"In vitro hyper-responsiveness to TNF-alpha contributes to adipokine dysregulation in omental adipocytes of obese subjects."

Maury, Eléonore ; Noel, Laurence ; Detry, Roger ; Brichard, Sonia

ABSTRACT

Context: In obesity, adipocyte hypertrophy and macrophage infiltration lead to overproduction of proinflammatory adipokines, which play a crucial role in the metabolic syndrome. The molecular mechanisms underlying this overproduction are still unsettled. The role of TNF-alpha also remains controversial in human obesity. Objective: We revisited the contribution of TNF-alpha to adipokine dysregulation in central obesity. We more particularly assessed the involvement of TNF-alpha vs. other stromal-vascular cell (SVC)-secreted factors and searched for potential differential responses to TNF-alpha between adipocytes of lean and obese individuals. Design and participants: Primary cultures of omental adipocytes from obese and non-obese age- and sex-matched subjects were used. For some experiments, we generated media previously conditioned by SVC, which mimic adipocyte microenvironment. Results: Adipocytes of obese subjects mainly overexpressed adipokines, in comparison to those of lean ones...

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In Vitro Hyperresponsiveness to Tumor Necrosis Factor-\(\alpha\) Contributes to Adipokine Dysregulation in Omental Adipocytes of Obese Subjects

Eléonore Maury, Laurence Noël, Roger Detry, and Sonia M. Brichard

Endocrinology and Metabolism (E.M., L.N., S.M.B.) and Surgery Units (R.D.), Faculty of Medicine, University of Louvain, B-1200 Brussels, Belgium

Context: In obesity, adipocyte hypertrophy and macrophage infiltration lead to overproduction of proinflammatory adipokines, which play a crucial role in the metabolic syndrome. The molecular mechanisms underlying this overproduction are still unsettled. The role of TNF-\(\alpha\) also remains controversial in human obesity.

Objective: We revisited the contribution of TNF-\(\alpha\) to adipokine dysregulation in central obesity. We more particularly assessed the involvement of TNF-\(\alpha\) vs. other stromal-vascular cell (SVC)-secreted factors and searched for potential differential responses to TNF-\(\alpha\) between adipocytes of lean and obese individuals.

Design and Participants: Primary cultures of omental adipocytes from obese and nonobese age- and sex-matched subjects were used. For some experiments, we generated media previously conditioned by SVCs, which mimic adipocyte microenvironment.

Results: Adipocytes of obese subjects mainly overexpressed adipokines, in comparison with those of lean ones, when cultured in SVC-conditioned media. This was abrogated by immunoneutralization of TNF-\(\alpha\), indicating that among the numerous factors secreted by SVCs, TNF-\(\alpha\) is a crucial contributor to adipokine dysregulation. Accordingly, adipocytes of obese subjects overproduced adipokines in response to direct exposure of TNF-\(\alpha\). This hyperresponsiveness was mediated by TNF-\(\alpha\) receptor 1 and hyperactivation of the nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) pathway. Correspondingly, NF-\(\kappa\)B activity was increased in adipocytes of obese subjects and correlated with adipocyte size, adipokine expression, and \textit{in vivo} insulin resistance. Eventually adipokine overexpression in adipocytes of obese subjects was prevented by NF-\(\kappa\)B inhibitors.

Conclusions: In obesity, TNF-\(\alpha\), i.e. over other SVC-secreted factors, a crucial determinant of adipokine dysregulation acts on enlarged adipocytes, which are hyperresponsive to this triggering signal. This ultimately exacerbates adipokine production, inflammation, and the metabolic syndrome. (\textit{J Clin Endocrinol Metab} 94: 1393–1400, 2009)
ulated upon activation normal T cells expressed and secreted (RANTES), one interleukin (IL-7), one tissue-inhibitor of metalloproteinases (TIMP-1) and one megakaryocytic growth factor [thrombopoietin (TPO)] (5). Several pieces of evidence indicate that these adipokines may contribute to obesity-linked disorders. Thus, all of them may play a role in cardiovascular disease via different mechanisms, whereas some may be involved or associated with insulin resistance/type 2 diabetes (GRO, MIP-1 β, RANTES and TIMP-1) or worsen obesity itself (TIMP-1) (reviewed in Ref. 5). In our previous work, enhanced expression of these adipokines in adipocytes did correlate with several features of the metabolic syndrome (5), but the mechanisms underlying their overproduction in obesity are still unknown.

