

Adipose Tissue IL-6 Content Correlates with Resistance to Insulin Activation of Glucose Uptake both *in Vivo* and *in Vitro*

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Obesity and type 2 diabetes are associated with insulin resistance, the mechanisms of which remain poorly understood. A significant correlation between circulating IL-6 level and insulin sensitivity has recently been found in humans. Because adipose tissue could be a significant source of IL-6, we analyzed the relationship between the levels of adipose tissue IL-6 and insulin action *in vivo*, during a hyperinsulinemic normoglycemic clamp, and *in vitro* by measuring glucose transport in adipocytes from 12 obese subjects with ($n = 7$) or without ($n = 5$) diabetes. We observed an inverse correlation between adipose tissue IL-6 content and maximal insulin-responsive-

ness measured *in vivo* ($P < 0.02$) and *in vitro* ($P < 0.02$). Conversely, there was no significant correlation between these two later parameters and adipose tissue leptin or tumor necrosis factor- α protein contents. Furthermore, we showed, for the first time, the presence of immunoreactive IL-6 receptors in the plasma membrane of human abdominal sc adipocytes. This suggests that locally secreted IL-6 could act on adipocytes by an autocrine/paracrine mechanism. In conclusion, increased IL-6 production by sc adipose cells might participate to the insulin-resistant state observed in human obesity. (*J Clin Endocrinol Metab* 87: 2084–2089, 2002)

OBESITY AND TYPE 2 diabetes are associated with impaired insulin action on peripheral target tissues (*i.e.* adipose tissue and skeletal muscle) (1, 2). The mechanisms that could explain such a link are not clearly elucidated, although several cytokines expressed in adipose tissue such as leptin, TNF- α and IL-6 could be involved (3). Leptin is primarily produced by adipocytes and has been shown to impair the metabolic action of insulin in isolated rat adipocytes (4). However, more recent studies suggest that leptin improves insulin sensitivity (5, 6). TNF- α has been found to induce insulin resistance *in vitro*, and its expression is high in adipose cells from animal models of insulin resistance (7). In human, increased expression of its gene has been documented in the sc abdominal adipose tissue depot, and studies have shown that the expression or production of this cytokine was directly related to the degree of obesity (8, 9), whereas others did not find such an association (10). More recently, TNF- α secretion has been shown to be correlated with insulin resistance measured *in vitro* (11) and *in vivo* (12). However, it has been shown that human sc adipose tissue did not release a significant amount of TNF- α *in vivo* (13) and a relationship between adipose-derived TNF- α and *in vivo* insulin sensitivity in human has not been observed in different

clinical studies (10, 14, 15). Therefore, the relation between adipose tissue TNF- α and insulin resistance remains largely debated in humans.

Otherwise, the sc adipose tissue was shown to secrete large amount of IL-6, and this secretion was correlated with the body mass index (BMI) of the subjects (13). Interestingly, such a correlation between circulating IL-6 levels and BMI was consistently found in different studies, suggesting a link between obesity and circulating IL-6 (12, 16–18). Moreover, we and others recently observed a significant correlation between circulating IL-6 levels and insulin sensitivity assessed by the fasting insulin resistance index or by an iv glucose tolerance test with minimal model analysis (12, 18, 19). We also showed that circulating IL-6 levels might reflect, at least in part, adipose tissue IL-6 production (18). Therefore, IL-6 might be involved in the resistance to insulin observed in obesity and type 2 diabetes with obesity in humans. However, little is known about IL-6 expression in adipose tissue and its relation with adipose tissue insulin resistance. We thus studied the relationship between adipose tissue IL-6 levels and maximal insulin-stimulated glucose uptake *in vivo* and *in vitro* in a group of obese subjects with or without type 2 diabetes.

Subjects and Methods

Subjects

Twenty-four Caucasian volunteers [nine lean nondiabetic (age range, 25–53 yr; BMI, 18.0–25.3 kg/m²), seven nondiabetic obese (age range, 26–58 yr; BMI, 29.1–44.3 kg/m²), and eight type 2 diabetic obese subjects

Abbreviations: APAAP, Alkaline phosphatase-anti-alkaline phosphatase; BMI, body mass index; CRP, C reactive protein; GIR, glucose infusion rates; HbA1c, glycated hemoglobin; IL-6R α , IL-6 receptor α ; NEFA, nonesterified fatty acids.

