Effect of hyperinsulinemia and very-low-calorie diet on interstitial cytokine levels in subcutaneous adipose tissue of obese women

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The primary cause of increased levels of circulating adipokines in obesity is unknown. In obese prediabetic patients, before hyperglycemia is developed, chronic hyperinsulinemia is observed, often associated with increased IL-8, TNFα, and IL-6 circulating levels (2, 3, 11). Therefore, increased plasma insulin levels might contribute to elevated systemic and local production of proinflammatory cytokines. Insulin, a key regulator of glucose and lipid metabolism in AT, has been found to increase mRNA expression and release of IL-6 and MCP-1 from human adipocytes and 3T3-L1 cells (8, 34). In AT explants, insulin stimulated secretion of plasminogen activator inhibitor 1 (PAI-1), IL-1β, IL-6, and TNFα deteriorate insulin signaling pathways via downregulation of IRS-1 and GLUT4 expression (23, 28, 30).

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perfused with physiological saline with 0.1% bovine serum albumin to-gether in a continuously stirred solution (stock solution) with known flow rates. Catheter inserted in situ in AT or in vitro in the tube were collected. Apparatus, Les Ulis, France). The samples of dialysate flowing out of the connected to a syringe and perfused with a microdialysis pump (Harvard Microdialysis). The cutoff of probes was 100 kDa molecular mass (20 were replaced by physiological saline. In the subgroup of six women, infusion. The infusion rate was adjusted according to arterialized for 180 min. Euglycemia (at the level of the individual fasting blood

**Materials and Methods**

Subjects, dietary protocol and clinical examination. Thirteen obese premenopausal women (age 48 ± 8 yr, BMI 35.3 ± 5.7 kg/m²) were recruited for the euglycemic-hyperinsulinemic clamp study. All subjects were drug free and healthy, as determined by medical history and laboratory findings. All patients had had a stable weight for at least 3 mo prior to inclusion. A subgroup of six women (age 37 ± 5 yr, BMI 36.7 ± 6.1 kg/m²) underwent a 1-mo dietary intervention program, during which they received 800 kcal/day (liquid formula diet; Redita, Promil, Czech Republic). Patients consulted a dietitian once a week. Subjects were examined at 8:00 AM in the fasting state. Body weight and waist and hip circumferences were measured, and body composi-tion was assessed by bioimpedance (QuadScan 4000; Bodystat, Douglas, UK). In the subgroup of six women who underwent the dietary intervention, the above-mentioned clinical evaluation was repeated at the end of the diet. The study was approved by Ethics Committee of the Third Faculty of Medicine, Charles University in Prague, and all subjects gave their informed consent before the start of the study.

Euglycemic-hyperinsulinemic clamp. The euglycemic-hyperinsul-inemic clamp was performed according to de Fronzo’s method (5). A catheter for insulin and glucose infusions was inserted into an ante-cubital vein, and a second catheter for blood sampling was placed in a dorsal vein of the ipsilateral hand. The hand was kept in a heated box to provide arterialization of venous blood. Priming plus continuous infusion of crystalline human insulin (Actrapid Human; Novo Nordisk, Bagsvaerd, Denmark), 40 mU·m body area^{-1}·min^{-1}, was given for 180 min. Euglycemia (at the level of the individual fasting blood glucose concentration) was maintained by a variable 20% glucose infusion. The infusion rate was adjusted according to arterialized plasma glucose levels measured every 5–10 min (Glucose Analyzer, Beckman Instruments, Fullerton, CA). The control experiment was carried out in the same way, but the infusions of insulin and glucose were replaced by physiological saline. In the subgroup of six women, the clamp was performed before and at the end of the dietary intervention.

Microdialysis. For all experiments, commercially available CMA/20 polyethylenesulfone (PES) microdialysis catheters were used (CMA Microdialysis). The cutoff of probes was 100 kDa molecular mass (20 mm length, 0.5 mm diameter of membrane). The inlet of the probe was connected to a syringe and perfused with a microdialysis pump (Harvard Apparatus, Les Ulis, France). The samples of dialysate flowing out of the catheter inserted in situ in AT or in vitro in the tube were collected.