Obesity is associated with several endocrine abnormalities [in particular hyperinsulinemia, enhanced glucocorticoid turnover, and altered sympathetic tone (6, 7)]. The abnormal hormonal milieu may be involved in adipokine dysregulation (8–10), but the inflammatory state per se may also contribute (11). When we compared the relative contribution of hormones and TNF-α, we found that TNF-α was the most potent inducer of adipokine changes. Yet the role of TNF-α remains controversial in human obesity (12). The aim of the present work was therefore to revisit the contribution of TNF-α to adipokine dysregulation in human central obesity. We focused on the involvement of TNF-α vs. other stromal-vascular cell (SVC)-secreted factors and searched for potential differential responses to TNF-α between adipocytes of lean and obese individuals in vitro.

Subjects and Methods

Subjects

OAT was obtained from obese and nonobese age- and sex-matched subjects undergoing abdominal surgery after an overnight fast (Table 1). Obesity was defined as a body mass index (BMI) of 30 kg/m² or greater. All cases were elective procedures to correct benign conditions (colonic diverticulosis, evagination, and overweight status treated by vertical banded gastroplasty) or malignant disease (carcinoma of colon). For cancer cases, adipose samples were collected at a site far from the tumor itself; these cases had no evidence of disseminated disease and the operation was curative. In those conditions, localized colorectal cancer did not modify mRNA levels of the investigated adipokines (supplemental Table 1, published as supplemental data on The Endocrine Society’s Journals Online Web site at http://jcem.endojournals.org).

For each patient, blood was collected in the fasting state before surgery. As shown in Table 1, obese subjects exhibited several clinical or laboratory features of the metabolic syndrome. When compared with age- and sex-matched controls, they were characterized by significantly higher blood pressure; higher blood glucose, hyperinsulinemia, and insulin resistance; abnormal lipid profile; increased plasma leptin; and decreased plasma adiponectin concentrations.

No patient had a history of diabetes or exhibited overt bowel inflammatory disease and none had undergone any significant weight change over the last 3 months. Patients receiving hormones (e.g., insulin, glucocorticoids) or nonsteroid antiinflammatory drugs or taking any medications known to influence fat mass or metabolism (e.g., thiazolidinediones, systemic and nonspecific modulators of adrenergic receptors etc.) were excluded. Three subjects in each group were receiving antihypertensive treatment (amlodipine, a calcium channel blocker).

For each culture, adipocytes from only one subject were used. Due to the limited tissue availability, not all data could be generated from all patients. However, when subgroups of obese and nonobese subjects were compared, each subgroup was always representative of the respective whole group (i.e., similar clinical/laboratory characteristics: BMI, age, sex ratio, insulin levels, etc.).

Clinical and laboratory characteristics of lean and obese subjects

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Nonobese</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>55.6 ± 2.3</td>
<td>52.0 ± 3.1</td>
</tr>
<tr>
<td>Sex ratio (men/women)</td>
<td>17/11</td>
<td>16/11</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.8 ± 0.5</td>
<td>39.3 ± 1.5</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>121 ± 4</td>
<td>141 ± 3</td>
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<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>73 ± 3</td>
<td>86 ± 2</td>
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<td>Glucose homeostasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/liter)</td>
<td>5.2 ± 0.1</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Insulin (mU/liter)</td>
<td>2.5 ± 0.1</td>
<td>11.8 ± 2.9</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.6 ± 0.1</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol (mmol/liter)</td>
<td>2.2 ± 0.2</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Total cholesterol/HDL</td>
<td>3.3 ± 0.2</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/liter)</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.2</td>
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<tr>
<td>Adipokines and inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>10.3 ± 1.8</td>
<td>27.7 ± 4.6</td>
</tr>
<tr>
<td>Adiponectin (μg/ml)</td>
<td>10.0 ± 1.2</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>hsCRP (mg/liter)</td>
<td>7.0 ± 1.2</td>
<td>10.4 ± 1.0</td>
</tr>
</tbody>
</table>

Clinical and laboratory parameters were obtained in the fastest state before surgery. Values are means ± SEM for 28 lean and 27 obese subjects. For cultures of adipocytes, due to the limited availability of OAT, not all data could be generated from all patients. However, when subgroups of obese and nonobese subjects were compared, each subgroup was always representative of the respective whole group (i.e., similar clinical/laboratory characteristics: BMI, age, sex ratio, insulin levels, etc.).