(age range, 41–60 yr; BMI, 28.6–38.7 kg/m²) participated in this study. In each subject, body weight was stable for at least 12 months before participation in the study, and none was engaged in any type of exercise program or was excessively sedentary. Type 2 diabetic patients were treated by sulfonylurea therapy, which was withdrawn at least 2 wk before the study. None was treated by insulin. All studied subjects were chemically and clinically euthyroid and were without renal or hepatic disease. All of the subjects participated in the clamp study. Blood samples were drawn at 0800 h after an overnight fast, and serum was stored at –80 C until analysis. In addition to the clamp study, insulin-stimulated glucose transport in adipocytes from sc abdominal adipose tissue was assessed in 12 of the obese subjects, diabetic (n = 7) or not (n = 5), and compared with their adipose tissue cytokines content. All subjects gave their written informed consent before their participation in the study, which was approved by the ethical committee of Assistance Publique-Hôpitaux de Paris and performed according to the French legislation (Huriet law).

Glucose clamp

Insulin action was measured in each subject after an overnight fast with the hyperinsulinemic normoglycemic glucose clamp technique as previously described (20). A primed continuous infusion of insulin (Umuline, Lilly, Paris, France) was subsequently given at two different rates (40 and 400 mU/m²·min). The continuous infusion was maintained for 100 min at each insulin infusion rate (20). Plasma glucose was maintained at 4.8 mmol/liter by a variable-rate iv 30% dextrose infusion. The plasma glucose concentration was measured every 5 min (Beckman Glucose Autoanalyzer II, Beckman Coulter, Inc., Fullerton, CA), and the glucose infusion rates (GIRs) were calculated according to established algorithms (21) using a portable PC microcomputer (Toshiba 3100 SX) that automatically controlled the peristaltic pump (Infusomat, Secura Braun, Melsungen, Germany). Plasma glucose was allowed to decrease from hyperglycemic to normoglycemic levels during the first step in diabetic patients, and the glucose infusion was not started until the plasma glucose concentration had declined to 4.8 mmol/liter, which required less than 60 min of insulin infusion. During the second plate, the mean glucose infusion rate in the last 30 min of 400 mU/m²·min insulin infusion was used to assess the maximal whole-body glucose disposal rate expressed as mg/m²·min, an estimate of maximal peripheral insulin action. Blood samples were taken every 10 min throughout the last 30 min of the hyperinsulinemic normoglycemic clamp for plasma insulin measurements.

Adipose tissue preparation

After an overnight fast, percutaneous mini-liposuction of sc abdominal adipose tissue under local anesthesia using 2–5 ml 1% lidocaine was performed as previously described (22). For protein measurements and immunohistochemical study, one adipose tissue specimen was blotted on sterile cloth and cleaned of blood and immediately frozen in liquid nitrogen and stored at –80 C until analysis. For glucose transport, adipocytes were isolated by collagenase digestion as previously reported (23).

Glucose transport

Glucose transport was measured by the method described by Kashiwagi *et al.* (24) in which the uptake of D-U⁻¹⁴C-glucose is determined at tracer concentrations. To measure basal and maximally stimulated glucose transport rates, adipose cells were incubated in the absence and presence of insulin (100 nM) for 60 min at 37 C in Krebs-Ringer phosphate buffer (pH 7.4) with 5% BSA. Thereafter, 4.5 μmol/liter of D-U⁻¹⁴C-glucose (304 μCi/μmol) was added to each tube, and the incubation was continued for 30 min before terminating by centrifugation through dionylphthalate oil. The glucose transport was expressed as clearance rate in fl/cell·sec.

Immunohistochemistry method

Cryostat sections (6 μm thick) were cut from frozen sc adipose tissue from three of the subjects and stored at –20 C until use. One visceral adipose tissue sample from one obese type 2 diabetic and sc adipose

