Expression of Lipolytic Genes in Adipose Tissue Is Differentially Regulated During Multiple Phases of Dietary Intervention in Obese Women

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Summary
The aim of this study was to investigate the time-course of the expression of key lipolysis-regulating genes in the subcutaneous adipose tissue (SCAT) during different phases of a 6-month dietary intervention. Fifteen obese women (BMI 34.7±1.0 kg·m⁻²) underwent a 6-month dietary intervention consisting of 1 month very low calorie diet (VLCD), followed by 2 months low calorie diet (LCD) and 3 months weight maintenance diet (WM). At each phase of the dietary intervention, a needle microbiopsy of the abdominal SCAT was obtained to evaluate mRNA expression of key lipolysis-regulating genes and a hyperinsulinemic euglycemic clamp (HEC) was performed. Dietary intervention induced a body weight reduction of 9.8% and an improvement of insulin sensitivity as assessed by a HEC. Compared to pre-diet levels, mRNA levels of the adrenergic β₂-receptor in SCAT were higher at the end of VLCD and not different at the end of LCD and WM. In contrast, the expression of the adrenergic α₂-receptor was lower at the end of VLCD and LCD compared to the pre-diet levels and did not differ at WM. Adipose triglyceride lipase and hormone-sensitive lipase levels were lower than the pre-diet levels at the end of LCD only, while phosphodiesterase-3B and the insulin receptor levels did not change throughout the dietary intervention. The results suggest that the regulation pattern of the genes that are involved in the control of lipolysis is different at the respective phases of the dietary intervention and depends on the duration of the diet and the status of energy balance.

Key words
Insulin • Adipocytes • Hypocaloric diet

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Introduction
Traditional weight-reducing hypocaloric diets as well as bariatric surgery interventions lead to an improvement in metabolic status of obese subjects already during the early stages of the intervention. It has been suggested that the early improvement in metabolic status is associated with calorie restriction per se – eventually combined with a mild initial weight loss – while the later beneficial metabolic effects are linked to sustained weight loss only (Hensrud 2001, Gumbs et al. 2005). Adipose tissue lipolysis has been suggested as one
of the candidate mechanisms underlying the obesity-related metabolic disturbances, namely insulin resistance (Reynisdottir et al. 1994a,b). Moreover, impaired catecholamine-mediated lipolysis was demonstrated in first-degree relatives of obese subjects (Hellström et al. 1996a) which suggests an association between blunted catecholamine-induced lipolysis and excessive accumulation of body fat.

Therefore, in the present study we aimed to follow the evolution of whole-body insulin sensitivity and the evolution of expression of genes involved in lipolysis regulation in adipose tissue (AT) during a dietary intervention (DI) which consisted of multiple phases. The first phase of the diet consisted of one month severe calorie restriction using a very low calorie diet (VLCD) of 3000 kJ/d. The subsequent phase consisted of 2 months low calorie diet (LCD) with a calorie intake of 5000-6000 kJ, followed by a 3 month weight maintenance diet (WM). This model provides an opportunity to study the “lipolysis – metabolic status – energy status” relationship.

Previous studies that reported mRNA data of genes involved in lipolysis regulation in AT during dietary interventions yielded various results, possibly related to differences in duration and severity of the diet as well as to the characteristics of the study populations. For example, the mRNA and protein levels of hormone-sensitive lipase (HSL) in subcutaneous AT (SCAT) of obese subjects were reduced after 8-12 weeks of diet (Reynisdottir et al. 1995, Jocken et al. 2007), while no differences or an increase in the HSL protein level were reported for dietary interventions with a shorter duration (4 weeks of VLCD) (Hellström et al. 1996b, Stich et al. 1997). Capel et al. (2009) showed that the regulation of adipocyte and macrophage genes during a multi-phase diet was dependent on the phase of the diet, namely the metabolism-related genes predominantly expressed in adipocytes were downregulated during energy restriction and upregulated during weight stabilization, while the genes related to immune functions that were predominantly expressed in macrophages were not changed or upregulated during energy restriction and downregulated during weight stabilization. Similar results were demonstrated regarding the regulation of lipolysis in situ: in obese women it was shown that the responsivenes of SCAT to adrenergic regulation of lipolysis varied during the respective phases of the diet (Koppo et al. 2012).

