CLINICAL STUDY

Secretion of adiponectin multimeric complexes from adipose tissue explants is not modified by very low calorie diet

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Abstract

Objective: Adiponectin is a protein abundantly secreted by the adipose tissue (AT). Plasma adiponectin levels are decreased in obese, insulin-resistant, and type 2 diabetic patients. Various multimeric complexes, i.e. high-, middle-, and low-molecular weight isoforms (HMW, MMW and LMW), are present in plasma. Here, we investigated the effect of weight reducing diet on the distribution of adiponectin isoforms in plasma and on their secretion in AT explants from obese subjects.

Design: A total of 20 obese subjects (age 37.8 ± 7.3 years, body mass index 33.9 ± 5.0 kg/m²) underwent eight weeks of very low-calorie diet (VLCD). A needle biopsy of subcutaneous abdominal AT and blood samples were taken before and after dietary intervention. AT explants were incubated in culture medium for 4 h. ELISA assay and western blot analyses were used to identify adiponectin complexes in culture media and in plasma.

Results: The distribution of adiponectin polymers in plasma was different from that secreted in human AT explants. Before VLCD, the relative amount of HMW isoform was 75.5 ± 9.1% of total adiponectin in culture media and 52.2 ± 11.2% in plasma. Despite the diet-induced weight loss and improvement of insulin sensitivity, VLCD neither induced change in total adiponectin level nor in the ratio of HMW to total adiponectin in plasma and in culture media of AT explants.

Conclusions: The profile of adiponectin polymeric isoforms secreted by AT explants into culture media differs from the plasma profile. A dietary intervention leading to weight loss and improvement of insulin sensitivity was not associated with modifications of AT secretion of total or HMW adiponectin.

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Introduction

Adipose tissue (AT) is an endocrine organ capable of secreting a number of adipokines that play a role in the regulation of AT and whole-body metabolism. One of them is adiponectin, a protein abundantly expressed and secreted by adipocytes, its level in plasma accounting for 0.01% of total plasma proteins. Adiponectin appears to have an important physiological role in relation to insulin sensitivity and diabetes (1) acting as an insulin sensitizing agent and a protective factor for the cardiovascular system (2). Data from animal models show that adiponectin exerts its effects on energy homeostasis and glucose and lipid metabolism primarily through phosphorylation and activation of adenosine monophosphate-activated protein kinase in liver and skeletal muscle (3). By contrast to other known proteins produced by AT, plasma adiponectin levels are reduced in obese humans as well as in insulin resistant, diabetic, and coronary heart disease patients (1, 4–6). This suggests a possible obesity-induced impairment in adiponectin production by AT.

Adiponectin is present intracellularly and in the circulation in various multimeric complexes (7, 8). It is synthesized as a single 30 kDa polypeptide that is assembled into different molecular weight isoforms. The basic adiponectin multimer consists of low-molecular weight (LMW) trimers, formed through hydrophobic interactions. The oxidizing environment within the lumen of the endoplasmic reticulum favors disulfide bond formation through which trimers can associate into middle-molecular weight (MMW) hexamers and high-molecular weight (HMW) multimers composed of 12–18 monomers (9, 10). Mechanisms that regulate formation and distribution of adiponectin complexes in AT remain poorly known so far.

Nevertheless, it has been suggested, that the HMW form of adiponectin is predominantly responsible for its
biological effects in liver, muscle, and endothelial tissues (10, 11). Several recent studies have demonstrated that the plasma levels of the HMW form and the ratio of HMW to total adiponectin are more closely associated with glucose tolerance and insulin sensitivity than total adiponectin (10–15). Clinical studies showed that patients with type 2 diabetes mellitus (T2DM) and coronary heart disease have selective reduction of HMW polymers (16–18) and that this decrease is reversed following moderate weight reduction and/or treatment with thiazolidinediones (TZD) (11, 18–20). Several authors have raised the possibility that TZDs that are PPARγ activators widely used to ameliorate insulin resistance and improve glucose tolerance in T2DM, may exert their effects through increased production and secretion of adiponectin, especially the HMW form, from adipocytes thus elevating plasma adiponectin levels (21–24). However, studies investigating changes in adiponectin multimeric complexes in plasma following dietary interventions have yielded contradictory results; some showed no changes in their distribution (19), while others found increased quantity of some of the multimeric complexes (12, 25) or only increase of HMW form (26). Moreover, the mechanisms governing production of the different adiponectin polymers in humans and variations in AT and plasma levels remain elusive.