tissue samples from two nonobese nondiabetic subjects obtained during surgery were also studied. After thawing, unfixed slides, as well as slides fixed in cold acetone, were rehydrated by immersion in 20% AB human serum in 0.05 M Tris-buffered saline (pH 7.6), then incubated 1 h at room temperature with the anti-IL-6 receptor α (IL-6Rα) (Santa Cruz Biotechnology, Inc., Tebu, France) at a dilution of 1:50. IL-6Rα is a rabbit polyclonal IgG specific for an epitope mapping at the carboxy terminus of human IL-6Rα. The results were verified by using the anti-IL-6Rα M91, which was kindly gifted by J. Brochier from INSERM U-475 (25). An immunoenzymatic method was performed using alkaline phosphatase-anti-alkaline phosphatase (APAAP) complexes (Dakopatts, Trappes, France). Briefly, slides were successively incubated for 30 min with mouse antirabbit Ig antibody diluted 1:50, rabbit antimouse antibody diluted 1:20, and the APAAP complexes diluted 1:50, respectively. A single amplification was performed by adding sequentially the rabbit antimouse antibody for 10 min and the APAAP complexes for 10 min. Sections were incubated with Fast Red salt-TR-Naphtol AX-TR phosphate (Sigma, Saint-Quentin Fallavier, France) in the presence of levamisole to block endogenous activity, then counterstained with aqueous hematoxylin and mounted in Immumount (Shandon, Cergy-Pontoise, France). A negative control was obtained by omitting the primary antibody.

Cytokine measurements

Serum levels of leptin, TNFα, and IL-6 were determined by ELISA (Quantikine leptin, Quantikine High Sensitivity TNFα, and Quantikine IL-6, R&D Systems, Oxford, UK). The same ELISA kits were used to determine the immunoreactive leptin, TNFα, and IL-6 protein content in adipose tissue samples after homogenization of 200 mg of frozen tissue in 400 μl of a buffer (pH 7.4) containing 10 mmol/liter of Tris-HCl, 250 mmol/liter of sucrose, and a cocktail of protease inhibitors (Complete, Roche Molecular Biochemicals, Burlington, NC) as previously reported (18).

Analytical methods

Venous blood samples were taken between 0800 and 0900 h after an overnight fast. Blood glucose was analyzed with a glucose oxidase method (Beckman Glucose Autoanalyzer II, Beckman Coulter, Inc.). Glycated hemoglobin (HbA1c) concentrations (normal range, 4.0–5.6%) were determined by using the Diamat high-pressure liquid-chromatographic system (Bio-Rad Laboratories, Inc., Ivry sur Seine, France). Serum insulin concentrations were measured using commercial RIA kits (Bi-Insulin IRMA, ERIA-Pasteur, Paris, France; normal range, 5–18 μU/ml). Nonesterified fatty acids (NEFA) were determined with a commercially available kit (NEFAC test; Wako Chemicals, Neuss, Germany). C reactive protein (CRP) was assessed by immuno-nephelometry on Behring Nephelometer 2 (Dade-Behring, La Défense, France).

Statistical analysis

Results are means ± SEM, and statistical significance was determined using ANOVA and the Kruskal-Wallis nonparametric test, followed by a Fisher protected least significant difference test for pair-wise differences. Correlations between factors were identified by Spearman's nonparametric test. *P* values of less than 0.05 were considered significant.

Results

Insulin resistance and circulating cytokine levels

The characteristics of the three groups of subjects are summarized in Table 1. When compared with the lean controls, as expected, all obese subjects had higher fasting plasma insulin. The type 2 diabetic group had higher fasting plasma glucose and HbA1c levels than the other two groups. As indicated by the clamp studies (GIRs), the group of type 2 diabetic subjects was more insulin-resistant than the group of obese nondiabetics that was more insulin-resistant than the lean control group. Serum leptin, TNF-α, and IL-6 levels are shown in Table 1. We found a 3- and 4-fold increase of

TABLE 1. Clinical and metabolic characteristics of the subjects (n = 24)

	Lean control subjects n = 9	Obese nondiabetic patients n = 7	Obese diabetic patients n = 8
Age (yr)	37 ± 3	44 ± 2	49 ± 2 ^a
Gender (M/F)	3/6	2/5	5/3
BMI (kg/m ²)	21.5 ± 0.8	35.2 ± 2.3 ^c	33.4 ± 1.6 ^c
Waist/hip ratio	0.78 ± 0.03	0.93 ± 0.04 ^b	1.01 ± 0.03 ^c
Glucose (mmol/liter)	5.2 ± 0.2	5.4 ± 0.2 ^f	11.6 ± 1.2 ^c
Insulin (μU/ml)	6 ± 1	12 ± 2 ^d	19 ± 2 ^c
NEFA (μmol/liter)	480 ± 42	745 ± 52 ^b	788 ± 60 ^b
HbA1c (%)	4.7 ± 0.1	4.9 ± 0.1 ^f	7.2 ± 0.6 ^c
GIR (mg/m ² ·min)	538 ± 29	407 ± 35 ^{b,f}	241 ± 19 ^c
Leptin (ng/ml)	6.0 ± 1.7	36.2 ± 7.2 ^{c,e}	14.6 ± 4.0
IL-6 (pg/ml)	0.53 ± 0.06	1.72 ± 0.53 ^a	2.02 ± 0.30 ^b
TNFα (pg/ml)	3.63 ± 0.39	4.12 ± 0.75	3.69 ± 0.92
CRP (mg/liter)	0.9 ± 0.3	4.3 ± 0.9 ^b	3.5 ± 0.7 ^b