Determination of in vitro recovery of IL-6 and MCP-1 at different flow rates. The microdialysis probes (n = 4) were immersed together in a continuously stirred solution (stock solution) with known concentration of either IL-6 or MCP-1 (1,500–2,000 pg/ml) and perfused with physiological saline with 0.1% bovine serum albumin (BSA) at a flow rate of 1 μl/min. After 3 h of equilibration, the probes were perfused at flow rates of 0.5, 1, 2, 3.5, and 5 μl/min, respec-tively. Samples (60–100 μl) of dialysate were collected and immediately frozen at −80°C until analysis. The ratio of the cytokine concentration in the dialysate to the known concentration in the stock solution was taken as a relative recovery [RR (%) = dialysate concentration/ cytokine stock solution concentration × 100].

In vitro time course of IL-6 and MCP-1 in dialysate and test of nonspecific binding. To evaluate the time course of reaching a steady state of IL-6 and MCP-1 concentrations in the dialysate, the probes were immersed in IL-6 and MCP-1 stock solution (see previous paragraph) and perfused at a flow rate of 1 μl/min for 5 h. The samples of dialysate were collected every hour and frozen at −80°C. In addition, another experiment was performed to test the time course of the change in dialysate concentration in response to an acute change of the cytokine concentration in the stock solution. Additional IL-6 (800 pg/ml) was spiked into the stock solution after 4 h of previous infusion, and the dialysate was collected every hour for the next 4 h. Concomitantly, a control sample from the stock solution was taken every hour and frozen for further analysis.

To test the role of the nonspecific binding of cytokines to the tubing and probes, the surfaces were saturated by rinsing with 1% BSA in saline for 1 h. Two probes (one saturated and one nonsaturated) were immersed in the stock solution of cytokine, perfused with 0.1% BSA in physiological saline, and sampled as described above.

**Determination of in vivo relative recovery of cytokines through microdialysis probes.** For determination of in vivo RR (%) of seven investigated cytokines (IL-1β, IL-6, IL-8, IL-10, TNFα, MCP-1, and PAI-1), a “zero-flow” method was applied in four subjects (17, 31). The zero-flow method derives the absolute concentration of the given substance in the interstitial space from concentrations in dialysate at different flow rates of the microdialysis perfusion. RR was calculated from the absolute concentration of the respective cytokine in the interstitial space [RR (%) = cytokine concentration in dialysate × interstitial concentration × 100]. The microdialysis probe was inserted without anesthetics into abdominal SCAT and perfused with 4% dextran (Rheodextran 70; Infusia, Czech Republic) in saline. After 3 h of equilibration, the perfusate flow rates were changed to 0.5, 1, 2.5, and 5 μl/min, and samples were collected so that a volume of 60 μl of dialysate was obtained. The samples were kept frozen at −80°C until analysis.

**In vivo time course of cytokines during hyperinsulinemic clamp and in control condition.** The in vivo kinetics of the seven investigated cytokines were evaluated during a euglycemic-hyperinsulinemic clamp and in control condition. In the control experiment, the microdialysis probe was inserted without anesthetics into abdominal SCAT and perfused with 4% dextran (Rheodextran 70) in saline at a flow rate of 1 μl/min. Dialysates were collected at 30-min intervals for 7.5 h and frozen at −80°C. The infusion of physiological saline was started at the 210th min of the experiment and lasted 3 h. In the euglycemic-hyperinsulinemic clamp experiment, the microdialysis protocol was identical to that of the control experiment, but at the 210th min of the experiment the hyperinsulinemic clamp (see above) was started and was carried out for 3 h.

**Determination of plasma and dialysate concentrations.** Plasma glucose was determined with the glucose oxidase technique (Biotrol kit, Paris, France). Plasma insulin was measured using an Immunotech Insulin Iirma kit (Immunotech, Prague, Czech Republic). Dialysate and plasma samples were analyzed using xMAP technology on the Luminex 100 instrument or using ELISA. Plasma levels of IL-1β, IL-6, IL-8, IL-10, and TNFα were determined using a High Sensitivity Human Cytokine Lincoplex Kit (Lincos-Millipore), and MCP-1 and PAI-1 were quantified by ELISA (Biosource-Invitrogen, Carlsbad, CA, and R&D Systems, Minneapolis, MN, respectively).