The main aim of this study was to investigate whether very low-calorie diet (VLCD)-induced weight loss had an impact on the production and release of specific adiponectin polymers by AT. The pattern of adiponectin isoforms secreted by AT explants derived from needle biopsies of abdominal subcutaneous AT was investigated before and after the diet and compared with the distribution of adiponectin polymers in plasma.

**Material and methods**

**Subjects**

A total of 20 obese subjects (17 women and 3 men; age 37.8 ± 7.3 years, body mass index (BMI) 33.9 ± 5.0 kg/m²) participated in this study. All subjects were drug-free and did not suffer from any disease except for obesity. Their body weight had been stable for at least three months before the beginning of the intervention.

Individuals were investigated before and after the weight reduction program (one woman did not complete the weight-reduction program) at the Department of Sports Medicine of the Third Faculty of Medicine of Charles University, Prague. Written informed consents were obtained from all subjects. The study was performed according to the Declaration of Helsinki and approved by the Ethical committee of the Third Faculty of Medicine, Charles University, Prague, Czech Republic.

**Clinical protocol**

Subjects were investigated at 0800 h after an overnight fast before and at the end of 8 weeks’ VLCD. The VLCD was designed to provide 800–1000 kcal/day (liquid formula diet: Modifast, Novartis Nutrition GmbH).

Body height, weight, waist, and hip circumference were measured. Body composition was assessed using multifrequency bioimpedance (Bodystat, Quad scan 4000, Isle of Man, British Isles). Coefficients of variation (CV) of fat mass and fat-free mass were 1.7% and 0.8% respectively. Blood samples were collected from an indwelling polyethylene catheter inserted into an antecubital vein for plasma metabolite and hormone determinations. After blood collection, plasma was separated by centrifugation (10 min, 190 g, 4 °C) and stored at −80 °C until analysis.

At baseline and at the end of the dietary intervention, a needle biopsy of subcutaneous abdominal AT was performed as previously reported (27). Plasma glucose was determined by the glucose oxidase technique (Beckman Instruments, Fullerton, CA, USA). Plasma insulin concentration was measured by RIA kit (Immunotech, Prague, Czech Republic). Homeostasis model assessment of insulin resistance index (HOMA-IR) was calculated as follows: (fasting insulin in mU/ml) × (fasting glucose in mmol/l)/22.5.

**AT explants**

AT from biopsies was washed in saline. AT pieces, i.e. explants (400 mg) were incubated in 4 ml Krebs/Ringer phosphate buffer (pH 7.4) supplemented with 4 g/l of BSA and 1 g/l glucose for 4 h at 37 °C in a shaking water bath with air as the gas phase (28, 29). After 4 h of incubation, 2 ml of the medium was taken from each sample, frozen in liquid nitrogen, and stored at −80 °C until analysis.

**Study of the time-course of adiponectin secretion in AT explants**

The kinetics of total adiponectin secretion was investigated in AT explants from 6 subjects with broad range of BMI who did not participate in the dietary intervention study. These subcutaneous AT samples were obtained during the surgery from abdominal region after obtaining written informed consent. Explants were incubated as described above and samples of media were taken in seven time-points: 0 h – immediately after addition of medium and after 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h of incubation. Total adiponectin was analyzed by ELISA (BioVendor Laboratory Medicine, Brno, Czech Republic).

**Determination of adiponectin polymers**

Plasma samples and media from explants were diluted according to the manufacturer’s instructions.
Adiponectin (multimeric) enzyme-immunnoassay kit from ALPCO Diagnostics (Salem, NH, USA) was used for quantification of adiponectin polymeric complexes. In addition to the determination of total adiponectin, this ELISA kit enables direct measuremnt of HMW + MMW and HMW alone using two protease treatments to digest LMW and LMW + MMW isoforms of adiponectin. MMW and LMW adiponectin were then calculated by subtraction. The dynamic range of this kit is 0.075–4.8 ng/ml. Intraassay CV for total adiponectin, MMW and HMW was 5.3%, 4.1% and 3.3% respectively. Adiponectin concentration in plasma samples as well as in culture media from each subject before and after the diet was analyzed on the same plate. Percentage of adiponectin isofrom to total adiponectin was calculated for each subject and time point.

