The Rise of Soluble TWEAK Levels in Severely Obese Subjects After Bariatric Surgery May Affect Adipocyte-Cytokine Production Induced by TNFα

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Context: Soluble TNF-like weak inducer of apoptosis (sTWEAK) is generated by the intracellular proteolytic cleavage of full-length membrane-bound TNF-like weak inducer of apoptosis (mTWEAK). sTWEAK levels are reduced in diseases with an inflammatory component. Additionally, sTWEAK hampers TNFα activity in human cells.

Objectives: The objectives of the study were as follows: 1) to determine circulating sTWEAK in severe obesity and after bariatric surgery; 2) to study m/sTWEAK and its receptor fibroblast growth-factor-inducible 14 (Fn14) protein expression in sc adipose tissue (SAT) of severely obese subjects, in SAT stromal vascular fraction (SVF), and isolated adipocytes and in human monocyte-derived macrophages; and 3) to explore, on human adipocytes, the sTWEAK effect on TNFα proinflammatory activity.

Design: sTWEAK levels were measured in cohort 1: severely obese subjects (n = 23) and a control group (n = 35); and in cohort 2: (n = 23) severely obese subjects before and after surgery. The m/sTWEAK and Fn14 expressions were determined in SAT biopsies, SVF, and isolated adipocytes from severely obese and control subjects and in human monocyte-derived macrophages. In human primary cultured adipocytes, sTWEAK pretreated and TNFα-challenged, IL-6, IL-8, and adiponectin protein and gene expressions were determined and nuclear factor-κ B and MAPK signaling analyzed.

Results: sTWEAK levels were reduced in severely obese subjects. After surgery, sTWEAK levels rose in 69% of patients. mTWEAK protein expression was increased in SAT and SVF of severely obese subjects, whereas Fn14 was up-regulated in isolated adipocytes. M2 human monocyte-derived macrophages overexpress mTWEAK. In human adipocytes, sTWEAK down-regulates TNFα cytokine production by hampering TNFα intracellular signaling events.

Conclusion: The decrease of sTWEAK in severely obese patients may favor the proinflammatory activity elicited by TNFα. (J Clin Endocrinol Metab 98: E1323–E1333, 2013)
NF-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily of cytokines, is a multifunctional cytokine that controls many cellular activities including proliferation, migration, differentiation, apoptosis, angiogenesis, and inflammation (1). High levels of TWEAK mRNA have been found in many tissues such as lymph nodes, pancreas, intestine, heart, brain, lung, ovary, vasculature, and skeletal muscle and at low levels in the liver, lung, thymus, kidney, and adipose tissue (2–5).

TWEAK is initially expressed as a full-length transmembrane protein of 27 kDa (mTWEAK) from which a soluble variant of 18 kDa (sTWEAK) can be generated intracellularly by proteolytic processing (6). Both isoforms, mTWEAK and sTWEAK, are biologically active and can bind to fibroblast growth factor-inducible 14 (Fn14), its only bona fide signal transducing receptor (6).

Reduced circulating TWEAK (sTWEAK) levels have been found in diseases with increased cardiovascular risk and low-degree chronic inflammation, such as type 2 diabetes, atherosclerosis, or chronic renal failure (3, 7, 8). Obesity is a highly prevalent chronic disease in which inflammatory events (systemic and local in the adipose tissue) play a key role in its associated comorbidities (9). In obesity, more adipose tissue infiltrating macrophages with an altered polarizing state have been reported (10, 11). An imbalance between M1 (proinflammatory) and M2 (antiinflammatory) macrophage types has been claimed to be an important determinant of the inflammatory component of the adipose tissue in obesity (12–17).

To date, TNFα has focused the attention as a preponderant inflammatory cytokine, with important implications both at local and systemic levels in obesity and related diseases (18). TWEAK and TNFα are cytokines that coexist within the context of obesity, and sTWEAK has been described to interfere with TNFα signaling in cellular systems such as human epithelial cancer cell lines, human synoviocytes, and mouse cerebral cortical neurons (19–21).