In the dialysate, IL-1β, IL-6, IL-8, IL-10, TNFα, MCP-1, and PAI-1 were measured by a Human Adipocyte Lincoplex Kit (Lincos-Millipore). IL-6 and MCP-1 in samples from in vitro microdialysis were analyzed by ELISA (Endogen-Pierce, Thermo Fisher Scientific and Biosource–Invitrogen, respectively).

**Statistical analysis.** Data are presented as means ± SD. Statistical analysis was performed using SPSS 12.0 for Windows (SPSS, Chi-cago, IL). Responses to the euglycemic-hyperinsulinemic clamp and/or to control intervention were expressed as the percent increase over the baseline levels at the 180th min of the hyperinsulinemic clamp or of the control experiment (infusion of physiological saline). The differences in the responses between the experimental and control conditions and/or between the prediet and postdiet conditions were assessed by Wilcoxon’s signed rank test. Correlations were assessed...
RESULTS

In vitro relative recovery of IL-6 and MCP-1 at different flow rates. In vitro preliminary experiments assessing RR (%) of several cytokines were performed before starting the clinical study. Concentrations of IL-6 and MCP-1 in dialysate outflowing from the probes immersed in stock solution with known concentration of the respective adipokine were determined at different flow rates of perfusion. RR was calculated as indicated (see MATERIALS AND METHODS). The relationship between RR and the perfusion flow rates is presented in Fig. 1A. A flow rate of 1 μl/min was chosen as the best compromise between the magnitudes of RR and flow rate for all further experiments. In vitro RRs of IL-6 and MCP-1 at a flow rate of 1 μl/min were 15 ± 5 and 41 ± 5%, respectively.

Time course of IL-6 and MCP-1 concentrations in dialysate in vitro. The time course of reaching a steady state of cytokine concentrations in dialysate was tested. Dialysate concentrations of IL-6 and MCP-1 were determined in samples collected every hour during a 5-h perfusion of microdialysis probe immersed in stock solution and perfused at a steady flow rate (1 μl/min). The levels of both cytokines were increasing with time and reached a steady state after 2–3 h from the start of perfusion (Fig. 1B). Response to an acute change in the surrounding environment was tested by adding IL-6 (800 pg/ml) to the stock solution at the 4th hour of the experiment (Fig. 1C). The dialysate concentration increased within 1 h, reaching a new steady state within 1–2 h. When the nonspecific binding of proteins to surfaces of probes and tubing was blocked with 1% BSA, no marked change in the pattern of IL-6 time course was observed (data not shown).

In vivo RR of cytokines. To determine in vivo RR (%), the concentrations of investigated cytokines in dialysate at various flow rates (0.5–5 μl/min) were measured (Fig. 2). The pattern of relationship between IL-6 and MCP-1 concentrations and flow rate was similar to that observed in vitro. The absolute concentrations of the respective cytokines in the interstitial space were determined using the zero-flow method. RRs at the flow rate of 1 μl/min were calculated for each cytokine and are presented in Table 1.

Cytokine concentrations in dialysate in vivo in control condition. A microdialysis probe inserted in abdominal SCAT was perfused at the flow rate of 1 μl/min for 7.5 h. The concentrations of IL-1β, IL-6, IL-8, MCP-1, and TNFα were rising in the dialysate during first 2.5–3.5 h after insertion of the microdialysis probe (Fig. 3). A steady state was reached after that time. The levels of PAI-1 in the dialysate were decreasing with time. IL-10 was not measurable in the dialysate.

Concentration of cytokines in SCAT and in plasma. Interstitial concentrations of cytokines were calculated using mean RR of individual proteins in vivo (Table 1) and dialysate concentration in steady state. Compared with plasma levels, the interstitial SCAT concentrations of IL-1β, IL-6, IL-8, TNFα, and MCP-1 were 10–1,000 times higher (Table 2). IL-10 was not detectable in the dialysate, and the concentration of PAI-1 was ~40 times lower in SCAT than in plasma.

