Tumor Necrosis-Like Weak Inducer of Apoptosis as a Proinflammatory Cytokine in Human Adipocyte Cells: Up-Regulation in Severe Obesity Is Mediated by Inflammation But Not Hypoxia

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Context: Adipose tissue hypoxia and endoplasmic reticulum (ER) stress may link the presence of chronic inflammation and macrophage infiltration in severely obese subjects. We previously reported the up-regulation of TNF-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor-inducible 14 (Fn14) axis in adipose tissue of severely obese type 2 diabetic subjects.

Objectives: The objective of the study was to examine TWEAK and Fn14 adipose tissue expression in obesity, severe obesity, and type 2 diabetes in relation to hypoxia and ER stress.

Design: In the obesity study, 19 lean, 28 overweight, and 15 obese nondiabetic subjects were studied. In the severe obesity study, 23 severely obese and 35 control subjects were studied. In the type 2 diabetes study, 11 type 2 diabetic and 36 control subjects were studied. The expression levels of the following genes were analyzed in paired samples of subcutaneous and visceral adipose tissue: Fn14, TWEAK, VISFATIN, HYOU1, FIAF, HIF-1a, VEGF, GLUT-1, GRP78, and XBP-1. The effect of hypoxia, inflammation, and ER stress on the expression of TWEAK and Fn14 was examined in human adipocyte and macrophage cell lines.

Results: Up-regulation of TWEAK/Fn14 and hypoxia and ER stress surrogate gene expression was observed in subcutaneous and visceral adipose tissue only in our severely obese cohort. Hypoxia modulates TWEAK or Fn14 expression in neither adipocytes nor macrophages. On the contrary, inflammation up-regulated TWEAK in macrophages and Fn14 expression in adipocytes. Moreover, TWEAK had a proinflammatory effect in adipocytes mediated by the nuclear factor-κB and ERK but not JNK signaling pathways.

Conclusions: Our data suggest that TWEAK acts as a pro-inflammatory cytokine in the adipose tissue and that inflammation, but not hypoxia, may be behind its up-regulation in severe obesity. (J Clin Endocrinol Metab 95: 2983–2992, 2010)
We previously reported an increase of TWEAK and FasL expression in SAT and visceral adipose tissue (SAT) of severely obese subjects. In this study, there was a marked up-regulation of both TWEAK and FasL gene expression in severely obese type 2 diabetic patients. Likewise, severely obese type 2 diabetic patients are characterized by elevated adipocyte and macrophage cell lines.

TWEAK and its receptor, Fn14, is a key player in the inflammatory processes. TWEAK controls many cellular activities and is emerging as a new proinflammatory cytokine. A recent study has shown that TWEAK and Fn14 are associated with obesity. Recently, cellular inflammation has emerged as an important mediator of these dysfunctions.

In obesity, hypoxia appears in and local hypoxia. In obesity, hypoxia appears in clusters of adipocytes that become distant from the vasculature as adipose tissue expands. These hypoxic clusters of adipocytes that become distant from the vasculature as adipose tissue expands.

**TABLE 1. Clinical and anthropometrical characteristics of the studied cohorts**

<table>
<thead>
<tr>
<th>Obesity study cohort</th>
<th>Type 2 diabetes study cohort</th>
<th>Severe obesity study cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lean</strong> (BMI &lt;25 kg/m²)</td>
<td>Control (n = 36)</td>
<td>Type 2 diabetic (n = 11)</td>
</tr>
<tr>
<td>(n = 19)</td>
<td>(n = 15)</td>
<td>(n = 19)</td>
</tr>
<tr>
<td><strong>Age (yr)</strong></td>
<td>51.7 ± 16.0</td>
<td>57.1 ± 15.0</td>
</tr>
<tr>
<td><strong>Gender (n, male/female)</strong></td>
<td>13/6</td>
<td>16/12</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>23.6 (24.2)</td>
<td>27.2 (27.9)</td>
</tr>
<tr>
<td><strong>Waist circumference (cm)</strong></td>
<td>83.0 (90.0)</td>
<td>97.0 (100.0)</td>
</tr>
<tr>
<td><strong>Cholesterol (mm)</strong></td>
<td>5.2 ± 1.2</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td><strong>HDL-cholesterol (mm)</strong></td>
<td>1.5 ± 0.5</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td><strong>Triglycerides (mm)</strong></td>
<td>1.0 (1.6)</td>
<td>1.1 (1.5)</td>
</tr>
<tr>
<td><strong>Glucose (mm)</strong></td>
<td>4.8 ± 0.7</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td><strong>Insulin (μIU/ml)</strong></td>
<td>3.4 (6.7)</td>
<td>4.0 (7.2)</td>
</tr>
<tr>
<td><strong>HOMA-IR</strong></td>
<td>0.75 (1.83)</td>
<td>1.01 (2.09)</td>
</tr>
<tr>
<td><strong>sIL-6 (pg/ml)</strong></td>
<td>1.4 (2.5)</td>
<td>1.0 (2.2)</td>
</tr>
</tbody>
</table>