| Glucose (mmol/liter) | 5.2 ± 0.1 | 5.9 ± 0.2 |
| Insulin (mU/liter)  | 2.5 ± 0.1 | 11.8 ± 2.9 |
| HOMA-IR             | 0.6 ± 0.1 | 3.0 ± 0.7 |
| Lipids              |          |        |
| LDL cholesterol (mmol/liter) | 2.2 ± 0.2 | 2.8 ± 0.3 |
| Total cholesterol/HDL | 3.3 ± 0.2 | 4.3 ± 0.2 |
| Triglycerides (mmol/liter) | 1.0 ± 0.1 | 1.4 ± 0.2 |
| Adipokines and inflammation |          |        |
| Leptin (ng/ml)      | 10.3 ± 1.8 | 27.7 ± 4.6 |
| Adiponectin (μg/ml) | 10.0 ± 1.2 | 6.7 ± 0.7 |
| hsCRP (mg/liter)    | 7.0 ± 1.2 | 10.4 ± 1.0 |

Clinical and laboratory parameters were obtained in the fastest state before surgery. Values are means ± SEM for 28 lean and 27 obese subjects. For cultures of adipocytes, due to the limited availability of OAT, not all data could be generated from all patients. However, when subgroups of obese and nonobese subjects were compared, each subgroup was always representative of the respective whole group (i.e., similar clinical/laboratory characteristics).

Patients provided written informed consent and the study protocol had the approval of the local Ethical Committee of the Cliniques Universitaires Saint-Luc.

Culture of mature adipocytes freshly isolated from OAT

OAT biopsies were transported to the laboratory within 5–10 min after sampling (6). Samples were fractionated into adipocytes and SVC (that mainly contain macrophages, endothelial cells, and preadipocytes) by collagenase treatment, as reported (5, 6). This yields two high purity fractions (5). Adipocytes (1 ml packed cells) were cultured for up to 24 h, as described (5). Digestion and 24-h culture did not influence secretion and release of the studied adipokines (5).

For some experiments, SVC-conditioned media were generated. These media were collected after culturing SVC (500 ml per 3 g initial tissue) for 24 h, as described for adipocytes (5, 6). Aliquots of medium to serve as conditioned medium, in which mature adipocytes were cultured, were collected after culturing SVC (500 ml per 3 g initial tissue) for 24 h, as described for adipocytes (5, 6). Aliquots of medium were stored at −20 C and then mixed before use [2:1, (vol/vol)] with fresh medium to serve as conditioned medium, in which mature adipocytes were cultured.

In other experiments, human recombinant TNF-α (PreproTech, Rocky Hill, NJ), different antibodies directed against human TNF-α, human TNF-α receptor (TNFR) 1 or TNFR 2 (R&D Systems, Minneapolis, MN), or protease inhibitors [nuclear factor-κB (NF-κB) inhibitor BAY11-7085, c-Jun NH 2-terminal protein kinase (JNK) inhibitor SP600125, both from Calbiochem, La Jolla, CA] were added to the medium alone or in combination. The concentrations used were recommended by the manufacturer (antibodies) or similar to those reported by others (13, 14) and devoid of overt toxicity as assessed by measurement of low and unchanged release of lactate dehydrogenase in media and microscopical examination (6). At the end of the experiments, cells were frozen at −80 C. Average adipocyte volume in a sample was calculated using lipid weight (Folch’s method), cell number [estimated by DNA
INTERLEUKIN which cysteine residues are separated by one amino acid. Chemokine family with adjacent critical cysteine residues; C-X-C, second chemokine family in differentiated adipocytes from either lean (n picogram per microgram adipocyte DNA. Values are means after 24 h of culture, with or without 10 ng/ml TNF-

Effects of TNF-α on adipokine production by newly differentiated adipocytes. Human omental adipocytes that had been fully differentiated in vitro were cultured with increasing concentrations of TNF-α for 24 h. Adipokine mRNA levels were measured by RTQ-PCR, normalized to the levels of TATA box-binding protein (TBP; used as reporter gene) and presented as relative expression compared with untreated cells. Insets, Adipokines secreted in medium after 24 h of culture, with or without 10 ng/ml TNF-α, were measured by ELISA and expressed as picogram per microgram adipocyte DNA. Values are means ± SEM for seven independent cultures obtained from seven subjects. *, P < 0.05, **, P < 0.01 vs. untreated cells. C-C, Chemokine family with adjacent critical cysteine residues; C-X-C, second chemokine family in which cysteine residues are separated by one amino acid.

