The obese healthy paradox: is inflammation the answer?

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A paradoxical but common finding in the obesity clinic is the identification of individuals who can be considered ‘inappropriately’ healthy for their degree of obesity. We think that studying these obese but metabolically healthy individuals and comparing them with equally obese but insulin-resistant individuals could provide important insights into the mechanistic link between adipose tissue expansion and associated metabolic alterations. In the present study, we investigated whether there are differences in inflammatory and insulin signalling pathways in VAT (visceral adipose tissue) that could account for the metabolic differences exhibited by morbidly obese individuals who are either insulin-resistant (IR-MO) or paradoxically insulin-sensitive (NIR-MO). Our results indicate that there are pathways common to obesity and unrelated to insulin resistance and others that are discriminative for insulin resistance for a similar degree of obesity. For instance, all morbidly obese patients, irrespective of their insulin resistance, showed increased expression of TNFα (tumour necrosis factor α) and activation of JNK1/2 (c-Jun N-terminal kinase 1/2). However, the IR-MO group showed significantly elevated expression levels of IL (interleukin)-(1-β and IL-6 and increased macrophage infiltrates compared with non-obese individuals and NIR-MO. IκBα [inhibitor of NF-κB (nuclear factor κB)] α, the activation of ERK1/2 (extracellular-signal-regulated kinase 1/2) and NF-κB were discriminative of the state of insulin resistance and correlated with differential changes in IRS-1 (insulin receptor substrate 1) expression and Akt activation between IR-MO and NIR-MO individuals. Our results support the concept that NIR-MO individuals lack the inflammatory response that characterizes the IR-MO patient and that IL-6, IL-1β, ERK and NF-κB are important effectors that mediate the inflammatory effects promoting insulin resistance.

Key words: inflammation, insulin resistance, obesity, signal transduction.

INTRODUCTION

Obesity is becoming a major health problem worldwide despite medical and political efforts to control its increased prevalence. Despite the recent success in identifying the molecular mechanisms controlling energy homeostasis, the truth is that there are no efficient treatments besides surgical approaches. The failure to prevent or reverse the development of obesity makes it more urgent to devise strategies to contain what would be a more devastating second wave of cardiovascular complications. Thus an immediate challenge is to identify who among these individuals could provide important insights into the mechanistic link between adipose tissue expansion and associated metabolic alterations. In the present study, we investigated whether there are differences in inflammatory and insulin signalling pathways in VAT (visceral adipose tissue) that could account for the metabolic differences exhibited by morbidly obese individuals who are either insulin-resistant (IR-MO) or paradoxically insulin-sensitive (NIR-MO). Our results indicate that there are pathways common to obesity and unrelated to insulin resistance and others that are discriminative for insulin resistance for a similar degree of obesity. For instance, all morbidly obese patients, irrespective of their insulin resistance, showed increased expression of TNFα (tumour necrosis factor α) and activation of JNK1/2 (c-Jun N-terminal kinase 1/2). However, the IR-MO group showed significantly elevated expression levels of IL (interleukin)-(1-β and IL-6 and increased macrophage infiltrates compared with non-obese individuals and NIR-MO. IκBα [inhibitor of NF-κB (nuclear factor κB)] α, the activation of ERK1/2 (extracellular-signal-regulated kinase 1/2) and NF-κB were discriminative of the state of insulin resistance and correlated with differential changes in IRS-1 (insulin receptor substrate 1) expression and Akt activation between IR-MO and NIR-MO individuals. Our results support the concept that NIR-MO individuals lack the inflammatory response that characterizes the IR-MO patient and that IL-6, IL-1β, ERK and NF-κB are important effectors that mediate the inflammatory effects promoting insulin resistance.