BMI, Body mass index; FFA, free fatty acid; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; sIL-6, soluble IL-6; ns, not significant.

a Differences vs. lean.

b Differences vs. overweight.
Patients and Methods

Selection of patients
A total of 96 Caucasian subjects were recruited at the University Hospital Joan XXIII (Tarragona, Spain) and the Hospital Virgen de la Victoria (Malaga, Spain). All were of white origin and reported that their body weight had been stable for at least 3 months before the study. They had no systemic disease other than obesity or type 2 diabetes, and all had been free of any infections during the month before the study. Liver and renal diseases were specifically excluded by biochemical work-up. VAT and SAT biopsies were obtained from all 96 subjects. Samples were obtained during abdominal elective surgical procedures (cholecystectomy, surgery for abdominal hernia or during bariatric surgery). The hospitals’ ethics committees approved the study, and informed consent was obtained from all participants. For the obesity study, a cohort of 19 lean, 28 overweight, and 15 obese nondiabetic subjects were selected. For the severe obesity study, a cohort of 23 severely obese subjects was included in the study. For intergroup gene expression comparisons, we selected an age- and gender-matched population of 35 overweight subjects as a control group. None of the morbidly obese subjects were receiving insulin therapy or oral antidiabetic or antilipemic agents. For the type 2 diabetes study, patients were classified as having type 2 diabetes mellitus according to the American Diabetes Association criteria. According to these criteria, we selected 11 type 2 diabetic obese subjects and 36 nondiabetic obese subjects for the intergroup comparison. Pharmacological treatment of the patients with type 2 diabetes mellitus was: insulin, 9.1%; oral hypoglycemic agents, 54.5%; diet, 9.1%; statins, 63.6%; fibrates, 0%; blood pressure-lowering agents, 54.5%.

Collection and processing of biological samples
All patients had fasted at least 12 h before the surgical procedure. SAT was collected at the site of surgical incision from the abdominal wall, whereas VAT was obtained from the epiploon. Adipose tissue samples were immediately transported to the laboratory in ice, washed in PBS one times, frozen in liquid N2, and stored at −80 C.

Analytical methods
Glucose, cholesterol, and triglycerides plasma levels were determined in 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 were measured as described elsewhere (13).

Gene expression-relative quantification of adipose tissue samples
Four hundred to 500 mg frozen adipose tissue was used for RNA extraction. RNA was reverse transcribed to cDNA and the following genes were assayed using Taqman assays (Applied Biosystems, Foster City, CA): Fn14 (Hs00171993_m1); TWEAK (Hs00611242_m1); VISFATIN (Hs00237184_m1); heat shock protein (HYOU1) (Hs00197328_m1); fasting-induced adipose factor (Hs00211522_m1); hypoxia-inducible factor (HIF)-1a (Hs00153153_m1); vascular endothelial growth factor (VEGF) (Hs00900054_m1); glucose trans-
porter (GLUT)-1 (Hs00197884_m1); glucose-regulated protein, 78 kDa (GRP78) Hs99999174_m1; X-box binding protein (XBP)-1 (Hs00231936_m1). Real-time quantitative PCR was performed on the 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA). The cycle threshold (Ct) value for each sample was normalized with the expression of PPIA (Hs99999940_m1). SDS software 2.3 and RQ Manager 1.2 (Applied Biosystems) were used to analyze the results with the comparative Ct method (2^-ΔΔCt). All data were expressed as an n-fold difference relative to a calibrator (a mix of different depot samples).