Data are means ± SE. Statistical significance was determined using ANOVA and the Kruskal-Wallis test, followed by a Fisher protected least significant difference test for pair-wise differences.

^a $P < 0.05$ vs. control subjects.

^b $P < 0.01$ vs. control subjects.

^c $P < 0.001$ vs. control subjects.

^d $P < 0.05$ vs. diabetic patients.

^e $P < 0.01$ vs. diabetic patients.

^f $P < 0.001$ vs. diabetic patients.

IL-6 in obese nondiabetic and type 2 diabetic subjects, respectively, compared with controls, whereas no significant difference was observed between the three groups for TNF-α. Serum leptin levels were higher in the nondiabetic obese group, whereas no significant difference was observed between type 2 diabetic and control subjects. In line with the finding for IL-6, serum CRP levels were 4-fold increased in obese subjects whatever their diabetic status.

No significant correlation was found between serum leptin or TNF-α and insulin-stimulated glucose disposal *in vivo*. By contrast, we observed a significant inverse correlation between serum IL-6 levels and insulin-stimulated glucose disposal during the hyperinsulinemic normoglycemic clamp ($r = -0.602$; $P < 0.01$). In line with the data obtained for IL-6, circulating CRP levels were inversely correlated with insulin responsiveness *in vivo* ($r = -0.515$; $P < 0.05$). BMI was significantly correlated with IL-6 ($r = 0.585$; $P < 0.01$), leptin ($r = 0.634$; $P < 0.005$), and CRP ($r = 0.621$; $P < 0.005$), but not with TNFα. Otherwise, no significant correlation was observed between age and either IL-6 or CRP levels. This suggests a possible link between circulating IL-6 levels and insulin resistance in human. Finally, we found a correlation between NEFA levels and either circulating IL-6 or CRP levels ($r = 0.608$, $P < 0.01$; and $r = 0.503$, $P < 0.02$, respectively). By contrast no significant correlation was observed between NEFA and either TNFα or leptin.

Insulin resistance and adipose tissue cytokines

Because circulating cytokine levels are in part an indirect indicator of their release by adipose tissue, we directly measured their content in adipose tissue in 12 of the obese subjects, diabetic or not. Mean leptin content was 3.15 ± 0.07 ng/g adipose tissue (2.94–3.70), whereas mean TNF-α content was 8.2 ± 1.9 pg/g adipose tissue (1.0–21.2), and mean IL-6 content was 17.5 ± 3.2 pg/g adipose tissue (1.0–31.8).

There was no correlation between adipose tissue leptin and either TNF-α or IL-6 (data not shown). Similarly, no correlation was found between IL-6 and TNF-α. Although not significant, there was a tendency for a correlation between serum and adipose tissue IL-6 within the obese group ($r = 0.521$; $P = 0.08$). In these 12 obese subjects, glucose transport in adipocytes was enhanced by 2-fold by insulin from 43 ± 9 fl/cell·sec (7–122) to 92 ± 22 fl/cell·sec (17–284). Insulin-stimulated glucose transport in adipocytes was strongly correlated with the whole-body insulin-stimulated glucose disposal *in vivo* ($r = 0.769$; $P < 0.02$; $n = 12$).