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INTRODUCTION

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Accumulating evidence identifies inflammation as the potential link between adipose tissue expansion and cardiometabolic complications [1]. In fact, obesity is now considered to be a condition that facilitates the development of a low-grade inflammatory state characterized by increased plasma levels of pro-inflammatory cytokines such as TNFα (tumour necrosis factor α), ILs (interleukins) and cytokine-like proteins known as adipokines [2]. Some of these molecules seem to be secreted by adipocytes, whereas others are predominantly derived from adipose-tissue-infiltrated macrophages. This inflammatory cascade involves activation of a complex network of signalling pathways, including the activation of various serine/threonine kinases and transcription factors such as JNK (c-Jun N-terminal kinase), PKC (protein kinase C) and NF-κB (nuclear factor κB) [3].

One of the consequences of this state of inflammation is the development of insulin resistance as well as increased risk of developing Type 2 diabetes. The specific mechanisms linking inflammation and defects in insulin sensitivity have been partially characterized and have revealed an incomplete picture of a complex cross-talk integrating metabolic, nutritional and inflammatory signalling pathways, eventually leading to the development of obesity-induced insulin resistance [4]. The IRS (insulin receptor substrate)-1 protein is one of the defective molecular effectors associated with obesity-related insulin resistance. Under physiological conditions, insulin stimulates the tyrosine phosphorylation of IRS-1, leading to activation of downstream signals, including PI3K (phosphoinositide 3-kinase) and the Akt pathway [5]. However, in the context of overnutrition-induced insulin resistance, IRS-1 becomes the target substrate for multiple pro-inflammatory serine kinases [6,7]. In fact, there is evidence that TNFα phosphorylates and inhibits IRS-1 on serine residues, resulting in impaired insulin action [8–10]. In particular, JNK1 and IKKα [IκBα (inhibitor of NF-κB) kinase α]...
have been shown to phosphorylate Ser107 directly and inhibit IRS-1 signalling [7,9]. Other studies carried out on 3T3-L1 adipocytes have also implicated the activation of other signalling pathways, such as ERK (extracellular signal-regulated kinase) 1/2 [11] and p38 MAPK (mitogen-activated protein kinase), in the development of insulin resistance [12].

The mechanisms involved in obesity-induced insulin resistance are not completely elucidated. Furthermore, whereas the link between obesity and insulin resistance is well established at the epidemiological level, there is clinical evidence that there are obese individuals who are metabolically healthy that do not develop insulin resistance [13]. Thus we hypothesized that studying this specific group could provide some important insights into the specific mechanisms linking adipose tissue expansion, inflammation and insulin resistance.

In the present study, we have investigated the differences in inflammatory pathways and insulin signalling markers in the VAT (visceral adipose tissue) of equally obese individuals that differ in their degree of insulin resistance. The present study focused on the visceral adipose depot as the most likely to be involved in the metabolic disturbances associated with obesity [14], and, specifically, we have evaluated the existence of differences in macrophage infiltration, mRNA gene-expression profiles of pro-inflammatory cytokines and transcription factors such as TNFα, IL-1β, IL-6, NF-κB and IkBα. We have also measured the mRNA and protein levels of IRS-1, as well as the activation of downstream pathways, such as JNK, ERK, Akt and the transcription factor NF-κB.

**EXPERIMENTAL**

**Subjects**

The present study included 24 MO (morbidly obese) subjects [BMI (body mass index) 36 ± 6.8 kg/m²] (12 women and 12 men) who underwent bariatric surgery with mixed techniques, combining gastric reduction with an intestinal bypass, and 12 non-obese subjects (BMI 18.5–24.9 kg/m²) (six women and six men) with no alterations in lipid or glucose metabolism, as controls. Approval for the study was given by the ethics committee and all patients gave their informed consent. The MO patients that were selected had a similar clinical profile, but a different degree of insulin resistance. Any patients received oral antiobiotic agents or insulin therapy. The weight of all individuals had been stable for at least 1 month and none had associated renal pathology. The MO patients that were classified according to their insulin sensitivity. Specifically, patients having a HOMA-IR (homoeostasis model assessment of insulin resistance) score of <5 were considered to be the non-insulin-resistant (NIR-MO) group. This cut-off point was established from the mean ± 2S.D. of a healthy control population. The MO subjects with a HOMA-IR score of >8, was considered to be the insulin-resistant (IR-MO) group. Clinical details of patients included in the study are indicated in Table 1.