The physiological role of sTWEAK within the context of obesity or insulin resistance has been scarcely studied. In this sense, in vitro studies have shown that sTWEAK stimulus could regulate preadipocyte differentiation and can exert a weak proinflammatory activity over subcutaneous mature adipocytes without affecting lipolysis or glucose uptake (22–25). On the other hand, studies on the function of sTWEAK in human macrophages have suggested that sTWEAK balances TNFα activity by repressing the production of proinflammatory cytokines and attenuating the transition from innate to adaptive immunity (23).

Taking the above data into consideration and because, to our knowledge, no information exists on circulating sTWEAK in severe obesity, in this study we explored whether sTWEAK may participate in the inflammatory/antiinflammatory imbalance observed in severe obesity. We show that sTWEAK levels were decreased in severely obese patients and rose after weight loss. We found that in severely obese subjects, the mTWEAK protein expression in sc adipose tissue (SAT) is mainly due to the cells contained in the stromal vascular fraction (SVF) compartment. We also reveal that M2 class macrophages express mTWEAK at higher levels than M1 type. Finally, we report that in human primary cultured adipocytes, sTWEAK down-regulates inflammatory cytokines induced by TNFα, by hampering intracellular signaling events.

Materials and Methods

Subjects

Two different cohorts were studied.

Cohort 1

Cohort 1 included 23 severely obese subjects and an age- and gender-matched population of 35 overweight subjects who were recruited at the Hospital Clínico de Málaga and Hospital Universitari de Tarragona Joan XXIII (Spain), respectively (Table 1). None of the severely obese subjects were receiving insulin therapy or oral hypoglycemic or hypolipidemic agents. Subjects were of Caucasian origin and had no systemic or infectious disease other than obesity.

Cohort 2

A different cohort from the Hospital Clínico de Málaga was included in the study. Twenty-three severely obese patients, who underwent bariatric surgery, were prospectively recruited. Patients were studied before and 6 months after bariatric surgery (Table 2).

Abdominal SAT was obtained from 4 severely obese patients (undergoing bariatric surgery procedures) and from 4 control subjects (undergoing laparoscopic surgery procedures such as hiatus hernia repair or cholecystectomies). Adipocytes and SVF were isolated as described elsewhere (4) from the biopsies of 4 severely obese patients and 4 control patients. Biological samples were frozen in liquid nitrogen and stored at −80°C for later analysis.

The corresponding hospital ethics committees responsible for research approved the study, and informed consent was obtained from all participants.

Analytical methods

Glucose, cholesterol, and triglyceride plasma levels were determined by a Hitachi 737 autoanalyzer (Roche Molecular Biochemicals, Marburg, Germany) using the standard enzyme methods. High-density lipoprotein cholesterol was quantified after precipitation with polyethylene glycol at room temperature (PEG–6000; Merck Schuchardt OHG, Hohenbrunn, Germany). Plasma insulin, levels of soluble IL-6, and plasma free fatty acids (FFAs) were measured as described elsewhere (5). TNFα in serum (cohort 1) and in plasma (cohort 2) was measured with the human TNFα ultrasensitive kit (BLK Diagnostics International, Barcelona, Spain). Serum sTWEAK was measured using the hu-
man TWEAK instant ELISA kit (Bender Medsystems, Vienna, Austria). Leptin was analyzed using the enzyme immunoassay ELISA kit (Diagnostic Systems Laboratories, Webster, Texas). Adiponectin was analyzed using the enzyme immunoassay ELISA kit (DRG Diagnostics GmbH, Marburg, Germany). Plasma high-sensitive C-reactive protein (CRP) was measured using a highly sensitive immunonephelometry kit (Dade Behring, Marburg, Germany).

Reagents

TWEAK, TNFα, IL-4, IL-10, and interferon (INF)-γ were purchased from PeproTech Inc (Rocky Hill, New Jersey). Lipopolysaccharide was purchased from Calbiochem (BioNova, Barcelona, Spain). Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (BioNova, Barcelona, Spain). Phorbol 12-myristate 13-acetate (PMA) was purchased from PeproTech Inc (Rocky Hill, New Jersey). Lipopolysaccharide was purchased from Calbiochem (BioNova, Barcelona, Spain). Leptin was analyzed using the enzyme immunoassay ELISA kit (Diagnostic Systems Laboratories, Webster, Texas). Adiponectin was analyzed using the enzyme immunoassay ELISA kit (DRG Diagnostics GmbH, Marburg, Germany). Plasma high-sensitive C-reactive protein (CRP) was measured using a highly sensitive immunonephelometry kit (Dade Behring, Marburg, Germany).