Western blot analysis
Western blot (WB) analysis was performed as previously described (25, 30). Samples of plasma and culture media from explants (10 μl) were diluted with Laemml sample buffer (without β-mercaptoethanol and SDS) and proteins were separated by a 7% native PAGE under non-reducing and non-denaturing conditions. The resolved proteins were transferred to a nitrocellulose membrane, the blots were blocked for 1 h with 5% low-fat milk in a PBS with 0.5% Tween20 (PBS-T) and incubated overnight at 4°C with anti-human adiponectin antibody (Rabbit polyclonal antibody, Biovendor Laboratory Medicine Inc., Modrice, Czech Republic) diluted 1:1000 in 1% low-fat milk in PBS-T. The membrane was then washed twice (15 min) with PBS-T. A second incubation (45 min) was carried out with goat anti-rabbit IgG conjugated with HRP (Jackson Immuno Research Europe Ltd, Cambridge, UK) diluted 1:10000 in 1% low-fat milk in PBS-T. Band intensities were analyzed using AIDA2.0 software. Signal intensities from the duplicate samples were averaged and used for statistical analysis.

Statistical analysis
Statistical analysis was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). The differences between the pre-diet and post-diet values and between the concentrations in plasma and medium were tested using the Wilcoxon signed-rank test for paired observations for all the variables studied. Univariate correlations were analyzed using Spearman’s correlation test. Values are presented as means ± S.D. Differences at the level of \( P < 0.05 \) were considered statistically significant.

Results
Biochemical and anthropometric data
Anthropometric and biochemical characteristics of subjects before and after eight weeks of VLCD are shown in Table 1. The intervention resulted in a reduction in BMI by 10.2% (\( P < 0.001 \)). Waist and hip circumf-erences decreased by 7.8% and 6.0% (\( P < 0.001 \)) respectively and fat mass (kg) decreased by 18.8% (\( P < 0.001 \)). Total cholesterol as well as high density lipoprotein (HDL) cholesterol levels decreased after the diet. Improvement in carbohydrate metabolism was reflected by a diet-induced decrease of fasting plasma glucose, fasting plasma insulin, and HOMA-IR by 8.7%, 39.3%, and 43.4% (\( P < 0.05; \ P < 0.001; \ P < 0.001 \)) respectively.

Table 1 Anthropometric and biochemical parameters of subjects before and after eight weeks of dietary intervention.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before VLCD</th>
<th>After VLCD</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Weight (kg)</td>
<td>98.9 ± 15.7</td>
<td>87.9 ± 14.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>33.9 ± 5.0</td>
<td>29.9 ± 4.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>40.8 ± 9.9</td>
<td>32.5 ± 8.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>59.5 ± 9.3</td>
<td>57.2 ± 29.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>107.5 ± 16.4</td>
<td>97.3 ± 14.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>120.5 ± 9.4</td>
<td>112.3 ± 8.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Glucose fasting (mmol/l)</td>
<td>5.1 ± 0.4</td>
<td>4.7 ± 0.6*</td>
<td>0.002</td>
</tr>
<tr>
<td>Insulin fasting (mIU/l)</td>
<td>9.9 ± 4.5</td>
<td>5.4 ± 2.6†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.1 ± 1.1</td>
<td>1.1 ± 0.6†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.3 ± 0.1</td>
<td>1.2 ± 0.1*</td>
<td>0.018</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.5 ± 0.3</td>
<td>4.0 ± 0.2*</td>
<td>0.002</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.116</td>
</tr>
</tbody>
</table>

Data are shown as means ± s.d. * \( P < 0.01; \ T < 0.001 \) for comparison before versus after diet.
above-mentioned kinetics of adiponectin secretion from AT explants, concentration at 4 h of incubation was chosen as representative for analysis of adiponectin polymers secretion during the dietary study.