VAT biopsies were obtained from MO patients undergoing bariatric surgery procedures or laparoscopic surgery procedures (hiatus hernia repair or cholecystectomies) in lean subjects. Tissue samples were washed in physiological saline, immediately frozen in liquid nitrogen and stored at −80°C for the different assays described below.

**Nuclear and cytoplasmic extracts**

Cytoplasmic and nuclear extracts were prepared from VAT using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce). Tissue samples of 100 mg were homogenized in CER (cytoplasmic extraction reagent) I with protease inhibitors (Sigma), 100 mM PAO (phenylarsine oxide) (Sigma) and 1 mM sodium orthovanadate (Sigma) using an Ultra-Turrax T25 Basic instrument (IKA Werke). After 10 min at 4°C, CER II was added to the lysate. Samples were pelleted by centrifugation at 15 000 g for 10 min at 4°C. The supernatant (cytoplasmic lysates) was recovered and frozen at −80°C.

The pellets (nuclear lysates) were incubated on ice for 40 min in NER (nuclear extraction reagent) with protease inhibitors (Sigma), 100 mM PAO and 1 mM sodium orthovanadate. Samples were pelleted by centrifugation at 15 000 g for 10 min at 4°C. Nuclear lysates were recovered and frozen at −80°C.

Reagent extraction volume was concentrated using Nanosep centrifugal devices (Pall). Protein concentrations were determined using BCA (bicinchoninic acid) protein assay reagent (Pierce) with BSA as a standard. The fractionated proteins were analysed by Western blot and EMSA (electrophoretic mobility-shift assay).

**Western blot analysis**

Cytoplasmic cell lysates (25 μg) were separated by SDS/PAGE, and immunoblots were incubated with the following antibodies: human anti-(pSer112 Akt), anti-phospho-JNK1/2, anti-(phosphotyrosine IRS-1) (Santa Cruz Biotechnology) and anti-phospho-ERK1/2 (Cell Signaling Technology). The immunoblots were reprobed with human anti-JNK1/2, anti-Akt, anti-IRS-1 (Santa Cruz Biotechnology) and anti-ERK1/2 (Calbiochem). The equal amount of protein was detected by staining with Ponceau S (Sigma). Immunocomplexes were detected with appropriate horseradish-peroxidase-conjugated secondary antibodies and detected by ECL (enhanced chemiluminescence) (GE Healthcare). Protein levels were quantified using the image analysis software Quantity One, version 4.4.0 (Bio-Rad Laboratories). Results were calculated as IOD (integrated optical density) and expressed in AU (arbitrary units).

**Table 1 Baseline biological characteristics of the healthy subjects and MO patients**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>NIR-MO</th>
<th>IR-MO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female (n/n)</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
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<tr>
<td>Age (years)</td>
<td>40.66 ± 3.62</td>
<td>46.63 ± 4.53</td>
<td>37.00 ± 3.13</td>
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<tr>
<td>Weight (kg)</td>
<td>65.00 ± 3.32</td>
<td>144.84 ± 7.98</td>
<td>154.88 ± 5.34</td>
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<tr>
<td>Height (cm)</td>
<td>168.83 ± 0.03</td>
<td>161.67 ± 1.94</td>
<td>166.55 ± 2.26</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>22.57 ± 0.84</td>
<td>55.00 ± 2.20</td>
<td>55.85 ± 1.32</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>80.08 ± 3.18</td>
<td>141.88 ± 6.04</td>
<td>147.80 ± 4.70</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>91.54 ± 3.72</td>
<td>156.63 ± 6.7</td>
<td>156.64 ± 2.89</td>
</tr>
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<td>Waist/hip ratio</td>
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<td>0.91 ± 0.01</td>
<td>0.94 ± 0.03</td>
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<td>Serum insulin (units/ml)</td>
<td>7.19 ± 0.41</td>
<td>14.57 ± 1.73</td>
<td>45.46 ± 2.44</td>
</tr>
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<td>HOMA-IR score</td>
<td>1.44 ± 0.11</td>
<td>3.31 ± 0.32</td>
<td>11.48 ± 0.68</td>
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<td>Serum glucose (mg/dl)</td>
<td>80.25 ± 2.96</td>
<td>98.50 ± 4.18</td>
<td>104.18 ± 2.93</td>
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<td>Serum cholesterol (mg/dl)</td>
<td>196.00 ± 11.01</td>
<td>196.65 ± 13.78</td>
<td>196.57 ± 10.05</td>
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<td>HDL cholesterol (mg/dl)</td>
<td>58.75 ± 5.10</td>
<td>49.00 ± 5.91</td>
<td>41.18 ± 4.67</td>
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<td>LDL cholesterol (mg/dl)</td>
<td>117.75 ± 9.06</td>
<td>125.81 ± 13.13</td>
<td>118.02 ± 12.44</td>
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<td>Triacylglycerols (mg/dl)</td>
<td>78.93 ± 9.62</td>
<td>115.62 ± 13.98</td>
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<td>GGT (units/l)</td>
<td>22.97 ± 1.55</td>
<td>20.85 ± 2.59</td>
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<td>GPT (units/l)</td>
<td>39.67 ± 2.84</td>
<td>33.12 ± 4.82</td>
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<tr>
<td>GGT (units/l)</td>
<td>32.25 ± 4.89</td>
<td>52.12 ± 21.81</td>
<td>34.81 ± 4.06</td>
</tr>
</tbody>
</table>
EMSA