Table 1. Clinical and Anthropometrical Characteristics of Cohort 1

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 35)</th>
<th>Severely Obese (n = 23)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>44.5 ± 8.3</td>
<td>40 ± 10</td>
<td>ns</td>
</tr>
<tr>
<td>Gender, n, male/female</td>
<td>23/12</td>
<td>9/14</td>
<td>ns</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.2 ± 3.6</td>
<td>57.4 ± 7.3</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>90.8 ± 13</td>
<td>146.2 ± 23.5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>122 ± 13</td>
<td>140 ± 23.6</td>
<td>ns</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>70.6 ± 9.4</td>
<td>84.5 ± 10.2</td>
<td>.004</td>
</tr>
<tr>
<td>Cholesterol, mM</td>
<td>4.8 ± 0.3</td>
<td>5.2 ± 1.1</td>
<td>ns</td>
</tr>
<tr>
<td>HDL-cholesterol, mM</td>
<td>1.3 ± 0.3</td>
<td>1 ± 0.6</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>1.2 ± 0.7</td>
<td>1.4 ± 0.8</td>
<td>ns</td>
</tr>
<tr>
<td>LDL-cholesterol, mM</td>
<td>2.9 ± 0.9</td>
<td>3.5 ± 1.1</td>
<td>ns</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.3 ± 0.7</td>
<td>5.6 ± 1</td>
<td>ns</td>
</tr>
<tr>
<td>Insulin, μU/mL</td>
<td>5.9 ± 4.5</td>
<td>31.5 ± 18</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.4 ± 1.1</td>
<td>8.1 ± 5.9</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>1.9 ± 1.4</td>
<td>6.1 ± 4.5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>TNFα, pg/mL</td>
<td>2.4 ± 1.3</td>
<td>4.1 ± 2.4</td>
<td>.003</td>
</tr>
<tr>
<td>sTWEAK, pg/mL</td>
<td>587.8 ± 264.3</td>
<td>252.8 ± 386.5</td>
<td>&lt;.003</td>
</tr>
</tbody>
</table>

Abbreviations: DBP, diastolic blood pressure; ns, not significant; SBP, systolic blood pressure.

Clinical and analytical characteristics (units provided) of the severely obese patients (n = 23) before and 6 months after surgery. Patients were 7 men and 16 women aged 40.2 ± 10 years.