To further study the relationship between adipocyte cytokines and insulin action both *in vivo* in whole body and *in vitro* in adipocytes, we compared the results obtained from either the hyperinsulinemic normoglycemic clamp or glucose transport in adipocytes and the cytokine contents in adipose tissue. Our results showed no correlation between adipose tissue leptin or TNF-α and 1) insulin-stimulated glucose disposal *in vivo*, 2) insulin-stimulated glucose transport in adipocytes, or 3) any of the metabolic parameters measured in this study, *i.e.* fasting plasma glucose, insulin, NEFA, and HbA1c. Conversely, we found a strong inverse correlation between adipose tissue IL-6 content and 1) insulin-stimulated glucose disposal *in vivo* ($r = -0.750$; $P < 0.02$) (Fig. 1A), 2) basal ($r = -0.748$; $P < 0.02$), or 3) insulin-stimulated glucose transport in adipocytes ($r = -0.767$; $P < 0.02$) (Fig. 1B). In addition, fasting plasma glucose was correlated with adipose tissue IL-6 content ($r = 0.591$; $P = 0.05$). It is interesting to compare these values with those we have obtained previously in lean controls. In control subjects, adipose tissue IL-6 levels were very low (1.5 ± 0.8 pg/g adipose tissue; $n = 7$), whereas GIR was high (538 ± 29 mg/m²·min; $n = 9$) (Fig. 1A). Similarly, control insulin-stimulated glucose transport in adipocytes was in the high range (234 ± 90 fl/cell·sec; $n = 5$) (Fig. 1B).

Immunohistochemistry results

Anti-IL-6Rα immunostaining of adipose tissue revealed a thin positivity delineating adipocytes (Fig. 2, A and C) and a strong positivity of the blood vessel wall (Fig. 2A, top right). This immunostaining disappeared when the primary antibody was omitted for negative control (Fig. 2, B and D). Most adipocytes (60%) showed positive staining, however variable in intensity with the two specific IL-6Rα antibodies used. Immunostaining was reproducible by using different independent technical procedures. Analysis of different samples revealed that IL-6Rα was present on the plasma membrane from sc and visceral adipocytes. Although performed with a few numbers of subjects, IL-6Rα was observed in control, obese nondiabetic, and obese type 2 diabetic subjects (data not shown).

Discussion

In the present study, we report that the circulating IL-6 level was highly correlated with insulin responsiveness measured by the gold standard hyperinsulinemic glucose clamp, confirming our previous work (18). This result is also supported by the significant correlation observed between circulating CRP levels and insulin responsiveness, IL-6 being

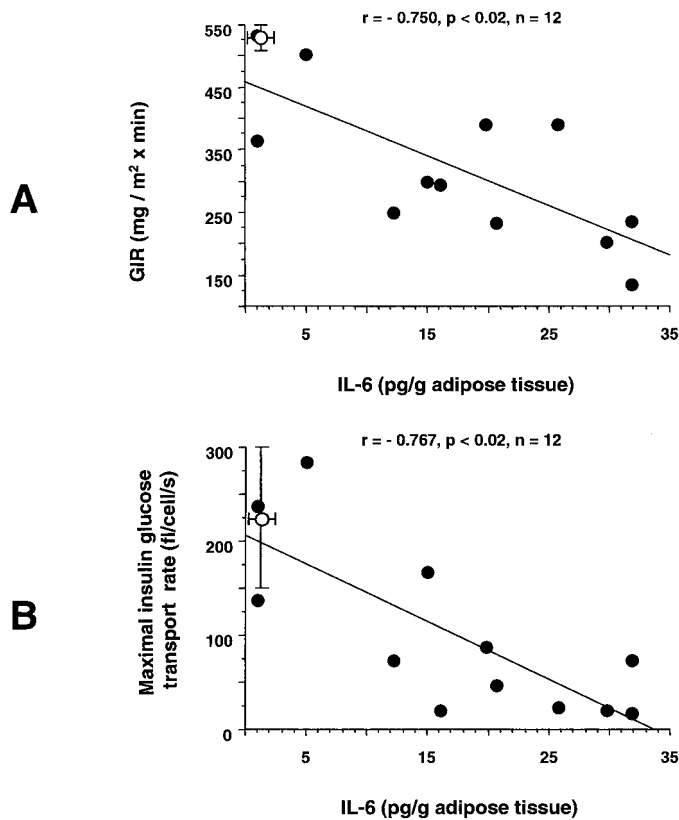


FIG. 1. Relationship between adipose tissue IL-6 protein content and GiR during the hyperinsulinemic normoglycemic clamp (A) and maximally insulin-stimulated glucose transport in isolated adipocytes (B) in individual obese subjects. The open circle indicates the mean (\pm SEM) values for lean control subjects to allow comparison, but these values have not been used in calculating the correlation coefficient rank between the two variables. Correlation was identified using Spearman's nonparametric test.