Nuclear extracts were tested for NF-κB-binding activity, employing a consensus oligonucleotides (5'-AGTTGAGGAGACTTCCCAAGC-3' and 3'-TCAACTCCCTGGAAGGTGCGG-5') (Santa Cruz Biotechnology), using the Digoxigenin EMSA kit from Roche Diagnostics. Nuclear protein (25 μg) was incubated with digoxigenin-labelled NF-κB oligonucleotide in binding buffer containing 100 mM Hepes (pH 7.6), 5 mM sodium EDTA, 50 mM (NH₄)₂SO₄, 5 mM DTT (dithiotheitol), 1% (w/v) Tween 20 and 150 mM KCl, together with 1 μg of poly(dI-dC)·(dI-dC) and poly-l-lysine to a final volume of 20 μL. After 15 min of incubation at room temperature (25°C), the protein–DNA complexes were resolved on a native 8% polyacrylamide gel in a 0.5× Tris/borate/EDTA buffer system and run at 200 V for 2 h. Gels were transferred on to nylon membranes in a semi-dry transfer system (Bio-Rad Laboratories) at 10 V and 300 mA for 30 min. The membranes were exposed to UV light in a transilluminator for 5 min, and incubated with alkaline-phosphatase-conjugated anti-digoxigenin antibody. Complexes were detected with CSPD® chemiluminescent substrate (Roche Diagnostics) and exposed to Hyperfilm (GE Healthcare) in a film holder for 4–16 h at room temperature.

Antibody supershift assays were performed by incubation of the nuclear proteins with 4 μg of affinity-purified polyclonal antibodies (Santa Cruz Biotechnology) against NF-κB p50 (H-119), NF-κB p65 (C-20) and NF-κB p52 (K-27) subunits for 30 min on ice before adding the labelled probe. Specific competition control of unlabelled oligonucleotides at 125-fold excess was added to the binding reaction mixture.

RNA extraction and quantitative real-time PCR

VAT RNA isolation was performed by homogenization with an Ultra-Turrax T25 Basic instrument using TRIzol® reagent (Invitrogen). Samples were purified using a RNeasy mini kit (Qiagen) and treated with DNase (RNase-free DNase Set; Qiagen). For first-strand cDNA synthesis, 1 μg of total RNA was reverse-transcribed using random hexamers (Roche Diagnostic) as primers and Transcriptor reverse transcriptase (Roche) reverse-transcribed using random hexamers (Roche Diagnostic) at 10 V and 300 mA for 30 min. The membranes were transferred on to nylon membranes in a semi-dry transfer system (Bio-Rad Laboratories) at 10 V and 300 mA for 30 min. The membranes were exposed to UV light in a transilluminator for 5 min, and incubated with alkaline-phosphatase-conjugated anti-digoxigenin antibody. Complexes were detected with CSPD® chemiluminescent substrate (Roche Diagnostics) and exposed to Hyperfilm (GE Healthcare) in a film holder for 4–16 h at room temperature.