Culture of adipocytes differentiated in vitro from omental precursor stromal cells

Omental preadipocytes were grown to confluence and then differentiated in vitro as described (Ref. 5, except that only 5 nM dexamethasone were used). A full stage of differentiation was achieved at d 12 (5), with was no morphological difference between adipocytes obtained from SVC of lean or obese subjects (not shown). Differentiated adipocytes were then cultured for 24 h without insulin and dexamethasone, which did not affect the expression of late adipocyte markers or the investigated adipokines (not shown). At d 13, the adipocytes were cultured with different hormones or TNF-α. At the end of the experiments, cells and media were saved. Because similar results were obtained with in vitro differentiated adipocytes from either lean (n = 3) or obese (n = 3 or 4) subjects, data were pooled for presentation.

RNA extraction and real-time quantitative PCR (RTQ-PCR)

Total RNA was extracted and reverse transcribed, and RTQ-PCR was performed with designed primers (supplemental Table 2), as described elsewhere (5). Relative changes in the expression level of one specific gene were presented as 2^ΔΔCt (5).

Quantification of adipokines in culture media

Adipokines were quantified by specific ELISAs as described (5).

NF-κB activity assay

The TransAM NF-κB p65 transcription factor assay kit was used (Active Motif, Rixensart, Belgium). Two hundred microliters of adipo-

cytes were thawed in the Complete lysis buffer provided by the manufacturer (Active Motif, Rixensart, Belgium), and whole-cell extracts were prepared after removal of the lipid supernatant. Binding of p65 NF-κB transcription factors was detected by ELISA. Signal intensity of NF-κB activation was proportional to adipocyte proteins (5–30 μg); approximately 10–15 μg adipocyte proteins were used per well. Data were expressed as OD and normalized on a per cell basis (per microgram DNA).

Other

 Plasma was obtained by refrigerated centrifugation and stored at −20 C. Measurements of glucose, insulin, lipids, adiponectin, and leptin, and calculation of the homeostasis model assessment-estimated insulin resistance index (HOMA-IR) were performed as described (5).

Results

Effects of hormones and TNF-α on adipokine production by newly differentiated adipocytes

We first examined the hormonal regulation of the investigated adipokines in human omental adipocytes that were fully differentiated. Insulin, dexamethasone, or isoproterenol (a β-adrenergic agonist used as a surrogate of catecholamines) induced only moderate (less than 2-fold) increases in GRO and TPO mRNAs, whereas the glucocorticoid and the β-agonist also slightly decreased MIP-1β and/or IL-7 (supplemental Table 3). Thus, hormones caused only modest changes in the expression of some adipokines.

By contrast, TNF-α induced a strong rise in the expression of all the investigated adipokines (except for TPO) (Fig. 1). This rise occurred in a dose-dependent fashion and amounted to 600-fold of control values for some adipokines (i.e. RANTES). These changes in mRNA abundance were accompanied by parallel changes in secretion levels (Fig. 1, insets). Because TNF-α induced potent changes on the investigated adipokines, we focused on its effects.

TNF-α production by OAT of lean and obese subjects

We first verified that TNF-α was actually increased in OAT of obese subjects. Adipose tissue TNF-α mRNA abundance was
Indeed 2-fold higher in obese than in lean subjects [relative expression: 1.99 ± 0.31 vs. 1.00 ± 0.28, n = 13 (obese) and 8 (lean); P < 0.05]. We next examined which subcellular fraction was the main contributor to TNF-α production (Fig. 2, A and B). Adipose tissue was fractionated into isolated adipocytes and SVCs, which were cultured independently for 24 h. TNF-α secretion by both fractions was higher in obesity. TNF-α mRNA levels of TNF-α and its receptors were quantified by RTQ-PCR, normalized to the levels of TATA box-binding protein and presented as relative expression compared with values obtained in adipocytes of lean subjects. Values are means ± SEM for eight lean and 13 obese subjects. *, P < 0.05, **, P < 0.01 vs. lean subjects; +, P < 0.05, 2+, P < 0.01 vs. adipocytes of the same subjects.

FIG. 2. Production of TNF-α (A and B) and expression of its receptors (C and D) in mature adipocytes and SVCs in human obesity. Adipocytes and SVCs were freshly isolated from OAT of lean and obese subjects and cultured independently for 24 h. TNF-α secreted in medium was measured by ELISA and expressed in picogram per microgram DNA in each cellular fraction. mRNA levels of TNF-α and its receptors were quantified by RTQ-PCR, normalized to the levels of TATA box-binding protein and presented as relative expression compared with values obtained in adipocytes of lean subjects. Values are means ± SEM for eight lean and 13 obese subjects. *, P < 0.05, **, P < 0.01 vs. lean subjects; +, P < 0.05, 2+, P < 0.01 vs. adipocytes of the same subjects.