<table>
<thead>
<tr>
<th></th>
<th>Before Surgery</th>
<th>6 Months After Surgery</th>
<th>P Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Change, %&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pearson Correlation, % TWEAK&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI, kg/m²</td>
<td>56 ± 6.6</td>
<td>43 ± 6.5</td>
<td>&lt;.001</td>
<td>−24.5 ± 7.1</td>
<td>R = −0.008; P = 0.976</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>143 ± 14.6</td>
<td>117.5 ± 13.7</td>
<td>&lt;.001</td>
<td>−18.1 ± 7.4</td>
<td>R = 0.208; P = .342</td>
</tr>
<tr>
<td>Cholesterol, mM</td>
<td>4.9 ± 1</td>
<td>3.4 ± 0.69</td>
<td>&lt;.001</td>
<td>−30.8 ± 11.6</td>
<td>R = −0.055; P = 0.802</td>
</tr>
<tr>
<td>HDL-cholesterol, mM</td>
<td>1.10 ± 0.3</td>
<td>0.92 ± 0.2</td>
<td>&lt;.001</td>
<td>−13.4 ± 15.3</td>
<td>R = −0.001; P = .998</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>1.6 ± 1</td>
<td>1.3 ± 0.5</td>
<td>.010</td>
<td>−7.9 ± 33.1</td>
<td>R = 0.065; P = .770</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.6 ± 1.1</td>
<td>4.8 ± 0.4</td>
<td>&lt;.001</td>
<td>−12.3 ± 15.3</td>
<td>R = 0.029; P = .897</td>
</tr>
<tr>
<td>FFAs, mM</td>
<td>0.56 ± 0.24</td>
<td>0.48 ± 0.17</td>
<td>.386</td>
<td>5.4 ± 56.4</td>
<td>R = −0.535; P = .018</td>
</tr>
<tr>
<td>Insulin, μU/mL</td>
<td>28.1 ± 16.4</td>
<td>11.3 ± 4.3</td>
<td>&lt;.001</td>
<td>−51.3 ± 23.9</td>
<td>R = −0.079; P = .734</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>7.2 ± 5</td>
<td>2.5 ± 1</td>
<td>&lt;.001</td>
<td>−58.3 ± 21.8</td>
<td>R = −0.103; P = .665</td>
</tr>
<tr>
<td>Leptin, ng/mL</td>
<td>82.8 ± 32.5</td>
<td>34.4 ± 37</td>
<td>&lt;.001</td>
<td>−61.8 ± 25.9</td>
<td>R = −0.075; P = .768</td>
</tr>
<tr>
<td>CRP, mg/mL</td>
<td>7 ± 6.2</td>
<td>1.6 ± 4.7</td>
<td>.001</td>
<td>−62.6 ± 32.3</td>
<td>R = −0.015; P = .954</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>3.1 ± 3</td>
<td>1.1 ± 1.7</td>
<td>.012</td>
<td>105.6 ± 767</td>
<td>R = −0.071; P = .786</td>
</tr>
<tr>
<td>TNFα, pg/mL</td>
<td>1.5 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>.033</td>
<td>−17.8 ± 14</td>
<td>R = 0.067; P = .820</td>
</tr>
<tr>
<td>sTWEAK, pg/mL</td>
<td>253 ± 110.4</td>
<td>319 ± 157</td>
<td>.029</td>
<td>34.8 ± 53.7</td>
<td>R = 1</td>
</tr>
</tbody>
</table>

Clinical and analytical characteristics (units provided) of the severely obese patients (n = 23) before and 6 months after surgery. Patients were 7 men and 16 women aged 40.2 ± 10 years.

<sup>a</sup> P Value for paired Student’s t test between T = 0 and T = 6.

<sup>b</sup> Percentage of change (T6−To/T6 = *100).

<sup>c</sup> Correlation analysis with percentage sTWEAK and clinical and anthropometrical variables R (Pearson correlation coefficient and P value).

<sup>d</sup> Plasma levels.

Cell culture and treatments

**Primary culture of human adipocytes**

Human preadipocytes were purchased from the European Collection of Cell Cultures (Salisbury, United Kingdom). Cells were cultured from Sigma-Aldrich (Barcelona, Spain). The following antibodies were used: antihuman TWEAK/TNFSF12 was purchased from R&D Systems (Barcelona, Spain); Fn14; p44/42 (ERK); stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK); p38 MAPK; phosphorylated (P)-p44/42 (ERK) (Thr202/Tyr204); P-SAPK/JNK (Thr183/Tyr185); P-p38 MAPK (Thr180/Tyr182); inhibitory-κB (IκB)-α; P-IκBα (Ser32) were obtained from Cell Signaling (Invitrogen, Spain); and anti-β2-actin antibody was obtained from Sigma-Aldrich.

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were differentiated into adipocytes as recommended by the European Collection of Cell Cultures. At day 14 of differentiation, cells were treated with 100 ng/mL TWEAK or left untreated. After 17 hours, the medium was replaced with a fresh medium. Cells were then either left unstimulated or stimulated for 8 hours with 50 ng/mL TNFα or with 50 ng/mL TNFα plus 100 ng/mL sTWEAK. Cells and cell culture supernatant were collected for further processing.