Cytokine determination

The quantitative determination of IL-1β and IL-6 were performed using the Milliplex High Sensitivity Human Cytokine Immunoassay (Millipore Corporation) according to the manufacturers’ protocols. Multianalyte profiling was performed on the Luminex-100 XMAPTM Technology (Luminex).

The xMAP technology (Luminex) combines the principle of a sandwich immunoassay with fluorescent bead-based technology, allowing individual and multiplex analysis in a single microtitre well [15]. Acquired fluorescence data were analysed by the Luminex 100 xMAP software (version 2.2).

Statistical analysis

All results are expressed as means ± S.E.M. Statistical analyses were carried out with the statistical software package SPSS (version 17.0). The data were analysed by one-way ANOVA. Post hoc statistical analysis was completed using a Duncan contrast statistic. Differences were considered statistically significant at P < 0.05. The Spearman correlation coefficient was calculated to estimate the linear correlations between variables. The rejection level for a null hypothesis was P < 0.05.

RESULTS

Pro-inflammatory cytokines TNFα, IL-1β and IL-6 in adipose tissue

We compared the mRNA expression levels of TNFα, IL-1β and IL-6 in VAT obtained from NIR-MO and IR-MO patients. Real-time PCR analysis showed that obesity is associated with high levels of TNFα mRNA expression in adipose tissue independently of their degree of insulin resistance (P < 0.05) (Figure 1A). Also, we found that NIR-MO patients expressed low levels of both IL-1β and IL-6 mRNA compared with IR-MO patients. Of interest, levels of IL-1β and IL-6 mRNA in IR-MO patients were not statistically different from the levels observed in the lean control.
Further morphological evidence of the involvement of macrophages was obtained using immunoreactivity for the CD68 marker. The number of macrophages was normalized to 100 adipocytes for comparison between patients. Our data confirm a 5-fold increase in the infiltration of macrophages in VAT of IR-MO patients compared with NIR-MO (24.5 ± 6.5 and 5.5 ± 2.1% respectively). As shown in Figures 2(G) and 2(H), we also observed that VAT from IR-MO characteristically showed macrophages surrounding adipocytes forming the typical crowns. Conversely, crown structures were hardly seen in the VAT from NIR-MO subjects (Figures 2E and 2F).

Expression and activation of inflammatory mediators JNK1/2, ERK 1/2 and NF-κB

After having demonstrated an increased inflammatory profile in adipose tissue from IR-MO patients, we then set out to investigate the signalling effectors that may affect insulin sensitivity. Our first candidate was JNK. Western blot analyses showed that all MO patients independently of their degree of insulin resistance had significantly elevated levels of JNK1/2 expression and phosphorylation compared with the control group. Of note, we did not observe significant differences between NIR-MO and IR-MO groups (Figure 3A). As shown in Figure 3(A), the increase in the phosphorylation of JNK1/2 observed in all MO group was probably due to the increase in its expression.

Our second candidate was ERK, as elevated activity of ERK in adipocytes of obese rodents and humans has been documented [16]. Indeed, there is evidence indicating that ERK may be a regulatory node controlling the inflammation signalling network. Thus we next studied the expression and activation of ERK1/2 in VAT of our three experimental groups. Our results indicated that ERK 1 and 2 isoforms were not phosphorylated in lean individuals. We found that ERK1/2 was activated in both obese groups (NIR-MO and IR-MO) compared with control subjects (Figure 3C); however, there was a significant increase in ERK phosphorylation in the IR-MO group compared with NIR-MO subjects.