Mature adipocytes freshly isolated from omental fat of lean or obese subjects were used in all subsequent culture experiments.

Effects of SVC-conditioned media on adipokine expression by mature adipocytes

Because TNF-α secretion is augmented in obesity and higher in SVCs, we examined whether media conditioned by SVCs mimicked the effects of TNF-α. Mature adipocytes isolated from lean and obese subjects were cultured in the presence of SVC-conditioned media. These media had been previously collected after culturing independently SVCs from lean or obese subjects; before use (i.e., after mixing with fresh medium), their final concentrations of TNF-α amounted to 0.08 ± 0.02 and to 0.22 ± 0.05 ng/ml when originally obtained from lean (n = 8) or obese (n = 13) individuals, respectively (P < 0.01). Conditioned media (from either lean or obese subjects) barely affected mRNA expression of the investigated adipokines in mature adipocytes of lean subjects. By contrast, in obese adipocytes, media conditioned by obese SVC induced an approximately 2-fold rise in gene expression of all the investigated adipokines (except for TPO whose expression was halved) (Fig. 3A). Thus, the effects of SVC-conditioned media on mature adipocytes reproduced those of TNF-α on in vitro differentiated adipocytes, and mature adipocytes of obese subjects were more responsive to these conditioned media.

Because SVCs release numerous regulatory factors, we examined whether the changes induced by SVC-conditioned media were specifically due to TNF-α. Adipocytes of additional obese subjects were cultured in media conditioned by obese SVCs, with or without anti-TNF-α antibody (Fig. 3B). The effects of obese SVC-conditioned media on adipokine mRNAs were largely prevented by immunoneutralization of TNF-α activity.

Eventually we directly tested TNF-α on mature adipocytes and compared the responses between lean and obese subjects (Fig. 4). Apart from for TPO, TNF-α did not cause any significant change of adipokine mRNAs in adipocytes of lean subjects even at the highest concentration used. By contrast, in obese subjects, significant increases were already detected at the lowest concentration (0.1 ng/ml) and mostly occurred in a dose-dependent fashion. These data indicate that adipocytes of obese subjects are hyperresponsive to TNF-α.

Mechanisms underlying the hyperresponsiveness of obese adipocytes to TNF-α

We studied TNF-α receptor and postreceptor signaling. We first investigated whether up-regulation of TNFR2 could mediate the hyperresponsiveness of obese adipocytes. However, only TNFR1 (but not TNFR2, data not shown) immunoneutralization did actually prevent most changes of adipokine mRNAs induced by TNF-α in adipocytes of obese subjects (Table 2).

We next explored which events downstream to TNFR1 could be involved. We studied the effects of NF-κB (BAY11-7085) and JNK inhibitors (SP600125) on TNF-α-induced changes of adipokine expression in mature obese adipocytes (Table 2). Blockade of NF-κB, but not the JNK pathway, did largely prevent alterations of adipokine mRNAs caused by TNF-α.

Because activation of NF-κB pathway may underlie the hyperresponsiveness of obese adipocytes to TNF-α, we directly measured NF-κB (p65) DNA binding activity in mature adipocytes of lean and obese subjects, in the absence or in the presence of TNF-α. TNF-α induced a slight but significant increase of NF-κB activity (~16%) in lean adipocytes (Fig. 5A). In obese adipocytes, both basal and TNF-α-stimulated values of NF-κB activity were about 75–95% higher than in lean adipocytes (Fig. 5A). When the increment of NF-κB activity induced by TNF-α (Δ) was expressed as percentage of the respective basal values within each group of lean and obese subjects, there was still a
2-fold difference between the two groups (Fig. 5B). This suggests that the hyperresponsiveness of obese adipocytes to TNF-α could be mediated by an increased responsiveness of NF-κB activity.