**Cell culture and stimuli**

SGBS preadipocyte cells were induced to differentiate to mature adipocytes as described (14). Differentiation was checked by accumulation of intracellular lipids by Oil Red O (Sigma-Aldrich, Barcelona, Spain). Adipocytes were cultured overnight in serum-free DMEM/F12 (1:1) (Invitrogen, Barcelona, Spain) medium before stimuli.

The human monocyctic THP-1 cell line (European Collection of Cell Culture, Wiltshire, UK) was used and cultured as described by Chacón et al. (15). Cells were serum deprived overnight before stimulation.

**Inflammatory stimuli**

Adipocytes were stimulated for 24 h with 10 and 100 ng/ml with human recombinant (hr) TWEAK (Peprotech, Barcelona, Spain) and 50 ng/ml TNFα (Peprotech). THP-1 cells were treated with 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 8 h.

**Hypoxia and ER stress stimuli**

Adipocytes were incubated at 37°C in a humidified atmosphere of 95% air-5% CO2 or placed into a hypoxic chamber (Billups-Rothenberg, Del Mar, CA) flushed with gas mixture consisting of 1% O2, 5% CO2, and 94% N2. Cells were incubated for 24 h at 37°C under these conditions. Chemical hypoxia was induced by treating cells with 200, 100, and 500 μM CoCl2 (Sigma-Aldrich) for 8 or 24 h.

ER stress was induced by the addition of 1–5 μg/ml of tunicamycin (Sigma-Aldrich) for 8 or 24 h.

Dimethylhydrodiphenyltetra-zoliumbromide-based in vitro toxicity assay kit and the lactate dehydrogenase-based assay kit were used as a measure of cell viability (Sigma-Aldrich) in response to hypoxia, TNFα, IL-6, and LPS treatment, as recommended by the manufacturers. Assays were performed in triplicates. We checked that the concentrations used and the length of incubations were nontoxic for SGBS and THP-1 cells (data not shown).

**Inhibitor experiments**

The signaling pathway was studied by using inhibitors concomitant with 100 ng/ml TWEAK stimulus for 8 h: 10 μM parthenolide, 10 μM U0126, and 10 μM SP600125 (Merck Chemicals, Barcelona, Spain).

RNA from THP-1 and SGBS mature adipocytes were extracted as described (15). Genes assayed were: TWEAK, Fn14, HIF-1α, and GRP78 using the probes mentioned above and the following probes for: MCP-1 (Hs00234140_m1), IL-6 (Hs00985641_m1), LEP (leptin; Hs00174877_m1), AP1 (a) (Sigma-Aldrich; Hs00605917_m1), and TNFα (Hs00174128_m1).

**Western blotting**

The Western blotting technique was performed as already described (16). A total of 40 μg of protein were subjected to 15% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-TWEAK goat polyclonal antibody (R&D Systems, Vitro S.L., Madrid, Spain) (1:1000) and anti-Fn14 affinity-purified antirabbit (Cell Signaling Technology, Beverly, MA). Rabbit and goat IgG conjugated with peroxidase was used as secondary antibody (1:5000). Placenta and human umbilical endothelial cell protein extracts were used as positive control. All blots were developed with SuperSignal West Femto chemiluminescent substrate (Pierce Biotechnology, Boston, MA) and visualized by VersaDoc imaging system and Quantity One software (Bio-Rad, Barcelona, Spain).

**Statistical analysis**

For clinical and anthropometrical variables, normal distributed data are expressed as mean value ± SD, and for variables with no Gaussian distribution, values are expressed as median (75th percentile). In vitro experimental results are presented as means ± so from three independent experiments performed in duplicates. Statistical significance was tested with the unpaired Student’s t test or Mann-Whitney U test for nonnormally distributed data or one-way ANOVA followed by the protected least-significant difference test or by Kruskal-Wallis U for nonnormally distributed data. P < 0.05 was considered significant.