We next investigated the NF-κB signalling pathway. Our results indicated that MO subjects had similar mRNA expression levels of the p65 subunit (RelA) compared with the non-obese subjects (Figure 3D). More interestingly, the mRNA expression levels of the IκBα subunit were significantly higher in IR-MO patients than NIR-MO and non-obese subjects (Figure 3F). This result was supported further by the confirmation that not only was the DNA binding of the transcriptionally active NF-κB (p65/p50) elevated in VAT of MO subjects compared with lean individuals, but also, more importantly, NF-κB activity was significantly higher in IR-MO than in NIR-MO patients (Figure 3E). Changes in NF-κB signalling and levels of adipokines seems to be co-ordinated as suggested by of the correlation between IL-1β and IL-6 with IκBα expression levels in VAT of both NIR-MO and IR-MO patients (Figure 4). This correlation was found to be maintained in both experimental groups separately (NIR-MO and IR-MO) and in the total MO group. All correlation coefficients (Rs) and P values for these correlations are stated in Figure 4.

Effects on markers of insulin signalling

Many studies have demonstrated that the nutritionally induced insulin resistance is associated with inhibition of specific post-receptor signal transduction steps, including inhibition of IRS-1 or -2 functions [1,17,18]. We studied the mRNA expression of both IRS-1 and IRS-2 in VAT from lean, NIR-MO and IR-MO subjects.

Macrophage infiltration in VAT

Given the increased expression of IL-1β and IL-6 in IR-MO adipose tissue, we hypothesized that these changes may be related to the degree of macrophage infiltration in adipose tissue. In agreement with this hypothesis, we found that the expression of macrophage markers CD11b, PLAUR, MCP-1 and CSF-3 was increased in the MO group compared with lean control (Figures 2A, 2B, 2C and 2D respectively). More importantly, our data also showed that the expression of macrophage markers was increased in the adipose tissue of IR-MO compared with NIR-MO patients (P < 0.05) supporting further the concept that macrophage infiltration may be an important determinant factor of the metabolic complications associated with obesity.
Real-time PCR analysis revealed elevated levels of mRNA of IRS-1 in NIR-MO individuals compared with lean control and IR-MO subjects (Figure 5A). Gene expression data were associated with appropriate protein changes as indicated by Western blot analysis of IRS-1 showing increased levels in NIR-MO patients compared with controls and IR-MO individuals (Figure 5B). These changes seemed to be specific for IRS-1, since no significant changes in IRS-2 mRNA were observed (results not shown). In addition, an increase in the tyrosine phosphorylation of the IRS-1 in NIR-MO group compared with lean and IR-MO subjects was observed (Figure 5B). This increase might probably be attributed to the changes in the IRS-1 protein expression.

In support for an increased insulin signalling activity in the NIR-MO population, compared not only with IR-MO patients, but also with lean insulin-sensitive individuals, we found an increased expression and activation of insulin-regulated Akt in our experimental groups. Western blot analyses showed that NIR-MO subjects had significant elevated levels of Akt expression and phosphorylation compared with non-obese and IR-MO subjects (Figure 5C). The elevated phosphorylation of Akt might be due to the changes in the expression levels of this kinase.

DISCUSSION

In the present study, we investigated the paradox of the inappropriately healthy MO subject and provide evidence supportive of the concept that a key factor linking the adipose tissue expansion and associated metabolic complications is the degree of inflammation of the adipose tissue. A particularly unique feature of our study is its focus on the visceral adipose depot. In fact, many studies focused on adipose tissue biology and the metabolic syndrome tend to focus on the subcutaneous depot given its relatively easy accessibility. However, accumulating evidence suggests that the visceral depot is the one that may contribute to the pathogenesis of obesity-associated insulin resistance. In this regard, our data provide direct evidence of differential macrophage infiltration degree, inflammatory state and insulin-resistance markers between VAT from NIR-MO and IR-MO individuals.

Our data also provided evidence that inflammation does not depend on the level of expansion of the adipose tissue. In fact, our data indicated that the degree of expansion is not the main determinant of associated inflammation. In some way, these data support our hypothesis stating that the main determinant for inflammation may be related to the remaining capacity for adipose expansion (see [19] for a review). Moreover, our data are consistent with the hypothesis that the effectors linking obesity and insulin resistance are inflammatory. The data supporting the hypothesis that pro-inflammatory networks are involved in the obesity-associated metabolic complications are supported by the fact that VAT from IR-MO individuals had more macrophages.