In the present study, we explored the lipolysis regulation at the transcriptional level and measured mRNA expression of key lipolytic genes in needle biopsy-derived samples of SCAT in a group of obese women that followed the above mentioned multiple-phase dietary intervention. In each individual, the SCAT samples were obtained at baseline and at the end of each dietary phase. At each phase of the diet, the whole-body insulin sensitivity was measured using a hyperinsulinemic euglycemic clamp. We demonstrated that the lipolysis is regulated at the transcriptional level during a multiple-phase dietary intervention and that the regulation during the early phase of the diet differs from that during the later periods. No straightforward relationship between the evolution of gene expression and that of insulin sensitivity was found.

Methods

Subjects
Fifteen obese premenopausal women were recruited for the study (Table 1). They all had a stable weight during the 3 months that preceded the study. Exclusion criteria were hypertension, diabetes, hyperlipidemia treated by drugs, drug-treated obesity, drug or alcohol abuse, pregnancy or participation in other studies. All subjects were fully informed about the aim and the protocol of the study and signed an informed consent approved by the Ethics committee of the Third Faculty of Medicine of the Charles University (Prague, Czech Republic).

Dietary intervention
During the first month of the dietary intervention program, subjects received a 3000 kJ/d VLCD (liquid formula diet; Redita, Promil, Czech Republic). During the next 2 months, a LCD was designed to provide 2500 kJ/d less than the individually estimated energy requirement based on an initial resting metabolic rate multiplied by 1.3 (the coefficient of correction for physical activity level; Toubro et al. 1996.). The final 3 months consisted of a WM diet during which subjects kept a stable weight. Subjects consulted a dietician once a week during the first 3 months and once a month during the WM phase. They provided a written 3-days dietary record at each consultation. Subjects were instructed not to change the level of their leisure time physical activity during the intervention. This was monitored by self-reported questionnaires.
Table 1. Subjects’ characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Pre-diet</th>
<th>VLCD</th>
<th>LCD</th>
<th>WM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>94.9 ± 3.4</td>
<td>87.9 ± 3.2</td>
<td>85.2 ± 3.2</td>
<td>85.6 ± 3.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.7 ± 1.0</td>
<td>32.1 ± 1.0</td>
<td>31.1 ± 1.0</td>
<td>31.3 ± 1.0</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>38.2 ± 2.7</td>
<td>32.7 ± 2.4</td>
<td>29.7 ± 2.0</td>
<td>30.6 ± 2.4</td>
</tr>
<tr>
<td>Fat free mass (kg)</td>
<td>56.8 ± 1.4</td>
<td>55.2 ± 1.5</td>
<td>55.6 ± 1.7</td>
<td>55.1 ± 1.5</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>102.9 ± 3.1</td>
<td>97.2 ± 3.3</td>
<td>95.4 ± 3.3</td>
<td>95.1 ± 3.2</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.8 ± 0.1</td>
<td>4.3 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.10 ± 0.09</td>
<td>0.94 ± 0.07</td>
<td>1.12 ± 0.08</td>
<td>1.22 ± 0.06</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.57 ± 0.1</td>
<td>1.12 ± 0.1</td>
<td>1.11 ± 0.1</td>
<td>1.07 ± 0.1</td>
</tr>
<tr>
<td>NEFAs (µmol/l)</td>
<td>653 ± 45</td>
<td>553 ± 50</td>
<td>515 ± 53</td>
<td>515 ± 53</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.6 ± 0.1</td>
<td>5.4 ± 0.2</td>
<td>5.5 ± 0.1</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Fasting insulin (mU/l)</td>
<td>12.4 ± 2.0</td>
<td>6.7 ± 0.6</td>
<td>5.6 ± 0.6</td>
<td>7.3 ± 0.7</td>
</tr>
<tr>
<td>GDR (mg/kg.min)</td>
<td>3.1 ± 0.4</td>
<td>3.9 ± 0.5</td>
<td>4.3 ± 0.5</td>
<td>4.3 ± 0.4</td>
</tr>
</tbody>
</table>

BMI: body mass index, HDL: high-density lipoprotein, NEFAs: non-esterified fatty acids and GDR: glucose disposal rate normalized to kg of body weight. * = significantly different from pre-diet values (P<0.05), ** = significantly different from VLCD (P<0.05), *** = significantly different from LCD (P<0.05).

Experimental protocol

On four occasions (i.e. before the start of the dietary intervention (baseline) and at the end of the VLCD, LCD and WM phase, respectively) subjects entered the laboratory at 8.00 a.m. after an overnight fast. A complete clinical investigation was performed, anthropometric parameters were measured and body composition was determined with multifrequency bioimpedance (Bodystat QuadScan 4000; Bodystat Ltd., Isle of Man, British Isles). Subsequently, subjects were placed in a semi-recumbent position. Venous blood samples were taken from an antecubital vein in order to determine the blood parameters related to lipid and carbohydrate metabolism. A needle micro biopsy of the abdominal SCAT (14-20 cm lateral to the umbilicus) was performed under local anesthesia (1% Xylocaine; AstraZeneca PLC, London, UK) as previously described (Klimcakova et al. 2006) to evaluate mRNA of key genes that are involved in SCAT lipolysis regulation. The samples were frozen immediately in liquid nitrogen and stored at –80 °C until analysis. Finally, a 3-h hyperinsulinemic euglycemic clamp (HEC) was performed to determine the glucose disposal rate.