**Results**

**TWEAK and Fn14 are overexpressed only in adipose tissue in severe obesity**

The main anthropometric and analytical characteristics of the obese, type 2 diabetes, and severely obese cohorts in our study are presented in Table 1. When we analyzed TWEAK and Fn14 gene expression in the SAT and VAT adipose tissue depots in the above-mentioned cohorts, only severely obese patients showed that both TWEAK and Fn14 were significantly overexpressed in both SAT and VAT depots when compared with their control group (Table 2). In obese and type 2 diabetic study cohorts, VAT and SAT TWEAK and Fn14 expression lev-

**TABLE 3.** Intragroup study of expression levels of TWEAK and Fn14 in paired SAT and VAT samples

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Overweight</th>
<th>Obese</th>
<th>Severely Obese</th>
<th>Type 2 Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWEAK</td>
<td>SAT &gt; VAT</td>
<td>SAT &gt; VAT</td>
<td>SAT = VAT</td>
<td>SAT = VAT</td>
<td>SAT &gt; VAT</td>
</tr>
<tr>
<td>Fn14</td>
<td>SAT &lt; VAT</td>
<td>SAT &lt; VAT</td>
<td>SAT = VAT</td>
<td>SAT &lt; VAT</td>
<td>SAT = VAT</td>
</tr>
</tbody>
</table>

P values are as indicated.
els did not change among obesity groups or with the presence of type 2 diabetes (Table 2).

Intragroup study of expression levels of TWEAK and Fn14 in paired SAT and VAT samples showed that in obese and severely obese subjects, TWEAK expression was similar in both depots (Table 3). In lean, overweight, and type 2 diabetic subjects, TWEAK expression was higher in SAT than VAT (Table 3). Fn14 was predominantly expressed in VAT samples in lean, overweight, and severely obese subjects, whereas in obese and type 2 diabetic subjects, the expression was similar (Table 3).

**Hypoxia and ER gene expression markers are highly expressed in SAT and VAT tissue of the severely obese subjects**

Hypoxia could be behind the overexpression of TWEAK and Fn14 in severe obese adipose tissue, and hence, we analyzed the relative expression levels in SAT and VAT depots from our obese and severely obese cohorts of widely accepted surrogate genes for hypoxia (2) and ER stress (4) listed in Table 4. Genes selected were: HIF-1α, a key transcription factor in hypoxia; VEGF, an angiogenic marker hypoxia regulated; VISFATIN, a proinflammatory and potentially insulin-mimetic adipokine regulated by HIF-1α; FIAF (fasting-induced adipose factor), involved in lipid metabolism and up-regulated by HIF-1α; GLUT-1, a glucose transporter gene found to be increased under hypoxia; and HYOU1, an oxygen-regulated gene. The markers of ER stress transducers selected were: XBP-1; X-box, a transcription factor that modulates the ER stress response; and GRP78, a glucose-regulated gene strongly related with hypoxia (see Supplemental Table 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org).

We observed that in the severely obese subjects, all selected genes were significantly up-regulated when compared with the control group (Table 4). Almost all analyzed genes (excepted FIAF, HYOU1, and GRP78) were expressed in significantly higher amounts in VAT than SAT depots.

In contrast, in the obesity study cohort, only VEGF was found to be up-regulated in lean subjects without differences for the rest of hypoxia and ER stress-explored genes (Table 4).

No significant differences in gene expression levels were found with the presence of type 2 diabetes for the selected hypoxia or endoplasmic reticulum gene markers (data not shown).