**Distribution of adiponectin isoforms in culture media from AT explants and in plasma of obese subjects**

To investigate the distribution of adiponectin polymeric isoforms secreted by human AT, AT explants obtained from obese subjects before the dietary intervention were incubated for 4 h and the quantity of different isoforms in culture media was determined by ELISA. In parallel, composition of adiponectin isoforms was measured by ELISA in plasma obtained from the same subjects before the diet. Data are shown in Fig. 2. Adiponectin multimeric ELISA assay used in this study is able to detect directly HMW form and total adiponectin only. Therefore, confirmatory non-denaturing WB analysis, which measures all adiponectin isoforms (HMW, MMW, and LMW) was performed in a subset of 14 plasma and culture media samples. Figure 3 shows representative WB of separated adiponectin isoforms from plasma and culture media.

In both, culture media and plasma, the most abundant isoform was the HMW form as assessed by ELISA (Fig. 2). While in media HMW represented $75.5 \pm 9.1\%$ of total adiponectin, in plasma the percentage was only $52.2 \pm 11.2\%$, i.e. $23.3\% (P<0.001)$ less than its relative quantity in culture media. When measurement was performed by semi-quantitative WB, the difference between the proportion of HMW adiponectin secreted by AT explants and HMW isoform in plasma was $43.9\%$ and the relative proportion of HMW to total adiponectin in media and plasma samples were $75.1 \pm 14.9\%$ and $31.2 \pm 12.5\%$ respectively. These results demonstrate the difference in the distribution of adiponectin multimers secreted by AT into culture media and in plasma.

**Effect of VLCD on adiponectin isoform distribution in culture media and plasma**

The effect of VLCD on the distribution of adiponectin isoforms in culture media and plasma is shown in Table 2. The diet did not induce any change in total adiponectin level either in culture media of AT explants or in plasma. Furthermore, the ratio of HMW to total adiponectin was not different before and after the diet both in plasma and in secreted media of AT explants. Results were similar, if just the subgroup of female subjects was considered.

**Discussion**

The major goal of this study was to investigate the effect of VLCD on the distribution of adiponectin isoforms in plasma and their secretion in the biopsy-derived AT

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**Figure 1** Kinetics of total adiponectin secretion from human adipose tissue explants. Adipose tissue explants ($n=6$) were incubated for various periods of time (0–24 h). Total adiponectin in samples of culture media was measured by ELISA.

**Figure 2** Adiponectin isoforms in culture media of adipose tissue explants and in the plasma of obese subjects ($n=20$). The quantities of adiponectin polymers are expressed as ratios to total adiponectin. ELISA assay for adiponectin multimer measurement was not sensitive enough to detect LMW isoform in culture media (ND, not detectable values).

**Figure 3** A representative example of the chemiluminescent detection of adiponectin multimeric complexes analyzed by western blot – comparison of adiponectin isoform profile in plasma and culture media after 4 h of AT explant secretion. Samples were run in duplicate.
Adiponectin is a protein secreted abundantly by AT and present in plasma at high levels in at least three isoforms (HMW, MMW, and LMW form). Despite many studies on physiological functions of adiponectin, little is known about the mechanism of adiponectin production and its release from adipocytes. The regulation of adiponectin multimeric isoforms might occur at the level of gene transcription (18), translation of mRNA into protein, as well as at the post-translational level, including protein modification, oligomerization, and secretion (31). Specific mutations in the adiponectin gene resulting in an impaired multimerization and/or impaired secretion of adiponectin from adipocytes lead to hypoadiponectinemia and may contribute to the phenotypic manifestation of insulin resistance and T2DM (10, 32–34). Therefore, oligomerization of adiponectin seems to be a critical process in the physiological action of adiponectin. Regulation of the production and secretion of different adiponectin complexes is thought to be tightly controlled, as presently there is no evidence of an exchange between the different forms of adiponectin, once they reach circulation (35).

Progress in the study of HMW adiponectin has been slowed down by the lack of commercially available assays to measure the individual polymers of adiponectin in large numbers of samples. A novel ELISA system for the selective measurement of human adiponectin multimers has recently been described (36). Using this method, Hara et al. (37) have reported that HMW adiponectin has better predictive power to detect both the presence of insulin resistance and the metabolic syndrome and is also more clinically useful to estimate the risk of coronary artery disease and atherosclerosis than total adiponectin (16).