**Human monocyte-derived macrophages**

Human peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Hypaque gradients (Amersham Bioscience, Barcelona, Spain). Monocytes were purified from PBMCs by magnetic cell sorting using CD14-microbeads (Milteny Biotech, S.L. Madrid, Spain). M1, M2a(IL-4), and M2c(IL-10) macrophages were generated in vitro according to the literature (11) by incubation of PBMCs in 1000 U/mL IFNγ, 10 ng/mL IL-4, and 10 ng/mL IL-10, respectively, for 5–6 days in RPMI 1640 supplemented with 10% fetal bovine serum.

In addition, THP-1 cells were polarized to M1, M2a, and M2c as described by Lepur et al (24). After treatments, all cells were collected for further processing.

**Immunoblot analysis**

Cellular proteins were submitted to SDS-PAGE, transferred to nitrocellulose membranes, and blocked. Blots were developed and quantified as described elsewhere (5).

**Gene expression and relative quantification**

RNA was extracted and quantified as described (4, 5). The TaqMan gene expression assays used were as follows: IL-6 (Hs00985641_m1), IL-8 (Hs00174103_m1), IL-1β (Hs01555410_m1), IL-10 (Hs00961622_m1), TNFα (Hs00174128_m1), TWEAK (Hs000611242_m1), MCP-1 (Hs00234140_m1), CD206 (Hs00267207_m1), ADIPOQ (Hs00605917_m1), PPARγ (Hs00234592_m1), and PPIA (Hs99999904_m1).

**Statistical analysis**

For clinical and anthropometrical variables, data are expressed as mean ± SD and for in vitro experimental results as mean ± SE. Differences in variables before and 6 months after surgery were analyzed by a paired Student’s t test. Cohen’s kappa agreement was calculated to detect positive or negative variations in circulating level changes before and after surgery between sTWEAK and adiponectin.

Differences among control and severely obese subject were tested with the Mann-Whitney U test for nonnormally distributed data or Student’s t test for normally distributed data. Spearman’s correlation coefficient was used to analyze the association between sTWEAK levels and clinical or metabolic parameters. The mixed linear model was used to determine whether any variable was affecting sTWEAK change between 0 and 6 months, considering the measures of each subject as a random effect and the time and the influence of variables as fixed. Gender, age, and homeostasis model assessment of insulin resistance (HOMA-IR) were considered to adjust the model. Finally, stepwise linear regression analysis was performed grouping patients at T 0 from cohort 1 and cohort 2 to determine the independence of the association between metabolic and clinically associated variables and sTWEAK levels. P < .05 was considered significant.

Statistical analysis was performed using version 15 of the Statistical Package for the Social Sciences (SPSS, Chicago, Illinois).

**Results**

**sTWEAK serum levels are reduced in severely obese patients and rise after bariatric surgery**

When we compared circulating serum sTWEAK levels in the cohort 1 of severely obese subjects vs a comparable control group (Table 1), we observed significantly lower levels of sTWEAK in severely obese patients compared with the control group (P < .003) (Figure 1A). No significant differences according to gender were found with respect to sTWEAK levels within the group of severely obese subjects or within the control group (P = .297 and P = .063, respectively) or after grouping all subjects from cohort 1 (P = .414). As expected, severely obese subjects showed a circulating proinflammatory profile with significantly higher levels of IL-6 and TNFα (Table 1).

We also measured sTWEAK in a different cohort before and after bariatric surgery (cohort 2, Table 2). At 6 months after surgery, sTWEAK levels were significantly elevated in 69% of the patients (P = .029) when compared with basal levels before weight loss (Table 2 and Figure 1B). No differences were found according to gender with respect to sTWEAK before (P = .698) or after surgery (P = .878). A mimetic effect of sTWEAK and adiponectin changes after weight loss were observed in some subjects (n = 17), with a tendency to be significant (κ = 0.415; P = .087) (25). Other clinical parameters, biochemical (glucose and lipid profile) and inflammatory, were found significantly decreased after surgery (Table 2). Correlation analysis with all studied variables at T = 0 and T = 6 showed that sTWEAK at T = 0 was negatively related to CRP at T = 6 (r = −0.537, P = .026), and sTWEAK at T = 6 significantly correlated with FFAs at T = 6 (r = −0.499, P = .025). In a bivariate correlation analysis, the percentage of change of sTWEAK was the only variable that significantly related with the percentage of change of FFA (r = −0.535, P = .018). Then we performed a paired t test to check whether FFA levels change significantly after surgery. Despite a clear trend to be lower, the differences were not statistically significant (P = .386). Finally, to test whether changes in FFAs were influencing sTWEAK changes, we used the mixed linear model, and we found that FFAs modulate the expression of sTWEAK (P = .021), meaning that an increase of 1 unit (1 mM) of FFAs decreases sTWEAK by −202.994 pg/mL [95% confidence interval (CI) (−373.167; −32.820)].