To address this issue, we have assembled a cohort of extremely well weight/metabolic state matched individuals and distributed them into three experimental groups, including two...
Obese populations differing in their level of insulin sensitivity. Comparison of the VAT of these experimental groups has revealed that IR-MO subjects exhibit a more pro-inflammatory profile than the NIR-MO subjects, and this pro-inflammatory profile is characterized by increased macrophage infiltration, increased levels of IL-1β, IL-6 and activation of specific pro-inflammatory signalling components ERK and NF-κB, which can, in our opinion, account for the increased insulin-resistance state that defined the experimental groups.

Our results indicate that TNFα mRNA levels are increased in parallel with adipose tissue mass expansion, regardless of the insulin sensitivity of the individual. In agreement with others [10,20], we have also found that obesity is associated with increased JNK1/2 expression and phosphorylation compared with non-obese patients. Nevertheless, levels of both TNFα and JNK1/2 did not allow the discrimination of NIR-MO from IR-MO individuals.

In searching for discriminative markers, we investigated IL-1β and IL-6. As in previous studies, we observed that expression of IL-1β is increased in the VAT of obese individuals [21]. However, our study indicated that changes in IL-1β are discriminative of the state of insulin resistance associated with obesity. In fact, our data provide convincing evidence that changes in IL-1β are restricted to the IR-MO group. Furthermore, no significant differences were observed in NIR-MO, which remained at levels comparable with those of lean individuals.

Another candidate we evaluated was IL-6. Some studies have suggested its involvement in the pathophysiology of insulin resistance. However, its specific role in modulating insulin sensitivity in different tissues such as liver, adipose tissue or skeletal muscle remains controversial [22]. In the present study, we provided evidence that IL-6 mRNA was increased in VAT of IR-MO patients compared with healthy NIR-MO subjects. In this regard, IL-6 and IL-1β followed a similar pattern of regulation as indicated by their correlative changes, that were observed in both NIR-MO and IR-MO subjects. Considered globally, our data indicate that IL-1β and IL-6 might act as important effectors of the insulin resistance associated with obesity. More importantly, we speculate that measurements of these adipokines might be of some use as diagnostic and therapeutic predictors of obesity-associated metabolic complications.

Our direct comparison between equally obese individuals with different degrees of insulin resistance also provided interesting data at the molecular level. For instance, we confirmed that elevated activity of ERK was observed in adipocytes of obese and insulin-resistant rodent and humans [23]. However, our data indicate that these effects on ERK signalling are more directly related to the degree of insulin resistance than to the expansion of fat mass. In fact, similar to IL-1β and IL-6, ERK was also more activated in IR-MO patients than in NIR-MO subjects.

How might these changes relate to the state of insulin resistance? It has been shown that IL-1β reduces IRS-1 expression...
Figure 4  Positive correlations among IL-1β, IL-6 and IkBα mRNA expression

Correlations (Rs) were assessed by Spearman product-moment correlation. Differences were considered significant if \( P < 0.05 \).

Figure 5  IRS-1 and Akt activation in VAT from non-obese, NIR-MO and IR-MO subjects