the major inductor of liver CRP synthesis. Interestingly, two recent studies reported such a relationship between circulating IL-6 level and insulin resistance, estimated either by the fasting insulin resistance index in a large population or by using the frequently sampled iv glucose tolerance test with the minimal model analysis in a smaller sample of subjects (13, 19). We found a significant relationship between both serum IL-6 or CRP levels and NEFA levels. This result is in line with the data of Kern *et al.* (12) suggesting that IL-6 could be related to insulin resistance, resulting in increased lipolysis. This is also supported by the fact that IL-6 is able to stimulate lipolysis in human breast adipocyte (26). Therefore, all together, these data emphasize the potential role played by cytokines from adipose tissue in insulin resistance in human. Because circulating IL-6 level is an indirect indicator of its release by adipose tissue, we investigated the relationship between adipose tissue IL-6 content and insulin action both *in vivo* and *in vitro* in humans. A recent study suggested that the relationship between circulating IL-6 and insulin action was mediated through adiposity (27). Accordingly, we observed that adipose tissue IL-6 content was very low in control samples. Therefore, to study the relationship between adipose tissue IL-6 content and insulin resistance independently of obesity, we studied obese subjects with

different degrees of insulin resistance. We found a significant correlation between adipose tissue IL-6 content and several metabolic parameters such as fasting plasma glucose, basal and insulin-stimulated glucose transport, and whole-body glucose disposal. These results suggest that IL-6 may play an important role in insulin action in these subjects. Moreover, our results suggested that IL-6 could act locally at the adipocyte level and that IL-6 could impair insulin action. Such an IL-6 action required IL-6 receptor to be present on sc adipocytes, which has never been reported. Interestingly, it has been shown recently that human breast adipocytes express IL-6 receptors (26). By using immunohistochemistry, we demonstrate, for the first time, that IL-6R α is present at the plasma membrane level in about 60% of the human abdominal sc adipocytes. These data suggest that, in humans, IL-6 secreted from abdominal sc adipocytes alter their metabolism by an autocrine or paracrine mechanism. However, when human breast adipocytes were differentiated *in vitro* from precursor cells, only a few percentage expressed IL-6 and IL-6R α (~2–3%). This could explain the poor inhibitory effect of IL-6 on insulin-stimulated glucose transport reported by Páth *et al.* (26). Further studies are required to clarify this point. A decreased expression of IL-6 and IL-6R α *in vitro* may be due to the fact that culture conditions do not fully mimic the *in vivo* situation (26).

It was recently shown that TNF- α production and glucose transport were highly correlated in human adipose tissue (11). The authors suggested that one third of the variance in insulin-stimulated glucose transport could be explained by variations in TNF- α secretion (11). In another study, a correlation was observed between TNF- α secreted *in vitro* from adipose tissue, but not plasma TNF- α level or adipocyte TNF- α mRNA, and insulin sensitivity (12). In the present study, we found no correlation between TNF- α adipose tissue protein content and either maximally insulin-stimulated glucose transport in adipocytes or whole-body glucose disposal during the hyperinsulinemic glucose clamp. These discrepancies may be explained by the different assays used to measure glucose transport in adipocytes, insulin resistance *in vivo*, TNF- α secretion and expression. Furthermore, Löfgren *et al.* and Kern *et al.* (11, 12) did not include type 2 diabetic subjects in their studies. Nevertheless, even if the role of TNF- α remains to be clarified, it seems likely that part of the variation in insulin-stimulated glucose transport may be imputed to IL-6 variation in adipose tissue.