Hyperinsulinemic euglycemic clamp

HEC was performed according to the DeFronzo method (DeFronzo et al. 1979). Priming plus continuous infusion of crystalline human insulin (Actrapid Human; Novo, Bagsvaerd, Denmark), 40 mU/m² body area · min, was given for 180 min. Euglycemia (the fasting blood glucose concentration) was maintained by a variable 20 % glucose infusion. The infusion rate was determined by measuring arterialized plasma glucose every 5 min (Beckman Glucose Analyzer; Beckman Coulter Inc., Fullerton, CA, USA).

Quantification of mRNA analysis

Total RNA was extracted from the adipose tissue biopsy samples with an RNeasy Mini kit (Qiagen, Valencia, CA) and reverse transcribed using random hexamers (Promega Corp., Madison, WI) and SuperScript II Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). Real-time quantitative PCR was performed with TaqMan probe-based gene expression assays (Applied Biosystems, Foster City, CA). An 18S ribosomal RNA was used as control to normalize gene expression (Ribosomal RNA Control TaqMan Assay kit; Applied Biosystems). Each sample was performed in duplicate and 10 ng cDNA was used as a template for real-time PCR. The relative expression was calculated using the ΔΔCt method (Livak and Schmittgen 2001).

Drugs and biochemical determinations

Plasma glucose was determined with a glucose hexokinase technique (Konelab 60i, Labsystems CLD, Konelab, Finland). Plasma insulin concentrations were
measured using a chemiluminescent immunometric assay (Immulite 2000 Insulin, DPC Czech sro, Brno, Czech Republic). Other laboratory analyses (lipids) were performed using standard biochemical laboratory methods.

**Statistical analysis**

All values are presented as means ± SEM. Statistical evaluation of the data was performed using ANOVA for repeated measures with an LSD post-hoc test (SPSS statistical software, version 19, SPSS Inc, Chicago). Correlations between mRNA gene expression and the diet-induced change in body fat mass were examined with the Pearson correlation coefficient. Significance was determined at P<0.05.

**Results**

**Anthropometric and plasma parameters of subjects during the different phases of the diet**

Pre-diet values and values measured at different phases during the weight reduction program (i.e. at the end of VLCD, LCD and WM) are reported in Table 1. Subjects’ body weight progressively decreased during VLCD and LCD and stabilized during WM. The loss in body weight was mainly due to a decrease in fat mass. Plasma triglycerides were significantly lower at the end of VLCD, LCD and WM compared to the pre-diet values. Similarly, baseline insulin levels were significantly lower at the end of VLCD, LCD and WM (Table 1). The glucose disposal rate (GDR) normalized to body weight significantly increased at the end of VLCD and the increase was maintained at the end of LCD and WM (Table 1).

**Effect of the dietary intervention on gene expression**

SCAT mRNA levels of key genes involved in adipose tissue lipolysis regulation, i.e. the adrenergic β2-receptor (ADRB2), adrenergic α2A-receptor (ADRA2A), adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), phosphodiesterase-3B (PDE3B) and insulin receptor (INSR), were quantified (Table 2). ADRB2 expression significantly increased at the end of VLCD when compared with the pre-diet baseline level and then, at the end of LCD and WM, returned to the pre-diet baseline levels. The expression of ADRA2A was significantly lower at the end of VLCD and LCD compared to the pre-diet baseline and increased back towards the pre-diet levels during WM. Expression of PDE3B and INSR did not significantly change throughout the intervention. The diet-induced adaptation of HSL and ATGL was similar: mRNA levels of the two lipases were significantly lower at the end of LCD compared with pre-diet levels and returned to the pre-diet levels at the end of WM. At the end of VLCD, ATGL levels were lower than the pre-diet levels but the difference was not significant (P=0.076).