Effect of hyperinsulinemia on dialysate and circulating levels of cytokines. In the group of 13 women, the insulin infusion during the clamp induced an increase of insulin plasma levels by 10.220.33.1 on June 15, 2017 http://ajpendo.physiology.org/ Downloaded from HYPERINSULINEMIA AND CYTOKINES E1156

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by Spearman’s correlation. The level of significance was set at \( P < 0.05 \).
The mean whole body glucose disposal rate was 3.13 ± 1.43 (mg·kg$^{-1}$·min$^{-1}$).

The effect of hyperinsulinemia on cytokine levels in these 13 obese women is shown in Table 3. During the euglycemic-hyperinsulinemic clamp, an increase of both interstitial and circulating concentrations of MCP-1 and IL-6 was higher than during the control experiment. IL-8 responded to hyperinsulinemia with a significant increase in SCAT but not in plasma. The responses of interstitial and circulating levels of IL-1β, PAI-1, IL-10, and TNFα were not significantly different compared with the control experiment.

In the dialysate, the relative variations of IL-6 levels in response to hyperinsulinemia correlated positively with those of IL-8 and MCP-1 ($r = 0.53$, $P < 0.05$ and $r = 0.59$, $P < 0.05$, respectively). There was a significant correlation between the changes of IL-6 and MCP-1 concentration in the dialysate during hyperinsulinemia and the changes in plasma ($r = 0.57$, $P < 0.05$ and $r = 0.73$, $P < 0.01$, respectively).

**Effect of VLCD on hyperinsulinemia-induced response of cytokines.** Anthropometric and biochemical parameters of six subjects undergoing dietary intervention are shown in Table 4. VLCD resulted in weight loss (12.5 ± 2.6%) accompanied by reduction of fasting plasma insulin, triglyceride, and cholesterol levels. During the euglycemic-hyperinsulinemic clamp, the subjects were clamped at their fasting blood glucose levels. Mean blood glucose levels during the last 30 min of the clamp were not different before vs. after the diet (Table 4). The coefficients of variation for the blood glucose levels during the clamp were 2.8 ± 1.5% before vs. 3.5 ± 1.7% after the diet. Insulin infusion induced a rise in plasma insulin levels in both conditions (for values see Table 4). Variations of plasma insulin levels were 6.4 ± 3.2% before and 4.5 ± 1.5%, respectively, after the diet. Insulin sensitivity was assessed by an index (M/ln INS$_{clamp}$) calculated as the whole body glucose disposal rate (M) per unit of steady-state plasma insulin (INS$_{clamp}$) [according to Murdolo et al. (18)]. Whole body glucose disposal rates were calculated using the mean exogenous glucose infusion rates during the last 30 min of the clamp. Insulin sensitivity was improved during the diet ($P = 0.03$; Table 4).

Plasma levels: the fasting basal levels of all of the measured circulating cytokines were not modified by VLCD (Table 5). The hyperinsulinemia-induced upregulation of IL-6 and MCP-1 plasma levels was enhanced at the end of VLCD compared with prediet results. Plasma IL-8 increased in response to hyperinsulinemia after VLCD, whereas no response was detected before the diet.
Dialysate: the significant prediet effect of hyperinsulinemia on IL-6, IL-8, and MCP-1 levels in dialysate was enhanced at the end of VLCD (Fig. 4). For TNFα and IL-1β, which were not affected by hyperinsulinemia before the diet, the upregulation of hyperinsulinemia in AT appeared after VLCD. No effect of hyperinsulinemia was observed for PAI-1 before or after the diet.

The dialysate concentrations of IL-6 correlated with dialysate concentrations of IL-8 and MCP-1 (r = 0.89, P < 0.05 and r = 0.83, P = 0.05, respectively) before and at the end of VLCD.

**DISCUSSION**

In this study, we determined in situ cytokine production by AT in response to hyperinsulinemia and its modulation during hypocaloric dietary intervention in obese women. The most important finding of our study is that experimental hyperinsulinemia increases AT levels and plasma levels of IL-6, MCP-1, and IL-8. Furthermore, this effect is enhanced following 4 wk of VLCD. Secondary, our study reported for the first time the SCAT interstitial concentration of several adipokines (IL-8, IL-1β, TNFα, and PAI-1).

