to a chronic accumulation of the receptor on the cell surface (13). All of these events would provide DM-2 patients with the ability to be hyperresponders to circulating TNF-α. In fact, DM-2 subjects showed an increased R2/R1 as a probable implication in response to glyceryl trinitrate, a nitric oxide donor, was associated with serum sTNFR1 concentration: the lower the sTNFR1 concentration, the lower the vasodilation. Because NO was recently demonstrated to enhance TNFR1 shedding (25), these findings merit further research.

From our findings, we suggest that the resistance to NO action in DM-2 patients may lead to decreased vasodilatory capacity, decreased TNFR1 shedding, and a subsequent increased R2/R1. The latter would potentiate the effects of TNF-α through an increased plasma half-life (4), finally leading to hypertension. Exercise would enhance NO action (19a), resulting in vasodilation, increased TNFR1 shedding, decreased R2/R1, and lowering of blood pressure. Our scheme is summarized in Fig. 5.

Some authors have found that decreased shedding of TNF-α receptors was mostly related to the development of disease than to elevated secretion of TNF-α itself (28). This suggests that the response of cells to TNF-α can be regulated by the number of remaining functional receptors on the cell surface, and this number will be greater if shedding is reduced. Thus the relatively high levels of unshed, functionally intact TNFR1 remaining on the cell surface may lead to greater responsiveness to TNF-α at physiological concentrations, resulting in increased sensitivity of the TNF-α-TNFR1 axis to TNF-α. Furthermore, sustained upregulation of human TNFR2 production in transgenic mice leads not only to an upregulated level of shed soluble receptors but also to a chronic accumulation of the receptor on the cell surface (13). All of these events would provide DM-2 patients with the ability to be hyperresponders to circulating TNF-α. In fact, DM-2 subjects showed an increased R2/R1 as a probable reflection of TNF-α action. To further test this hypothesis, we also measured in these same patients the plasma concentration of MMP-9. We found a positive relationship between the density of TNFR1 in circulating monocytes and circulating MMP-9 (r = 0.56, P = 0.01; data not shown). Because MMP-9 synthesis and secretion are under the control of TNF-α (35), these findings favor the hypothesis of increased sensitivity to TNF-α in the presence of unshed TNFR1.

In summary, our findings suggest that insulin resistance and hypertension are linked to altered TNFR1 and TNFR2 shedding, leading to increased R2/R1 in DM-2 patients. An increased R2/R1 lowers after decreasing blood pressure, which indicates that it is reversible and not genetically determined. Whether alterations in shedding of peripheral white blood cells is also found in another cell type remains to be established.

REFERENCES