Leptin is synthesized and secreted by adipose tissue, and this adipose production may account for about 99% of the circulating leptin level in human (3). Recently, leptin was shown to differently affect insulin sensitivity, whether it was tested in skeletal muscle or in adipocytes of rodents (4–6). Indeed, leptin improved insulin sensitivity in muscle (5, 6), whereas it impaired insulin action in isolated adipocytes (4). In the present study, we found no significant correlation between adipose tissue leptin protein content and insulin-stimulated glucose uptake *in vivo* and *in vitro*. Thus, the role played by leptin in insulin action in humans remains unclear and needs further investigation. Otherwise, our data indicate a strong correlation in obese subjects between IL-6 and insulin resistance in particular at the adipose tissue level, suggesting that IL-6 could modulate insulin signaling. However,

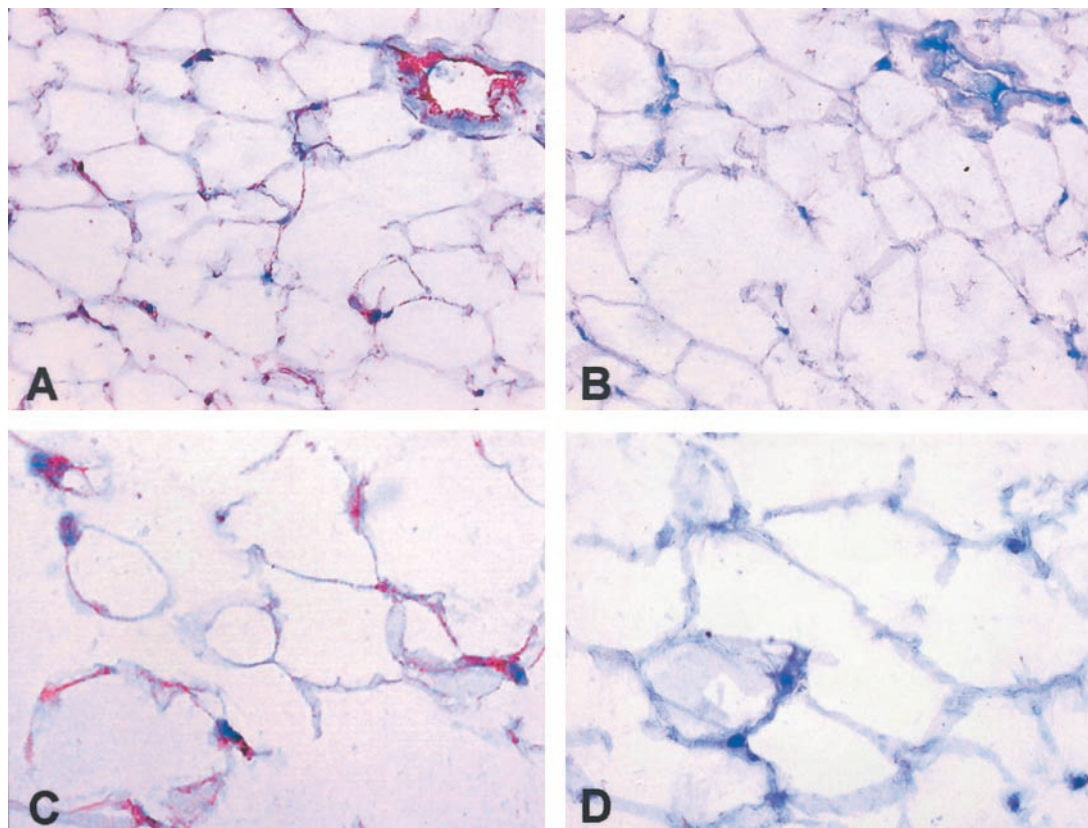


FIG. 2. Immuno-detection of IL-6 receptor in adipocyte plasma membrane from an obese subject. IL-6 receptor staining is red and counterstained is blue. A and B, There is a thin positive anti-IL-6 receptor immunostaining at the adipocyte plasma membrane and a strong positivity of the blood vessel wall (A, top right) in acetone-fixed adipose tissue sections. B and D, Negative controls.

little is known about IL-6 action in adipocytes at present. On the basis of the present study, it would be important to delineate the mechanisms whereby IL-6 impedes insulin action in adipocytes. It has been demonstrated recently that TNF- α counterregulates the insulin response in 3T3-L1 adipocytes by phosphorylating IRS-1 at Ser³⁰⁷ via the MAPK kinase/ERK pathway (28). Interestingly, IL-6 receptor signaling mediated via the signal transducing subunit gp130 involves several pathways, including the activation of ERK (29). Thus, it might be hypothesized that overproduction of IL-6 from adipose tissue could chronically activate the ERK pathway and induce IRS1 serine phosphorylation, therefore inhibiting insulin signaling. The links between IL-6 and insulin signaling have to be elucidated.

In conclusion, IL-6 might be an important local and systemic factor participating in the insulin-resistant state in human obesity.

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