**Inflammatory but not hypoxic or ER stress stimuli modulate Fn14 expression in human adipocytes**

In view of the above-mentioned results, we questioned whether inflammation, hypoxia, or hypoxia-induced ER stress could be behind the up-regulation of TWEAK and

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**TABLE 4. Gene expression analysis of surrogate hypoxia and ER stress genes in SAT and VAT samples in obesity (arbitrary units)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Severe obesity study cohort</th>
<th>Lean (n = 15)</th>
<th>Overweight (n = 28)</th>
<th>Severe obese (n = 23)</th>
<th>VAT</th>
<th>SAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>1.2 (2.5) 1.7 (0.7)</td>
<td>1.3 (1.3) 1.0 (0.9)</td>
<td>1.4 (1.3) 0.9 (1.0)</td>
<td>1.2 (1.9) 1.1 (1.2)</td>
<td>6.6 (27.8) 6.0 (25.6)</td>
<td></td>
</tr>
<tr>
<td>VISFATIN</td>
<td>2 (0.9) 2 (0.9)</td>
<td>1.1 (1.2) 1.0 (1.2)</td>
<td>1.0 (1.2) 1.0 (1.2)</td>
<td>1.0 (1.2) 1.0 (1.2)</td>
<td>6.2 (32.8) 6.0 (32.8)</td>
<td></td>
</tr>
<tr>
<td>FIAF</td>
<td>1.2 (1.0) 1.0 (0.9)</td>
<td>1.1 (0.9) 1.0 (0.9)</td>
<td>1.1 (0.9) 1.0 (0.9)</td>
<td>1.2 (1.0) 1.0 (0.9)</td>
<td>6.6 (27.8) 6.0 (25.6)</td>
<td></td>
</tr>
<tr>
<td>GLUT-1</td>
<td>1.0 (1.0) 1.0 (1.0)</td>
<td>1.1 (1.2) 1.0 (1.2)</td>
<td>1.1 (1.2) 1.0 (1.2)</td>
<td>1.2 (1.0) 1.0 (0.9)</td>
<td>6.6 (27.8) 6.0 (25.6)</td>
<td></td>
</tr>
<tr>
<td>GRP78</td>
<td>0.2 (0.2) 0.2 (0.2)</td>
<td>0.2 (0.2) 0.2 (0.2)</td>
<td>0.2 (0.2) 0.2 (0.2)</td>
<td>0.2 (0.2) 0.2 (0.2)</td>
<td>6.6 (27.8) 6.0 (25.6)</td>
<td></td>
</tr>
<tr>
<td>XBP-1</td>
<td>0.9 (0.9) 0.9 (0.9)</td>
<td>0.9 (0.9) 0.9 (0.9)</td>
<td>0.9 (0.9) 0.9 (0.9)</td>
<td>0.9 (0.9) 0.9 (0.9)</td>
<td>6.6 (27.8) 6.0 (25.6)</td>
<td></td>
</tr>
</tbody>
</table>

* Differences when comparing same depot between obesity group study (P < 0.05).}

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mRNA in the severely obese subjects. To this end we analyzed TWEAK and Fn14 expression in SGBS mature adipocytes under hypoxic conditions induced either chemically (CoCl₂) or by low oxygen tension (1% O₂).

In these conditions, whereas TWEAK and Fn14 gene expression was not affected (Fig. 1, A and C), IL-6 and LEP mRNA were up-regulated (Fig. 1, A, B, and D). TNFα gene expression was below detection levels (data not shown).

We found that HIF-1α gene expression was down-regulated by the hypoxic stimulus and GRP78 expression was not altered (Fig. 1A).

To analyze the effect of an ER stress stimulus on TWEAK and Fn14 expression in adipocytes, we used 1 μg/ml of tunicamycin for 24 h. We observed that tunicamycin had no effect on TWEAK or Fn14 expression. However, IL-6, LEP, and GRP78 expression were altered under these conditions (Fig. 1E).

In contrast, when we tested an inflammatory stimulus (50 ng/ml TNFα for 24 h), we found Fn14 expression significantly increased at mRNA (Fig. 1C) and protein levels (Fig. 1G).

Both stimuli together, hypoxia and inflammation, did not affect TWEAK gene expression and did not increase the inflammatory effect of TNFα over Fn14 gene expression (Fig. 1C). TNFα significantly increased HIF-1α and GRP78 (Fig. 1D).

Also, as an inflammatory stimulus, we tested IL-6 for 24 h, which produced a significant increase in Fn14 gene expression, whereas TWEAK gene expression was not altered (Fig. 1F).