In the present study, the ELISA assay was used to compare the distribution of adiponectin polymers in plasma and in the culture media of subcutaneous AT explants and to investigate the effect of weight loss induced by hypocaloric diet on adiponectin secretion. In our previous study, we had demonstrated that native-PAGE under non-reducing and non-denaturing conditions clearly separates adiponectin multimers into three forms: HMW, MMW, and LMW. (25). In this study, the WB results supported those obtained by ELISA and confirmed that HMW is the predominant form secreted from AT as previously shown using other methods (24).

Table 2 Quantity of adiponectin isoforms in plasma and culture media before and after very low-calorie diet (VLCD) measured by ELISA (n=20).

<table>
<thead>
<tr>
<th></th>
<th>Plasma (μg/ml)</th>
<th>Medium (ng/ml per μg DNA per 4 h)</th>
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<tr>
<td></td>
<td>Before VLCD</td>
<td>After VLCD</td>
</tr>
<tr>
<td>Total adiponectin</td>
<td>4.06 ± 2.49</td>
<td>4.15 ± 1.67</td>
</tr>
<tr>
<td>HMW/total %</td>
<td>52.2 ± 11.2</td>
<td>55.6 ± 13.8</td>
</tr>
<tr>
<td>MMW/total %</td>
<td>19.8 ± 10.3</td>
<td>19.8 ± 8.9</td>
</tr>
<tr>
<td>LMW/total %</td>
<td>27.9 ± 11.1</td>
<td>24.6 ± 14.6</td>
</tr>
</tbody>
</table>

Data are shown as means ± s.d. ND, not detectable values. *P<0.001 for comparison of % HMW to total adiponectin between plasma and culture medium samples.
Changes in adiponectin levels could arise besides alterations in synthesis, secretion, and/or clearance also from various lifestyle interventions. At least two different interventions that improve insulin action, long-term weight loss (either diet- or exercise-induced) (23, 25, 31) or TZD treatment (21, 22, 50) might elevate circulating adiponectin levels. Studies with TZDs suggest that regulation at the level of secretion of adiponectin complexes is an important mechanism for the regulation of adiponectin activity and selective improvement of HMW complexes in AT and plasma (11, 24). Recent studies have demonstrated that the PPARγ agonist-mediated transcriptional induction of the adiponectin gene is relatively modest (21) or even completely absent (51), which suggests that other mechanisms might be at play in explaining the increase in plasma adiponectin levels after TZD treatment. Critical intracellular molecular chaperones involved in the maturation of adiponectin complexes in the secretory pathway were described and may in fact represent the most relevant target genes for PPARγ agonists in the induction of adiponectin secretion (52, 53). In vitro studies on human and mouse AT and adipocytes reported significantly increased levels of secreted HMW adiponectin after pioglitazone treatment (24). Furthermore, weight reduction (26) or treatment of T2DM patients with the insulin-sensitizing agent rosiglitazone (52, 53) might elevate circulating adiponectin levels after TZD treatment. Critical intracellular molecular chaperones involved in the maturation of adiponectin complexes in the secretory pathway were described and may in fact represent the most relevant target genes for PPARγ agonists in the induction of adiponectin secretion (52, 53). In vitro studies on human and mouse AT and adipocytes reported significantly increased levels of secreted HMW adiponectin after pioglitazone treatment (24). Furthermore, weight reduction (26) or treatment of T2DM patients with the insulin-sensitizing agent rosiglitazone (52, 53). In vitro studies on human and mouse AT and adipocytes reported significantly increased levels of secreted HMW adiponectin after pioglitazone treatment (24). Furthermore, weight reduction (26) or treatment of T2DM patients with the insulin-sensitizing agent rosiglitazone (52, 53).

In summary, we have demonstrated here that the distribution of circulating adiponectin polymers is different from the distribution of polymers secreted from human AT explants of obese subjects. Adiponectin secreted from human AT consists predominantly of HMW isoforms. Eight weeks of VLCD promoting changes in body weight and insulin sensitivity did not induce changes in secretion and plasma levels of adiponectin isoforms.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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