The levels of the investigated adipokines were found to be considerably higher in SCAT than in plasma in the present study, except of PAI-1 (Table 2). Indeed, SCAT interstitial concentrations of IL-6 and MCP-1 were 1,000 and 50 times higher, respectively, than their plasma levels. The interstitial concentrations of these cytokines in our study (5.7 ± 3.0 ng/ml and 2.7 ± 1.9 for IL-6 and MCP-1, respectively) are similar to data of Sopasakis et al. (29), who reported a concentration of IL-6 of 6.3 ± 4.6 ng/ml, and Murdolo et al. (20), who found an MCP-1 level of 2.0–6.6 ng/ml in human SCAT. This indirectly confirms the reproducibility and feasibility of microdialysis technique in this type of investigations. The levels of IL-8, IL-1β, TNFα, and PAI-1 in the present work ranged from 30 to 1,800 pg/ml. These data are consistent with a role of these factors as paracrine/autocrine regulators within AT. However, the determinants of the high levels of these adipokines in SCAT when compared with circulating levels need further investigation. They imply the complex processes at the level of cellular translation, secretion, and/or clearance. One possible determinant could be the high net flux of NEFA within AT, as fatty acids are known to activate inflammatory pathways in macrophages, the cell type responsible for the majority of adipokine production (32, 33). Interestingly, the concentration of PAI-1 was lower in SCAT than in plasma. Although this protein was shown to be markedly secreted by AT (7), its

### Table 3. Relative change (%) of cytokine and chemokine concentrations in the dialysate and in plasma during hyperinsulinemic clamp and in control condition

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Adipose Tissue</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Plasma</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Clamp</td>
<td>P</td>
<td>Control</td>
<td>Clamp</td>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>76±7.4</td>
<td>108±88</td>
<td>NS</td>
<td>4.4±7.9</td>
<td>3.9±4.1</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>7.0±4.8</td>
<td>57±15</td>
<td>0.011</td>
<td>6.8±8.5</td>
<td>19.4±6.7</td>
<td>0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>0.9±19</td>
<td>35.0±14.7</td>
<td>0.05</td>
<td>8.3±3.3</td>
<td>6.5±5.3</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
<td>2.6±7.9</td>
<td>7.8±11.6</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>−35.0±10.1</td>
<td>−11.3±8.9</td>
<td>NS</td>
<td>−2.9±5.5</td>
<td>4.9±4.4</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.9±4.7</td>
<td>56.3±18.9</td>
<td>0.017</td>
<td>−3.9±3.3</td>
<td>40.0±16.2</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>−12.5±16.3</td>
<td>−15.4±7.8</td>
<td>NS</td>
<td>−38.5±11.9</td>
<td>−29.7±6.5</td>
<td>NS</td>
<td></td>
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</tbody>
</table>

Data are presented as means ± SD; n = 13. Relative change is expressed as % increase over baseline levels at the 180th minute of the hyperinsulinemic clamp or at the 180th minute of control experiment (infusion of physiological saline). NS, not significant; ND, not detected in dialysate. P, level of significance of the difference between the relative change during hyperinsulinemic clamp and control condition.

### Table 4. Anthropometric and biochemical characteristics of subjects undergoing the diet

<table>
<thead>
<tr>
<th></th>
<th>Before VLCD</th>
<th>After VLCD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight, kg</strong></td>
<td>98.8±18.4</td>
<td>90.5±17.4‡</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>36.7±6.1</td>
<td>33.7±5.6‡</td>
</tr>
<tr>
<td><strong>Fat mass, kg</strong></td>
<td>39.3±12.9</td>
<td>33.8±11.8‡</td>
</tr>
<tr>
<td><strong>Fat-free mass, kg</strong></td>
<td>59.5±7.2</td>
<td>56.7±7.2‡</td>
</tr>
<tr>
<td><strong>Waist, cm</strong></td>
<td>110±14</td>
<td>103±15‡</td>
</tr>
<tr>
<td><strong>Glucose, mmol/l</strong></td>
<td>5.4±0.3</td>
<td>5.1±0.3</td>
</tr>
<tr>
<td><strong>Insulin, mU/l</strong></td>
<td>16.8±9.6</td>
<td>6.4±2.9*</td>
</tr>
<tr>
<td><strong>NEFA, μmol/l</strong></td>
<td>628±169</td>
<td>669±91</td>
</tr>
<tr>
<td>b-Hydroxybutyrate, mmol/l</td>
<td>0.14±0.12</td>
<td>0.69±0.33†</td>
</tr>
<tr>
<td><strong>Triglycerides, g/l</strong></td>
<td>1.76±0.99</td>
<td>1.20±0.23*</td>
</tr>
<tr>
<td><strong>Total cholesterol, mmol/l</strong></td>
<td>4.81±0.99</td>
<td>3.83±0.77*</td>
</tr>
<tr>
<td><strong>HDL, mmol/l</strong></td>
<td>1.13±0.38</td>
<td>0.94±0.26*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; n = 6 subjects. VLCD, very low calorie diet. P values of the difference between the pre- and post-VLCD values: *P < 0.05; †P < 0.01; ‡P < 0.001.