There were positive relationships between values of NF-κB activity and mRNA abundance of each investigated adipokine in nonconditioned medium. Each histogram relative to conditioned media is actually the mean levels of TATA box-binding protein and presented as relative expression compared with values obtained this part of the experiment; the addition of control IgG did not modify adipokine expression levels (not shown). In each panel (A and B), adipokine mRNA levels were measured by RTQ-PCR, normalized to the

FIG. 3. Effects of media conditioned by SVCs on adipokine expression in mature adipocytes of lean and obese subjects (A) and prevention by TNF-α immunoneutralization (B). A, Mature adipocytes were freshly isolated from OAF of lean or obese subjects and cultured for 24 h in basal (nonconditioned) medium or in conditioned media. These conditioned media had been previously collected after culturing SVCs from other lean and other obese subjects for 24 h. More precisely, data were generated from adipocytes of three lean and three obese subjects, adipocytes of each individual being cultured in eight to 11 separate conditioned media (four to five obtained from lean SVCs and four to six from obese ones) and in conditioned medium. B, Mature adipocytes of additional obese subjects were cultured for 24 h, with or without 0.8 μg/ml anti-TNF-α antibody, in nonconditioned or obese SVC-conditioned media. Data were generated from adipocytes of four obese subjects, adipocytes of each individual being cultured in four separate conditioned media. Nonimmune IgG, used at the same concentration as anti-TNF-α, served as supplementary control in this part of the experiment; the addition of control IgG did not modify adipokine expression levels (not shown). In each panel (A and B), adipokine mRNA levels were measured by RTQ-PCR, normalized to the levels of TATA box-binding protein and presented as relative expression compared with values obtained with nonconditioned medium. Each histogram relative to conditioned media is actually the mean ± SEM for 12–16 experimental conditions. A, *, P < 0.05; **, P < 0.01 vs. media conditioned by lean SVCs; + +, P < 0.05; +, P < 0.01 vs. nonconditioned medium. B, *, P < 0.05 vs. nonconditioned medium without antibody; +, P < 0.05; + +, P < 0.01 vs. conditioned medium without antibody.

Discussion

TNF-α was overexpressed in omental adipose tissue of obese subjects, as originally described in sc fat (16, 17) and was more abundantly produced by SVCs than adipocytes, in agreement with pooled data from both adipose depots of obese subjects (18). Strikingly, TNF-α secretion by obese SVCs was 20-fold larger than that by lean adipocytes. This has to be ascribed to the altered cellular composition of the SV fraction in obesity, which is characterized by an increased number of macrophages and endothelial cells, two cell types that largely contribute to the production of proinflammatory factors (5, 19).

Exposure of mature adipocytes to SVC-conditioned media, especially from obese subjects, induced a pattern of adipokine gene expression similar to that observed after TNF-α treatment. One should expect corresponding changes of adipokine mRNAs and protein secretion, as previously shown (see Fig. 1). TNF-α immunoneutralization of these SVC-conditioned media specifically prevented adipokine mRNA changes, suggesting that TNF-α is a major SVC mediator of adipokine dysregulation in adipocytes. Coculture of murine 3T3-L1 adipocytes and macrophage cell line did result in marked up-regulation of proinflammatory adipokines, which was inhibited by a neutralizing antibody to TNF-α (13). By using a more physiologically relevant source of proinflammatory cytokines (i.e., SVCs), our data broaden the concept that, among all the numerous factors released by SVCs, TNF-α plays a crucial paracrine role in worsening adipocyte inflammation in humans. Hence, based on our original observations, TNF-α appears to be a potent and proximal molecule in the hierarchy of cytokines’ and chemokines’ networks of human adipose tissue.
Adipokine gene expression induced by either SVC-conditioned media or direct exposure to TNF-α were amplified in adipocytes of obese subjects compared with lean ones. This hyperresponsiveness of obese adipocytes to an inflammatory stimulus is a novel finding that sharply contrasts with the marked resistance to insulin, another hallmark of the metabolic syndrome. This hyperresponsiveness was already triggered by the lowest concentration of TNF-α used (0.1 ng/ml, Fig. 5), which was within the same range as that measured in SVC-conditioned media. Adipocyte size has recently been shown to be an important determinant of dysregulated adipokine expression and secretion, with the hypertrophic adipocytes shifting their immune balance toward the production of proinflammatory molecules (3–5). We extend these data, which had been previously obtained in the basal state, to adipocytes acutely challenged by a proinflammatory stimulus. This paradigm may perhaps explain why there was no difference in TNF-α response between adipocytes differentiated in vitro from stromal precursors of obese vs. nonobese donors as a similar degree of fat cell hypertrophy was achieved in both situations (our own data).