Bivariate correlation analysis grouping subjects from cohorts 1 and 2 at T = 0 (n = 81) showed that circulating sTWEAK correlates negatively with HOMA-IR (r =
circulating levels, we constructed a multiple regression model with sTWEAK as the dependent variable, including all the above-correlated variables as independent ones, and gender and age as confounding factors. The final model indicated that the increase of 1 unit of BMI (kilograms per square meter) decreases sTWEAK by $-7.73$ pg/mL (95% CI $-12.2; -3.26$).

Finally, to test the influence of sTWEAK, HOMA-IR, cholesterol, triglycerides, and HDL-cholesterol on BMI index we performed a regression analysis with BMI as the dependent variable. The result showed that BMI was predicted by HOMA-IR [$B = 1.85$ (CI 1.21; 2.5)] and HDL-cholesterol [$B = -11.51$ (95% CI $-19.6; -3.3$)]. The model had an adjusted $R^2$ coefficient of 0.478.

**mTWEAK is predominantly expressed in the SVF and Fn14 in the isolated adipocytes obtained from SAT of severely obese patients**

When we determined the protein expression of TWEAK isoforms and Fn14 in the SAT depot of control and severely obese patients, we observed that the expression of mTWEAK and Fn14 was higher in severely obese than in controls, whereas the expression of sTWEAK was undetectable in both groups (Figure 2A). Additionally, as already described, TNFα mRNA levels were up-regulated in the SAT samples of severely obese subjects, confirming the existing local inflammatory background in these patients (Supplemental Figure 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org) (26).

When we analyzed isolated adipocytes and SVF from SAT of severely obese and control subjects, we observed that mTWEAK and Fn14 protein were slightly overexpressed in SVF from severely obese subjects, and, in the adipocyte fraction of these subjects, the Fn14 receptor protein expression was also significantly elevated (Figure 2, B and C).
mTWEAK is overexpressed in M2a but not in M2c, M1, or unpolarized M0 monocyte-derived macrophages

We addressed the question of whether M1, M2a, or M2c macrophage types express TWEAK because there was no information regarding this issue, and these macrophage types have been described in the obese setting (16). We first determined TWEAK expression at the mRNA and protein level in THP-1 polarized macrophages to M1, M2a, and M2c. We observed that the M1 type had significantly up-regulated expression of IL-1α, IL-8, IL-6, MCP-1, and TNFα when compared with the unpolarized M0 type; in the M2a macrophage, IL-10 and CD206 were significantly up-regulated, whereas M2c showed a similar profile to M0, with a slightly significant up-regulation of TNFα. Interestingly, TWEAK mRNA was markedly up-regulated only in the M2a type (Figure 3A). To rule out that this finding is not cell type restricted, we also obtained M1, M2a and M2c type human monocyte-derived macrophages, and we observed that IL-6 mRNA was overexpressed in the M1 type, whereas CD206 was overexpressed in the M2a type (Figure 3C). Both M1 and M2a expressed similar mRNA levels of TWEAK (Figure 3C).

At the protein level, we showed an overexpression of mTWEAK only in the M2a type, in both THP-1 polarized macrophages, and human monocyte-derived macrophages (Figure 3B and D). Very low intracellular levels of sTWEAK were detected in each macrophage subtype (Figure 3, B and D).