### Table 5. Concentration of plasma cytokines, glucose and insulin at baseline and at the end (180th minute) of the euglycemic-hyperinsulinemic clamp before and after VLCD

<table>
<thead>
<tr>
<th>Cytokine, pg/ml</th>
<th>Before VLCD</th>
<th>After VLCD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1β</strong></td>
<td>1.18±0.98</td>
<td>1.22±1.03</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>4.2±2.5</td>
<td>4.8±2.5*</td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td>4.5±2.2</td>
<td>4.8±2.2</td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td>11.5±8.8</td>
<td>12.5±9.5</td>
</tr>
<tr>
<td><strong>TNFα</strong></td>
<td>3.8±1.7</td>
<td>3.9±1.4</td>
</tr>
<tr>
<td><strong>MCP-1</strong></td>
<td>132±76</td>
<td>184±117†</td>
</tr>
<tr>
<td><strong>PAI-1</strong></td>
<td>8,010±2,320</td>
<td>4,780±1,660</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; n = 6 subjects. *P < 0.05; †P < 0.05; significant change during the clamp. ‡P < 0.05; difference between the relative increase during the clamp when comparing pre- and post-VLCD.
circulating levels are probably derived from other tissues. Accordingly, measurement of arteriovenous differences revealed no release of AT-derived PAI-1 into circulation (39).

Elevated plasma insulin levels present in obesity might be one of the drivers of the enhanced production of cytokines that further participate in the development of metabolic disturbances. In this study, we observed an insulin-induced increase in IL-6, IL-8, and MCP-1 but not in TNFα or IL-1β in SCAT of obese subjects (Table 3). In accord with our observation, MCP-1 interstitial levels in SCAT were shown to increase in obese and in lean subjects (20). Similarly, IL-6 mRNA levels increased during hyperinsulinemia in healthy lean men (16). On the other hand, the results of in vitro experiments are less consistent. Addition of insulin to human AT explants enhanced the release of IL-8 and PAI-1 (10), while other studies found insulin-stimulated release of IL-8, VEGF, and PAI-1 in adipocytes but not in whole AT explants (6). In accord with the reported results (6), insulin did not significantly stimulate in vivo PAI-1 production in our study. Pathways that mediate the insulin effect on cytokine and chemokine production in AT are not understood. In human preadipocytes, insulin was proved to induce IL-6 expression and release through cGMP/PKG and MAPK kinases pathways (18). In 3T3-L1 adipocytes, chronic insulin treatment enhanced ROS production, which was followed by inhibition of insulin signaling and glucose uptake, increase of endoplasmic reticulum stress, and JNK pathway...
activation (9). In addition, insulin may act on the levels of translation/release of cytokines. These findings are further extended to the functional level by our observations, that insulin increases AT concentrations of IL-6, IL-8, and MCP-1, which are considered to mediate local as well as whole body proinflammatory and insulin sensitivity decreasing effects. It is important to note here that several cell types present in the AT (macrophages, preadipocytes, endothelial cells) are influenced by insulin and that they might stimulate direct production of adipokines or might secrete other signaling molecules and regulate production of adipokines in a paracrine way. The positive correlation between MCP-1 and IL-6 AT and plasma levels suggests that the processes from AT can be mirrored in the circulation and that insulin can contribute to enhanced circulating cytokine levels in obese subjects.