The primary molecular mechanisms whereby TNF-α triggers an excessive inflammatory response in hypertrophied adipocytes are still unsettled. We explored the signaling pathways at the receptors’ and postreceptors’ levels. In agreement with data obtained in whole adipose tissue (20, 21), TNF-αR2, but not TNF-αR1, was increased in adipocytes isolated from obese subjects. TNF-αR2 has been proposed to mediate some effects of TNF-α in chronic inflammatory states such as obesity in both rodents (22) and humans (20, 23, 24). However, our immunoneutralization experiments ruled out a role for TNF-αR2 in overstimulating adipokine production and clearly established that this effect of TNF-α was mediated by TNF-αR1, in line with most effects of TNF-α on adipose tissue (25). Dissection of downstream signals indicates a pivotal role for the transcriptional factor NF-κB in mediating TNF-α-induced adipokine production, in line with other reports (26–28). Yet the activity and the functional sig-

### TABLE 2. Blockade of TNF-αR1 or NF-κB and JNK pathways on TNF-α-induced adipokine expression in obesity

<table>
<thead>
<tr>
<th></th>
<th>GRO</th>
<th>RANTES</th>
<th>MIP-1β</th>
<th>TIMP-1</th>
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<tr>
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<td>1.00 ± 0.15</td>
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<td>TNF-α</td>
<td>2.16 ± 0.30a</td>
<td>2.45 ± 0.74b</td>
<td>2.02 ± 0.31a</td>
<td>1.98 ± 0.40a</td>
<td>0.50 ± 0.07a</td>
<td>2.57 ± 0.49b</td>
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<td>0.98 ± 0.19</td>
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<td>Control</td>
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<td>1.00 ± 0.43</td>
<td>1.00 ± 0.18</td>
<td>1.00 ± 0.08</td>
<td>1.00 ± 0.10</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.38 ± 0.64a</td>
<td>2.06 ± 0.30a</td>
<td>2.09 ± 0.30a</td>
<td>2.11 ± 0.46a</td>
<td>0.47 ± 0.14a</td>
<td>2.04 ± 0.19a</td>
</tr>
<tr>
<td>BAY11-7085</td>
<td>0.87 ± 0.31</td>
<td>0.81 ± 0.43</td>
<td>0.82 ± 0.42</td>
<td>0.84 ± 0.31</td>
<td>1.00 ± 0.18</td>
<td>1.30 ± 0.23</td>
</tr>
<tr>
<td>BAY11-7085 + TNF-α</td>
<td>1.16 ± 0.37d</td>
<td>1.16 ± 0.28d</td>
<td>1.23 ± 0.38d</td>
<td>1.10 ± 0.35d</td>
<td>1.19 ± 0.32c</td>
<td>1.48 ± 0.27d</td>
</tr>
<tr>
<td>SP600125</td>
<td>1.25 ± 0.52</td>
<td>0.79 ± 0.42</td>
<td>0.90 ± 0.40</td>
<td>1.06 ± 0.39</td>
<td>0.77 ± 0.38</td>
<td>1.30 ± 0.32</td>
</tr>
<tr>
<td>SP600125 + TNF-α</td>
<td>1.69 ± 0.45</td>
<td>1.68 ± 0.44a</td>
<td>1.52 ± 0.41</td>
<td>1.43 ± 0.55</td>
<td>0.73 ± 0.33</td>
<td>1.98 ± 0.26</td>
</tr>
</tbody>
</table>

Omental adipocytes were freshly isolated from obese subjects and cultured with or without test agents. For blockade of TNF-αR1, adipocytes were cultured with 10 ng/ml TNF-α or 5 μg/ml anti-TNF-α, alone or in combination, for 24 h. Nonimmune IgG, used at the same concentration as anti-TNF-α, served as supplementary control; the addition of control IgG did not modify adipokine expression levels (not shown). For blockade of NF-κB and JNK pathways, adipocytes were cultured with or without NF-κB and JNK inhibitors (10 μM BAY11-7085 and 10 μM SP600125, respectively) for 25 h; in the absence or presence of TNF-α (10 ng/ml) added for the last 24 h. Because the inhibitors were dissolved in dimethylsulfoxide, the same amount of dimethylsulfoxide was added to all the experimental conditions. mRNA levels are presented as relative expression compared with control adipocytes (without antibody/inhibitors and TNF-α). Results are the mean ± SEM for adipocytes of six to seven obese subjects.

*a* P < 0.05 for the effect of TNF-α vs. respective paired condition without TNF-α.

*b* P < 0.01 for the effect of TNF-α vs. respective paired condition without TNF-α.

*c* P < 0.01 for the effect of antibody/inhibitor on TNF-α-induced changes (antibody/inhibitor vs. no antibody/inhibitor).