**Associations between the diet-induced changes of clinical variables and mRNA levels**

No significant correlations between the pre-diet baseline mRNA expression of the genes explored and the diet-induced change in body fat mass during VLCD were observed: r = 0.076 for ADRA2A, r = –0.159 for ADRB2, r = –0.001 for PDE3B, r = –0.147 for INSR, r = –0.266 for HSL and r = –0.174 for ATGL. Similarly, no significant correlations between the pre-diet baseline mRNA expression of the genes and the diet-induced change in body fat mass during the entire 6-month intervention were observed: r = –0.018 for ADRA2A,
**Discussion**

Results of the present study demonstrate that multi-phase dietary intervention modifies the expression of key genes involved in SCAT lipolysis control and that the status of energy balance during the dietary intervention is an important determinant of the lipolysis genes regulation. These findings are in agreement with previous research in humans (Vítkova et al. 2007, Capel et al. 2009, Kováčiková et al. 2010) and in experimental models (Kosteli et al. 2010) that showed that the diet-induced changes of adipose tissue expression of genes related to metabolism or immune function are strongly dependent on the phase of the diet.

One of the regulators of lipolysis that received substantial attention in dietary intervention studies are catecholamines as they are believed to be one of the major players that regulate lipid mobilization in adipose tissue. Most of the studies that examined the lipolytic response to β-adrenergic stimulation after 3-12 weeks VLCD reported an increased lipolytic response *in situ* (Barbe et al. 1997, Flechtner-Mors et al. 1999, Sengenes et al. 2002) and *in vitro* (Stich et al. 1997, Wahrenberg et al. 1999). After 15-weeks LCD, the maximal lipolytic response to isoprenaline (a β-adrenoceptor agonist) was unchanged when compared to the pre-diet level, while the β-adrenoceptor lipolytic sensitivity was increased in isolated adipocytes (Mauriège et al. 1999). The α2-adrenoceptor mediated anti-lipolytic action in isolated adipocytes was found to be unchanged after 4-12 weeks VLCD (Kather et al. 1985, Stich et al. 1997, Wahrenberg et al. 1999) and to be reduced after 12-15 weeks LCD *in vitro* (Mauriège et al. 1999) and *in situ* (Stich et al. 2002). Results of the above mentioned single-phase dietary intervention studies were in agreement with a recent study in which the SCAT lipolysis was examined during a long-term multi-phase diet (Koppo et al. 2012). The authors reported that the adrenaline-induced SCAT lipolysis *in vivo* was enhanced during the calorie-restricted phases of the diet and returned back to the pre-diet levels during the WM phase. The diet-induced changes in SCAT lipolysis were rather related to a decreased α2-adrenergic regulation than to an increased β-adrenergic regulation (Koppo et al. 2012). Changes in postreceptor signaling pathways as well as changes in density of the adrenoceptors should be considered as possible mechanisms that underlie the changes in lipolytic responsiveness during the dietary intervention. It was found that in SCAT of obese women the β-adrenoceptor density increased after VLCD compared with the pre-diet levels, while the α2-adrenoceptor density remained unchanged (Mauriège et al. 1999, Wahrenberg et al. 1999). On the transcriptional level, it was reported that, after 12 weeks of LCD, SCAT ADRA2A mRNA levels decreased and ADRB2 mRNA levels did not change compared with the pre-diet condition in obese women (Stich et al. 2002). The present study investigated the changes in adrenoceptor gene expression during different phases of the dietary intervention in the same individual. The lipolytic ADRB2 gene was up-regulated after VLCD, and not after LCD and WM, while the anti-lipolytic ADRA2A gene was down-regulated after VLCD and LCD. After WM, the adrenoceptor mRNA levels returned back to the pre-diet values. Although there are many intermediate steps between the adrenergic regulation of SCAT lipolysis *in vivo* and the transcriptional level of the adrenoceptor gene expression, these findings parallel the results of our previous study mentioned above (Koppo et al. 2012) in which adrenergic regulation of lipolysis was investigated *in situ* using microdialysis. Indeed, in that study, the α2-mediated antilipolytic action was reduced *in situ* at VLCD and LCD, similarly to the evolution of ADRA2A mRNA levels. In contrast, the β-adrenoceptor-mediated stimulation of lipolysis *in situ* increased at VLCD and went back to pre-diet levels, similarly to ADRB2 mRNA levels.