It is well known that some cytokines may help to polarize macrophages toward M1 and M2 types (12); we then checked whether sTWEAK treatment could have an effect on macrophage polarization. When sTWEAK was added to the polarization treatments of THP-1 cells, no polarizing effect was observed because the mRNA expression profile of the selected cytokines remained the same (Supplemental Figure 2).

sTWEAK reduces TNFα induction of IL-6 and IL-8 cytokine in primary cultures of human adipocytes

A down-regulation of the TNFα inflammatory effects by sTWEAK have been described in different cell types in the setting of autoimmune and chronic inflammatory diseases (19–21). We then explored whether sTWEAK might also interfere with TNFα proinflammatory effects in adipocytes because the involvement of TNFα in the inflammatory events that take place in obesity is well established (27). For this purpose we analyzed human primary cultured adipocytes using a previously described experimental procedure, in which cells pretreated with sTWEAK or left untreated were further challenged with TNFα (19).

When human primary cultured adipocyte cells were pretreated with sTWEAK, we observed a significant reduction (P < 0.005) in the TNFα-induced IL-6 and IL-8 mRNA and protein expression profile (Figure 4, A and B). No changes were observed for adiponectin gene or protein expression (Figure 4, A and B) or for PPARγ gene expression (Figure 4A).
Adipocytes remained greater than 90% viable at the end of the different treatments (data not shown).

**sTWEAK inhibits TNFα cellular signaling in primary cultures of human adipocytes**

To explore a possible inhibition of the TNFα inflammatory cascade by sTWEAK, we analyzed the activation status of the nuclear factor-kB (NF-κB) and MAPK signaling pathways, well-known mediators of TNFα signaling in adipocytes (27). For this purpose, sTWEAK pretreated primary cultured adipocytes were further challenged with TNFα for 5 seconds, 15 seconds, 30 seconds, 2 hours, and 8 hours. Pretreatment of adipocytes with sTWEAK lowered the activity of NF-κB by significantly reducing IκBα phosphorylation at 5 seconds, which was followed, as expected, by a reduction in total IκBα. Regarding MAPK signaling cascades, we observed that sTWEAK pretreatment significantly reduced the activation of JNK at 10 seconds and 15 seconds, and ERK1/2 protein kinase at 5 and 10 seconds, whereas p38 phosphorylation was only moderately reduced but did not reach significance (Figure 5).

**Discussion**

In the present study, we report, for the first time, that sTWEAK circulating levels are significantly decreased in severely obese patients compared with a control group. On the other hand, sTWEAK levels rise after bariatric surgery in 69% of patients. The observation of lower levels of sTWEAK in severely obese subjects is in agreement with decreased sTWEAK circulating levels found in patients with other chronic inflammatory conditions such as...
type 2 and type 1 diabetes, heart failure, or atherosclerosis, all of which are associated with an increased cardiovascular risk (3, 7, 28, 29).

The improvement in the metabolic and inflammatory profile after massive weight loss leads to a recovery of the circulating levels of antiinflammatory molecules; such is the case of adiponectin. Since we observed that sTWEAK levels also rise after surgery, this may suggest that sTWEAK could play an antiinflammatory role. However, to confirm this association, we are aware that a larger sample size will be necessary. Interestingly, we also found that FFA modulates the expression of sTWEAK, which datum may indicate that lipotoxicity could be an important factor that may be regulating sTWEAK levels.

There is no clear explanation for decreased sTWEAK levels in severely obese patients. In general, the mechanisms leading to reduced levels of sTWEAK in diseases associated with cardiovascular risk are not known. In the case of severe obesity, we found that the main determinant of the sTWEAK circulating levels was BMI in an inverse-dependent manner. However, the low levels of mRNA of TWEAK expression detected in subcutaneous and visceral adipose tissue (5) suggest that the contribution of adipose tissue to sTWEAK levels should be minor. Thereafter the circulating sTWEAK levels may be decreased in severe obesity, possibly as a consequence of the metabolic derangement of other tissues such as vasculature and skeletal muscle, which are both involved in the development of obese comorbidities, and in which TWEAK expressed at higher levels has been found (2).

Many cytokines of the TNF family exist in the form of transmembrane proteins and, after proteolytic cleavage, are secreted as soluble form. Typically both forms activate their receptors efficiently, however, sometimes with a different intensity (30–32). In the case of TWEAK, mTWEAK induces a superior response than sTWEAK in vitro (33).