*d* P < 0.05 for the effect of antibody/inhibitor on TNF-α-induced changes (antibody/inhibitor vs. no antibody/inhibitor).
Here we compared NF-κB activity in mature adipocytes and relationships with BMI, adipocyte size, and insulin resistance (IR). Mature adipocytes of lean and obese subjects were cultured with or without 10 ng/ml TNF-α for 4 h. NF-κB (p65) DNA-binding activity was quantified by ELISA in whole-cell protein extracts. Measurements were expressed as OD units and normalized on a cellular basis (per microgram adipocyte DNA). Upper panels. Data are presented as percentages of values in untreated lean adipocytes (A) or increments induced by TNF-α (stimulated minus basal values) (B, in percent of the respective basal values in each group). Results are the mean ± SEM for adipocytes of six lean and seven obese subjects. *, P < 0.05; **, P < 0.01 for the effect of TNF-α vs. respective untreated conditions; +, P < 0.05 for the effect of obesity vs. respective lean conditions. Lower panels. Relationships between adipocyte NF-κB or Δ NF-κB activity and BMI, adipocyte size, or HOMA-IR indices. Stimulated values of NF-κB activity (percent of untreated lean data) are shown in C and in D, but similar correlations were obtained with the basal values (not illustrated). Δ NF-κB activity values are presented as raw data in E. Correlation analysis was performed in the six lean (A) and seven obese subjects (B) studied together. Dotted lines correspond to the 95% confidence interval for r.

Significance of NF-κB in obese adipocytes remains to be elucidated. Here we compared NF-κB activity in adipocytes of lean vs. obese subjects and newly showed that both basal and TNF-α-stimulated NF-κB activities were higher in adipocytes of obese subjects than in those of lean subjects. Accordingly, an increase in constitutive NF-κB activity has been reported in 3T3-L1 cell line during adipocyte differentiation and hypertrophy (29). In our study, NF-κB activity did correlate with the expression of each adipokine and adipocyte size, reinforcing the concept that adipocyte hypertrophy plays a crucial role in triggering inflammation. Eventually the response of NF-κB activity to TNF-α also correlated with insulin resistance in vivo, supporting the idea that NF-κB is an important molecular link between inflammation and insulin resistance in human obesity. Once activated, this process can be self-perpetuating in obese subjects through a positive feedback loop due to TNF-α hyperresponsiveness. Although TNF-α is an established link between insulin resistance and inflammation in obese rodent models, whether such a link exists in humans is still debated. Our work may support this idea, but our in vitro results need to be cautiously interpreted in so far as their translation into human physiology is considered. The few TNF-α neutralization studies, which addressed in vivo whether TNF-α is involved in insulin resistance of obese subjects yielded negative results, possibly because of their limited power (30–33). Alternatively, this lack of benefit could be due to the fact that TNF-α predominantly acts in an autocrine/paracrine fashion on production/target tissues in obesity. However, anti-TNF-α treatments of inflammatory diseases such as rheumatoid arthritis have clearly shown a role for TNF-α in systemic insulin sensitivity in humans (34–36).

In obesity, the hyperresponsiveness of hypertrophied adipocytes to TNF-α coupled to an oversecretion of this cytokine by SVsCs appears to be teleologically meaningful. This amplified paracrine cross talk involving TNF-α may be viewed as an ultimate attempt to control adiposity. Enhanced TNF-α (action/production) aims at limiting further weight gain through lipolysis and insulin resistance, impaired preadipocyte differentiation, and increased adipocyte apoptosis (37). However, these effects of TNF-α occur at the expense of worsening insulin resistance and inflammation. This insulin-resistant and inflammatory state may further be amplified by the overproduction of the investigated adipokines (5), thereby linking obesity to its comorbidities.

In conclusion, TNF-α that, among all the numerous factors secreted by SVsCs, is a crucial determinant of adipokine dysregulation in obesity acts on enlarged adipocytes, which are hyperresponsive to this inflammatory triggering signal. This in vitro adipocyte hyperresponsiveness results in adipokine overproduction through TNFR1 and hyperactivation of NF-κB. This amplified paracrine cross talk between adipocytes and SVsCs in human obesity ultimately leads to worsening of the inflammatory state and the metabolic syndrome.

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Address all correspondence and requests for reprints to: S. M. Brichard, Unité d’Endocrinologie et Métabolisme, Université Catholique de Louvain/Unité d’Endocrinologie et Métabolisme 5530 Avenue Hippocrate 55, B-1200 Brussels, Belgium. E-mail: sonia.brichard@uclouvain.be.

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