(A) mRNA expression levels of IRS-1. Total mRNAs were extracted from adipose tissue and subjected to real-time PCR amplification using TaqMan® technology suitable for relative genetic FASN expression quantification. Results are means ± S.E.M. for 12 subjects. Significant differences (Duncan; \( P < 0.05 \)) are indicated by different letters. (B) Expression and phosphorylation of IRS-1. Cell lysates from VAT were prepared, and cytoplasmic proteins (100 μg/lane) were separated by SDS/PAGE (6 % gels) and then transferred on to nitrocellulose membranes. The membranes were probed with human anti-(phosphotyrosine IRS-1) and anti-IRS-1 antibodies. The blots are representative of two samples in each group with similar results. Results in the histogram are mean ± S.E.M. relative IOD values of the ratio of phosphotyrosine IRS-1 to IRS-1 of the respective blots for 12 subjects of each group. Significant differences (Duncan; \( P < 0.05 \)) are indicated by different letters. (C) Expression and phosphorylation of Akt. Cytoplasmic proteins (25 μg/lane) were separated by SDS/PAGE (8 % gels) and then transferred on to nitrocellulose membranes. The membranes were probed with human anti-(pSer472 Akt) and anti-Akt antibodies. The blots are representative of two samples in each group with similar results. Results in the histogram are mean ± S.E.M. relative IOD values of the ratio of pSer472 Akt to total Akt of the respective blots for 12 subjects of each group. Significant differences (Duncan; \( P < 0.05 \)) are indicated by different letters.
and prevents Akt activation through a mechanism that is partly mediated by ERK activation [24]. Our results indicate that IRS-1 expression and Akt activation were increased in VAT from the NIR-MO patients whose adipose tissue had low levels of IL-1β, IL-6 and ERK activation. Conversely, the IR-MO group was characterized by high expression of IL-1β, IL-6, and decreased IRS-1 and Akt expression and activation compared with NIR-MO patients. Considered globally, these data suggest an important role of IL-1β, IL-6 and ERK activation as discriminators between NIR-MO and IR-MO individuals and as important determinants of inhibition of insulin signalling in human VAT.

IRS-1 is a relevant modulator of insulin signalling in adipose tissue [25]. Our data suggest that IRS-2, whose expression was unchanged in our three experimental groups, may not be an important determinant of obesity-associated insulin resistance, at least in VAT. Interestingly, our data also suggest that our NIR-MO patients may be in a state of facilitated insulin signalling. In fact, NIR-MO patients had increased IRS-1 mRNA expression in VAT compared not only with obese insulin resistant individuals, but also even with lean individuals. This maintained insulin signalling may contribute to the paradox of inappropriately healthy carbohydrate metabolism in these individuals.

Our IR-MO group showed a decrease in the expression of IRS-1. Moreover, in the present study, we found an inverse correlation in vivo between the levels of IL-1β and those of IRS-1, but not with IRS-2, which corroborate recent in vitro studies, showing that IL-1β promotes a decrease in the amount of IRS-1, but not IRS-2, expression in both 3T3-L1 and human adipocytes [24]. Our data outline the relevance of IRS-1 as a potential mode of control in obesity-associated insulin resistance.

Our results suggest that obesity-associated insulin resistance is related to inflammation as indicated by nuclear accumulation of NF-κB. This agrees with the observation that obesity is associated with an increase in NF-κB DNA-binding activity [26]. In addition, our results indicated that NIR-MO individuals have low levels of NF-κB activation compared with IR-MO patients. In contrast, expression of p65 (RelA) subunit mRNA levels does not seem to be affected by the degree of obesity and/or insulin resistance, as the three groups (non-obese, NIR-MO and IR-MO) exhibited similar levels. These results agree with the recent observation that circulating mononuclear cells of obese individuals presented elevated levels of p105 NF-κB subunit mRNA, but similar levels of p65 (RelA) subunit mRNA when compared with non-obese subjects [27]. We also observed significantly higher levels of IkBα in VAT from IR-MO individuals than in NIR-MO and non-obese subjects. Those data are in accordance with previous observations, indicating that NF-κB activation was positively correlated with an increase in IkBα mRNA levels in circulating mononuclear cells of obese patients [28,29]. Altogether, these results suggest that NF-κB may be an important effector of the pro-inflammatory response associated with obesity-related metabolic complications.

Considered globally, our results support the concept that the development of insulin resistance associated with obesity depends on the activation of the inflammatory cascade and that IL-6, IL-1β, ERK and NF-κB seem to be important effectors mediating the inflammation effects promoting insulin resistance.

**AUTHOR CONTRIBUTION**

All authors gave substantial contributions to the conception and design of the study, analysis and interpretation of data, drafting and critically revising the article into an important intellectual content, and gave the final approval of the present version of the manuscript.

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