Another important lipolysis-regulating pathway is that of insulin. In the present study mRNA levels of PDE3B and INSR were explored. The anti-lipolytic effect of insulin is mediated through activation of PDE3B; more specifically, insulin activates phosphatidylinositol 3-kinase serine kinase, the kinase that activates PDE3B,
through binding to INSR (Rondinone et al. 2000). The activation of PDE3B promotes cAMP degradation, which in turn inactivates protein kinase A (PKA) that reduces phosphorylation of lipases and perilipins. In the present study, no changes in mRNA levels of PDE3B and INSR were observed throughout the dietary intervention. It cannot be excluded that a change in the activation of PDE3B might have occurred. Nevertheless, in situ data also suggest a lack of change in insulin sensitivity of adipose tissue during this dietary intervention: it was reported that, during a hyperinsulinemic euglycemic clamp performed at different time points of a multi-phase diet, the decrease in SCAT diacylglycerol was similar at the respective phases of the diet. This demonstrated that the anti-lipolytic effect of insulin did not change throughout the dietary intervention during which subjects’ whole-body insulin sensitivity improved (Koppo et al. 2012).

In the present study we also focused on the two lipases that are involved in the lipolytic pathway. ATGL and HSL are cytosol lipases that hydrolyze intracellular triacylglycerols (TAGs) into glycerol and NEFA. HSL has been considered to be the enzyme that catalyzes the rate-limiting step of adipose tissue lipolysis. However, more recently it was reported that the overexpression of ATGL may increase basal lipolysis while its inhibition suppresses stimulated lipolysis (Bezaire et al. 2009). Regarding the factors that influence HSL gene expression, it was reported that glucose deprivation (Raclot et al. 1998) as well as a sustained activation of the PKA pathway (Plée-Gautier et al. 1996) down-regulated the HSL gene. Taking into account that during the calorie-restricted phases of the diet, glucose intake is limited and the stimulated SCAT lipolysis (and thus the activation of the PKA pathway) is enhanced, it can be hypothesized that the HSL gene would be down-regulated during the calorie-restricted phases. Indeed, mRNA levels of HSL were significantly lower at the end of LCD compared with pre-diet levels. Interestingly, the pattern of evolution of ATGL mRNA levels during this dietary intervention paralleled the evolution of basal lipolysis in situ observed in our previous microdialysis study (Koppo et al. 2012). In contrast, no such parallel was found for the pattern of evolution of mRNA HSL: it was different when compared with either basal lipolysis or adrenaline-stimulated lipolysis in situ.

As mentioned above, results of this study and of previous ones show that the diet-induced changes of adipose tissue gene expression are dependent on the phase of the diet. Capel et al. (2009) showed that the regulation of adipocyte and macrophage genes during a multi-phase diet was dependent on the phase of the diet. The metabolism-related genes (predominantly expressed in adipocytes) were downregulated during energy restriction and upregulated during weight stabilization, while the genes related to immune functions (predominantly expressed in macrophages) were not changed or upregulated during energy restriction and downregulated during weight stabilization (Capel et al. 2009). Furthermore, recent studies showed the dependence of lipolysis on the phase of the hypocaloric diet in mice (Kosteli et al. 2010) and in humans (Koppo et al. 2012). In the former study (Kosteli et al. 2010) it was suggested that lipolysis drives the adipose tissue macrophage accumulation since the peak in macrophage number coincided with the peak in adipose tissue lipolysis. Furthermore, after an extended period of weight loss, adipose tissue lipolysis decreased, as did the macrophage content. It would be tempting to hypothesize that the same regulatory effect could occur in humans. It was reported that markers of macrophage infiltration in human SCAT show a similar time-pattern, i.e. a slight or no increase during one month of VLCD and a decrease under the pre-diet levels at the end of a 6 month diet (Capel et al. 2009, Kováčiková et al. 2010). This would suggest that the biological role of SCAT lipolysis goes beyond serving as an energy substrate and that it may play a role in the regulation of macrophage infiltration and the inflammation of the adipose tissue.

Similarly to the studies of Mutch et al. (2007, 2011) it might be hypothesized that the baseline levels of the lipolytic genes or their diet-induced changes are
predictors of the diet-induced loss in fat mass. We tested specifically whether subjects with higher “responsiveness”, i.e. larger modifications of mRNA levels of the respective genes during the initial phase of the diet (i.e. VLCD), would be those with higher diet-induced fat loss either during VLCD or during the entire 6-month intervention. This association was not confirmed in the present study. Similarly, no associations between the pre-diet mRNA expression of lipolysis-regulating genes and the diet-induced loss in fat mass during either VLCD or entire intervention were observed.

In conclusion, this study demonstrates that the dietary intervention associated with an improvement in insulin sensitivity modifies the mRNA expression of key genes that are involved in SCAT lipolysis regulation. The pattern of this diet-induced modification varies during the course of the diet and is related to the status of energy balance. No straightforward relationship between the evolution of expression of the respective genes and that of insulin sensitivity was found.

Conflict of Interest
There is no conflict of interest.

Acknowledgements
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References