VLCD enhanced responses of adipokines to hyperinsulinemia during clamp in SCAT (IL-1β, IL-6, IL-8, TNFα, and MCP-1; Fig. 4) as well as in plasma (IL-6, IL-8, MCP-1; Table 5). The enhanced responsiveness to insulin-mediated stimulation of cytokine release was associated with the diet-induced decrease of fasting plasma insulin levels, and, consequently, the baseline values of the investigated cytokines in SCAT interstitial space and/or in plasma were not modified. It may appear that the enhanced responsiveness of cytokine release to insulin parallels the diet-induced enhancement of whole body insulin sensitivity of glucose uptake (35). In this study, the insulin sensitivity expressed as M/I/NINSclamp increased (P = 0.03) during VLCD. The increase in whole body glucose disposal rate (M) did not reach significance (P = 0.1); this might have been influenced by the number of subjects in this study; moreover, it is to be noted that the response of glucose disposal rate to the hypocaloric diet is variable and some studies report no increase during the diet (12, 13, 25). The findings in our prospective study are in line with results of the cross-sectional study of Ruge et al.(24). They found a higher responsiveness of plasma IL-6 to hyperinsulinemia in healthy lean subjects compared with insulin-resistant subjects. Moreover, the hyperinsulinemia-induced rise of IL-6 correlated negatively with BMI and with fasting plasma insulin. However, the mechanism underlying the enhanced responsiveness of cytokine release is not clear; sensitization of some of the pathways possibly mediating the insulin effect on cytokine release (i.e., the cGMP/PKG and MAPK kinase pathways mentioned above) as well as sensitization of translational pathways and/or mechanisms involved in the release of cytokines into the interstitial space may play a role.

The investigation of cytokines secreted by AT has been limited so far to in vitro studies or in vivo animal studies. Microdialysis is a powerful approach that enables direct determination of the interstitial levels of substances in vivo in various tissues in humans (21, 22, 27, 29). Nevertheless, this method has some limitations that should be considered. One of the possible limitations is a low recovery of large molecules in the dialysate. In this study, the zero-flow method was used to assess the recovery of selected cytokines (17, 31) (Figs. 1 and Fig. 2). A recovery between 20 and 55% was observed (Table 1). These recoveries are similar to or slightly higher than those previously published (1, 37). In contrast to previous studies (1, 21, 37), we compared the recovery from in vivo and in vitro experiments; the recovery rates were similar in vivo and in vitro. It should be emphasized here that relative recovery is not only a function of molecular weight, since the recoveries of IL-6 and MCP-1 are quite different, although their molecular weights are very similar. This difference is probably inherent in the shape of molecules with different gyration radius (1). Another limitation of microdialysis may be a local injury following insertion of the probe, which might be reflected by a transient rise of cytokines and chemokines in the interstitial space. However, in human skeletal muscle (27) and also in AT (20), the microtrauma was found to be present only for a short period of time after insertion of the probe; thus, it is unlikely that it contributed to a gradual increase of interstitial levels of cytokines. Furthermore, the increase of cytokine/chemokine levels in the dialysate observed for 3 h after the start of microdialysis is also found in vitro (Fig. 1, B and C). Hence, other underlying mechanisms than local inflammation should be considered. We suggest that the progressive increase of concentration at the beginning of the microdialysis studies may have been partially caused by unspecific interactions and partially by steric hindrance of the probe (causing the slow diffusion of proteins through the membrane).

In conclusion, the present study shows that several AT-derived cytokines/chemokines can be detected in SCAT by using the microdialysis method. Upregulation of the interstitial levels of IL-6, IL-8, and MCP-1 by hyperinsulinemia was demonstrated in humans, and this effect was enhanced by a weight-reducing hypocaloric diet.

These findings indicate that the increased insulin levels in obese and diabetic subjects might be one of the drivers of the enhanced production of adipocytokines in AT and, consequently, of the evolution of the whole body proinflammatory state. Elucidation of mechanisms underlying the insulin effect on cytokine release in AT and of the clinical impact of the diet-induced sensitization of AT adipokine release to insulin action warrants further studies.

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