Conclusion: 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-immunoassay 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 measurement 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 LMW 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 isoform 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 Laemmli 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% goat anti-rabbit IgG conjugated with HRP (Jackson Laboratory Inc., Modrice, Czech Republic) diluted 1:1000 in 1% low-fat milk in PBS-T. Band detection was done on a FujiFilm LAS 1000 detection system (Fuji Photo Film Co., Ltd, Tokyo, Japan). Band 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 circumferences 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.

**Kinetics of adiponectin secretion from human AT**

In order to determine the time-course of adiponectin secretion by AT explants, a kinetic analysis was performed in six subcutaneous AT samples from two lean, two overweight, and two morbid obese women (see Material and methods).

Kinetics of total adiponectin secretion from AT explants during 24 h is shown in Fig. 1. All AT samples showed similar pattern of adiponectin secretion. During the first 6 h, there was an increase of adiponectin in culture media. Afterwards, adiponectin secretion reached a steady state. The biggest rise in adiponectin secretion was observed during the first hour of incubation. The kinetics appeared to be close to linearity in the interval between 1 h and 6 h. The linear regression coefficient in that time interval was similar in lean, overweight, and obese subjects (lean, \( r = 0.892 \); overweight, \( r = 0.846 \); obese, \( r = 0.931 \)). Given the

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

<table>
<thead>
<tr>
<th></th>
<th>Before VLCD</th>
<th>After VLCD</th>
<th>( P ) value</th>
</tr>
</thead>
<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 (mU/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. *\( P < 0.01; † P < 0.001 \) 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 ± 9.1% of total adiponectin, in plasma the percentage was only 52.2 ± 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 ± 14.9% and 31.2 ± 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 Explants.
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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<tbody>
<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.

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, translation of mRNA into protein, as well as at the post-translational level, including protein modification, oligomerization, and secretion. 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. 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 isoforms 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.

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. Using this method, Hara et al. 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.

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. 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. However, differences exist in relative amounts of each isoform measured by ELISA and WB respectively. They are probably due to methodological differences, e.g. indirect assessment of LMW isoform concentration in the ELISA assay and the semiquantitative character of WB analysis. In addition, our data suggest that the profile of adiponectin polymeric isoforms secreted from subcutaneous AT is clearly different from that in plasma.

Adiponectin complexes are stable in serum and do not interconvert post-secretion. Thus, one can speculate that adiponectin is assembled in cells primarily into HMW isoform prior to secretion and then in extracellular space or in plasma it can be modified into smaller molecular weight forms. There are many proteins present in plasma acting as regulators of protein size and multimer formation. Protein disulfide bond reductase thrombospondin-1 (THBS1), secreted from endothelial cells reduces the average multimer size of the von Willebrand factor and impairs the efficacy of this protein. THBS1 was recently found as a true adipokine produced by adipocytes and increased gene expression of THBS1 in AT and its elevated plasma protein levels were observed in obese, insulin resistant, and diabetic patients and animal models of obesity. This leads us to hypothesize the existence of proteins with reductase activity acting on adiponectin multimeric isoforms. Another plausible hypothesis on adiponectin distribution in plasma is that the subcutaneous AT secretes primarily HMW adiponectin and MMW and LMW isoforms in plasma can proceed from different sources other than the AT itself. The differences between adiponectin production in subcutaneous and visceral fat depot may also contribute to the above-mentioned differences between secretion in subcutaneous AT and plasma as both fat depots contribute to the circulating levels of adiponectin. In fact, different protein production or gene expression of many adipokynes, including adiponectin, was demonstrated in visceral when compared with subcutaneous fat. The results are not unequivocal: lower adiponectin protein and mRNA levels in visceral versus subcutaneous AT were evidenced in several studies while opposite results were reported by other authors. To date, no study on adiponectin isoforms secretion by visceral AT explants has been reported. Thus, we can speculate that the differences between distribution of adiponectin polymers in plasma and subcutaneous AT explants may partly reflect differences between adiponectin polymers secretion in visceral and subcutaneous AT. Furthermore, the effect of the dietary intervention might be different in visceral AT.
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 (11) preferentially increased the HMW form of adiponectin, but not the other two oligomeric complexes.

This study shows that during VLCD, the secretion of total and HMW adiponectin is not modified. To date, few studies have examined the effects of the diet induced weight loss on plasma total adiponectin or its isoforms with conflicting results (12, 19, 25, 26, 54). The results of the latter studies might suggest that adiponectin levels are modified after long-term extensive weight loss (e.g. induced by bypass surgery (55, 56)) whereas, no change is observed when moderate weight loss is induced by lifestyle intervention (57–60). This adaptation may be related to a relatively low turnover and high plasma adiponectin concentration (29). Moreover, our data reveal that during the VLCD, total and HMW adiponectin are not major determinants of diet-induced improvement in insulin sensitivity.

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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Effect of diet intervention on adiponectin polymers secretion

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