Inflammatory stimuli but not hypoxia or ER stress stimuli modulate TWEAK expression in macrophages

Macrophages are an important component of adipose tissue milieu in severe obesity and have been shown to contribute to the inflammatory state observed in obesity. To analyze the effect on TWEAK and Fn14 of a hypoxic or an inflammatory stimulus over human macrophages, we tested THP-1 human macrophage cell line under a chemical hypoxic stimulus. We found that hypoxia, induced chemically with 100 μM CoCl₂, did not change the expression levels of TWEAK and Fn14 (Fig. 2, A and B). We decided to use a dose of 100 ng/ml LPS as an inflammatory stimulus on phorbol-12-myristate-13-acetate-pretreated THP-1 cells instead of TNFα because doses of...
TNFα ranging from 0.1 to 50 ng/ml for 8 h produced no significant effect on cytokine stimulation (data not shown). Both LPS and TNFα are known to activate in THP-1 overlapping signaling pathways (17, 18), although with different intensities. We observed that an inflammatory stimulus induced by LPS significantly up-regulated TWEAK and HIF-1α, and down-regulated Fn14 (Fig. 2B).

IL-6 and TNFα mRNA were found both significantly up-regulated under inflammation induced by LPS, but chemical hypoxia up-regulated only IL-6 (Fig. 2C).

An ER stress stimulus induced by 5 μg/ml tunicamycin for 8 h significantly increased IL-6, TNFα, and GRP78 but did not alter TWEAK gene expression. Fn14 mRNA was found significantly down-regulated by tunicamycin (Fig. 2D).

IL-6 stimuli over THP-1 macrophage significantly up-regulated only TWEAK gene expression levels (Fig. 2E).

**TWEAK acts as proinflammatory cytokine over human adipocyte cells**

To explore the role of TWEAK over adipocytes, we stimulated SGBS adipocytes with 10 or 100 ng/ml hrTWEAK for 24 h. TWEAK stimulation led to a significant increase in mRNA levels of IL-6 and MCP-1 (Fig. 3A). Fn14, APM1, and LEP gene expression were un-
changed under these conditions. TNFα mRNA levels were undetectable (Fig. 3A).

To determine which signaling pathway was switched on, we used parthenolide as NF-κB inhibitor, U0126 as MAPK kinase-ERK1/2 inhibitor, and SP600125 as c-Jun N-terminal kinase (JNK) inhibitor. We observed that inhibition of NF-κB and MAPK kinase-ERK1/2 reduced the gene expression of IL-6 and MCP-1, whereas JNK inhibition did not reduce the expression of IL-6 and increased MCP-1 expression (Fig. 3B).

**Discussion**

Within adipose tissue, a common feature of the inflammatory process associated with obesity is the altered expression of many cytokines and adipokines. We recently reported that the TWEAK/Fn14 axis may be a new component of the network that participates in the inflammatory imbalance observed in obesity and insulin resistance states (10). In this sense, Tweak and Fn14 expression have been detected in carotid atherosclerotic plaques, suggesting a pathological role of TWEAK/Fn14 axis in this obesity-related comorbidity (19).

In the present study, we showed that TWEAK/Fn14 up-regulation in SAT and VAT tissue was linked to severe obesity. One particularity of severe obesity is the presence of elevated systemic inflammation (11, 12). At tissue level, the precise mechanisms behind this inflammatory response as the adipose tissue mass expands are not well known. It has been suggested that hypoxia could be a key trigger of adipose tissue dysfunction because hypoxia generates tissue inflammation (2). In obese human subjects, decreased oxygen partial pressure within the adipose tissue was paralleled by an increase in the expression and secretion of the chemokine and markers of macrophage infiltration (20).

Interestingly, we found up-regulation of hypoxia response genes such as VEGF, GLUT1, FIAF, VISFATIN, and HIF-1α only in our cohort of severely obese patients. The up-regulation of HIF-1α observed in adipose tissue of our severely obese subjects is in accordance with previous reports (21). However, in view of our own and others’ *in vitro* studies that showed HIF-1α mRNA down-regulation in adipocytes (2), the high expression found in whole adipose tissue may be accounted for mainly by the cells in the stromovascular fraction, as has been previously suggested (22). No changes in hypoxia and ER stress genes were observed in our obese cohort; only VEGF was found overexpressed in the lean subjects, which is in accordance with a recent scientific report (20).