In this study, we have analyzed the expression of TWEAK protein isoforms in SAT in detail by isolating adipocytes from their SVF cells. Interestingly, adipocytes show negligible m/sTWEAK expression levels but a high expression of their receptor Fn14, whereas the SVF cells express mTWEAK only. Since macrophages are an important component of the SVF cells, this finding may point to macrophages as one of the cells that expresses this cytokine in the adipose tissue of severely obese subjects. This
observation could be of importance because in severe obesity, this tissue is highly infiltrated with the macrophages (up to 40%) (10).

In obesity, infiltrating macrophages have been described to fit within the M1 and M2 (a and c) type, and the M2 type has been described as the most abundant within the obese adipose tissue (12–17). It has been reported that macrophages can express mTWEAK on their surface (23), but to date, there has been no evidence in favor of a specific subtype. Here we report that M2a human monocyte-derived macrophages express higher levels of mTWEAK when compared with M2c, M1, and M0 type. Since phenotypes of macrophages that express TWEAK are not described in human adipose tissue, the impact of our findings needs to be corroborated in isolated macrophages from severely obese subjects.

It is well known that some cytokines may help to polarize macrophages toward M1 and M2 types (12). In this sense, we found that in our experimental conditions, sTWEAK has no effect over the different macrophage activation states, indicating that the presence of sTWEAK may not affect macrophage phenotype.

In addition, we have shown that treatment of human primary cultured adipocytes with sTWEAK significantly reduced the production of TNFα-induced IL-6, and IL-8 by down-regulating NF-κB and MAPK pathways with no apparent changes in the adiponectin gene or protein expression. Although it is known that NF-κB regulates the expression of IL-6 and IL-8 by directly binding to their promoter regions (34, 35), it does not regulate adiponectin expression directly. Adiponectin is in part regulated by peroxisomal proliferator-activated receptor-γ (36), which does not undergo any change in its expression either after sTWEAK pretreatment. This inhibitory/modulatory effect of sTWEAK on TNFα-activated cells is in line with what occurs in other pathologies such as rheumatoid arthritis (20), ischemic stroke (21), and in several tumor epithelial cells (19), suggesting a broader general competitive behavior of sTWEAK and TNFα. Both sTWEAK and TNFα cytokines coexist within the adipose tissue environment, and both have a proinflammatory potential, although at the same doses, TNFα has a much more potent and rapid inflammatory upshot than sTWEAK (37). In this sense, the low circulating levels of sTWEAK found in obese subjects may contribute to amplify the deleterious effect of TNFα in systemic and local specific tissues.

In conclusion, our observation of lower sTWEAK circulating levels in severely obese subjects may favor the
proinflammatory activity elicited by TNFα. Additionally, in human adipocytes, the modulator effect by sTWEAK over TNFα cytokine production broadens our knowledge of TNFα inflammatory response and could impinge on a better understanding of its adverse metabolic effects in obesity.

Acknowledgments

We gratefully acknowledge the collaboration of Dr Enric Caubet (Department of Surgery, Hospital St Pau i Sta Tecla, Tarragona, Spain). We thank the BioBanc of HUJ23 for providing human samples and Pilar Hernández (Statistics, Epidemiology, and Bioinformatics Unit, Institut d’Investigació Sanitària Pere Virgili of Tarragona, Tarragona, Spain).

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This work was supported by projects from the Fondo de Investigación Sanitaria: Grant PI 11/00049 (to M.R.CH), Grant PI08/0733 (to M.R.C.), Grant SAF2012-36186 (to S.F.-V.) and from the Project Consolider Ingenio Grant CSD-2010-00065 (to A.G.-E.). S.F.-V. acknowledges support from the “Miguel Català de Salut” tenure track program (Grant CP10/00438) from the Instituto de Salud Carlos III. M.R.C. and A.G.-E. are also cofinanced by Institut d’Investigació Sanitària Pere Virgili of Tarragona, Tarragona, Spain).

Disclosure Summary: The authors have nothing to disclose.

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