On the other hand, the proinflammatory milieu facilitated by hypoxia in adipose tissue may represent an important stimulus for macrophage attraction. It is known that adipose tissue in severe obesity is highly infiltrated (up to 40%) with macrophages; this in turn generates a vicious circle producing a high degree of tissue inflammation (22).

In view of the parallel up-regulation of TWEAK/Fn14 (this study and Ref. 10) and hypoxia-specific genes in severe obesity, we considered that hypoxia could be modulating TWEAK/Fn14 gene expression in adipose tissue of these patients. Our experimental data showed that hypoxia did not regulate *per se* TWEAK and Fn14 expression in SGBS adipocytes cells or THP-1 macrophage. Similarly to hypoxia, TWEAK and Fn14 expression in both adipocytes and macrophage cells was not changed by an ER stress stimulus, whereas changes in the production of inflammation-related cytokines in SGBS adipocyte cells were consistent with the original hypoxia hypothesis; IL-6 and LEP were found up-regulated and TNFα was not altered under hypoxic conditions (23, 24).

Inflammatory stimuli *in vitro* differentially increased the expression of TWEAK in macrophages and Fn14 in adipocytes. But both hypoxia and inflammatory stimulus strongly increased expression, in both adipocytes and macrophages, of IL-6, a potent inflammatory cytokine.
On the other hand, IL-6 circulating levels were highly increased in our severely obese subjects. Moreover, hypoxia has been described as a positive stimulus for IL-6 (25). Interestingly, when we stimulated adipocyte and macrophage cells with IL-6, we observed the same effects as depicted by the inflammatory stimuli of TNFα in adipocytes and LPS in macrophages: a specific up-regulation of TWEAK in macrophages and Fn14 in adipocytes. These findings suggest a possible paracrine action of TWEAK expressed by macrophages over Fn14 up-regulated receptor in adipocytes.

In adipose tissue fractions of severely obese subjects, expression of Fn14 was higher in the stromovascular fraction than in the mature isolated adipocyte (10). However, the precise contribution of adipocytes, macrophages and other cells to the production of TWEAK and Fn14 is difficult to assess quantitatively. It is not certain whether the process of preparation and subsequent incubation of the different cell fractions alters their relative rate of production and release of various adipokines. In addition, large adipocytes show the highest rates of production of inflammatory adipokines (26), but because of their greater fragility, they are likely to be underrepresented, or absent, from most cell fractionations.

What, in turn, could be the biological effect of TWEAK over adipocytes? Our experiments suggest an active participation of TWEAK in the inflammatory environment found in obesity because we show for the first time that TWEAK has a proinflammatory effect over human adipocytes in which it induces the expression of the proinflammatory cytokines IL-6 and MCP-1. What could the molecular mechanisms be by which adipocytokines sense and respond to TWEAK? In other cell types, the binding of TWEAK to its receptor, Fn14, has been shown to activate a limited set of signaling pathways involved in cell proliferation and differentiation, including NF-κB activation, which is associated with its proinflammatory effect (6): MAPK, JNK, ERK (27), and p38 MAPK (28, 29). Here we showed that activation of NF-κB and ERK1/2 signaling pathways was implicated in the proinflammatory process of TWEAK over human adipocytes. In THP-1 macrophage cells, TWEAK signal transduction has been shown to be NF-κB dependent and induced the degradation of inhibitory-κB (30).

In conclusion, in the present paper, we show, for the first time, that TWEAK acts as a proinflammatory cytokine in human adipocyte cells. We report an overexpression of TWEAK/Fn14 and hypoxia characteristic genes in both SAT and VAT adipose tissue depots of severely obese patients; however, both TWEAK and Fn14 genes were not hypoxia sensitive. The increment of TWEAK and Fn14 in severe obesity may be due to the inflammatory environment, in particular that caused by the presence of IL-6, a cytokine that could be playing a key role in TWEAK/Fn14 regulation.

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