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Serum concentrations and tissue expression of components of insulin-like growth factor-axis in females with type 2 diabetes mellitus and obesity: The influence of very-low-calorie diet

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ABSTRACT

We explored serum concentrations and mRNA expression of insulin-like-growth factor-1 (IGF-1) axis components in subcutaneous adipose tissue (SCAT) and peripheral monocytes (PM) of 18 healthy females, 11 obese non-diabetic females (OB) and 13 obese women with type 2 diabetes (T2DM) examined at baseline and after very-low-calorie diet (VLCD). T2DM women had decreased expression of IGF-1, IGF-1 receptor (IGF-1R), IGFBP-2 (IGF binding protein-2) and IGFBP-3 in SCAT and increased expression of IGF-1R in PM compared to control group. IGF-1R and IGFBP-3 mRNA expression in SCAT of OB was comparable to control group. In T2DM women VLCD increased serum levels and SCAT expression of IGFBP-2 and PM expression of IGFBP-3. We conclude that decreased IGF-1, IGF-1R and IGFBP-3 expression in SCAT and increased IGF-1R expression in PM of T2DM subjects might contribute to changes of fat differentiation capacity and to regulation of subclinical inflammation by PM, respectively. Increased SCAT and circulating IGFBP-2 and IGFBP-3 in PM might participate in metabolic improvements after VLCD.

1. Introduction

Previous studies have shown that type 2 diabetes and prediabetic states are associated with altered circulating levels of IGF-1 and its binding proteins (IGFBPs) (Rajpathak et al., 2009). Most of cross-sectional studies have found that free IGF-1 levels are elevated in patients with impaired glucose tolerance and T2DM, while total IGF-1 levels are within normal range (Frystyk et al., 1999) or slightly decreased (Rasmussen et al., 2006). IGFBP-1 levels were low in these patients (Dunger et al., 2004). Furthermore, increased circulating IGFBP-3 may be a risk factor for insulin resistance and T2DM (Rajpathak et al., 2009). In contrast to changes of circulating levels of IGF-1 and its binding proteins, little is known about the relationship between tissue mRNA expression of IGF-1 axis components and the disturbances in glucose metabolism which are closely interconnected with the development of subclinical inflammation.

In previous studies, low-grade subclinical inflammation arising in adipose tissue in obesity has been proposed to play an important role in the development of insulin resistance and diabetes (Wellen et Hotamisligil, 2003; Xu et al., 2003; Hotamisligil, 2006) with both adipocytes as well as adipose tissue macrophages contributing to this inflammatory process (Mraz et al., 2011; del Pozo et al., 2011). It has been suggested that most of the adipose tissue macrophages in fat are derived from circulating monocytes recruited to adipose tissue by various chemoattractants (Cancello et al., 2005). Our previous study has shown that the interplay between adipose tissue and circulating monocytes may be critically important in the development of the systemic subclinical inflammation and type 2 diabetes (Mraz et al., 2011). In the present work we therefore tried to explore, whether IGF-1 axis components contribute to dysregulation of glucose metabolism and the development of subclinical inflammation on the level of mRNA expression in peripheral monocytes and subcutaneous adipose tissue.

Previously, IGF-1 has been suggested as an important regulator of glucose and lipid metabolism in humans (Frystyk et al., 1997), a mediator of hormonal effects on the immune system (Koojiman et al., 1992b) and a potential modulator of responses to a variety

Abbreviations: VLCD, very-low-calorie diet; IGF-1, insulin-like growth factor-1; IGFBP, insulin-like growth factor binding protein; IGF-1R, insulin-like growth factor-1 receptor; PM, peripheral monocytes; SCAT, subcutaneous adipose tissue; T2DM, type 2 diabetes mellitus; GH, growth hormone.

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of immunological challenges (Stuart et al., 1991). Several studies have shown that IGF-1 has a regulatory role in inflammatory processes (Kluge et al., 1997; Che et al., 2002) and is, together with other IGF axis components, implicated in the regulation of adipocyte differentiation and lipid accumulation in vitro (Kloting et al., 2008).

In the current study, we hypothesized that IGF-1 axis components might have a role in the interplay between adipose tissue and peripheral monocytes in the development of subclinical inflammation in obesity and type 2 diabetes mellitus and might contribute both to underlying metabolic disturbances in T2DM patients and the positive metabolic effect of dietary intervention. To this end, we measured serum levels and subcutaneous adipose tissue (SCAT) and peripheral monocytes (PM) mRNA expression of selected components of the IGF-axis in obese non-diabetic and obese type 2 diabetic patients at baseline and studied its modulation by short-term dietary intervention in patients with type 2 diabetes.

2. Materials and methods

2.1. Study subjects

Thirteen obese women with type 2 diabetes mellitus (T2DM group), 11 obese non-diabetic females (OB group) and 18 healthy normal-weight age-matched women (C group) were included in the study. Because of possible gender-related differences in the studied factors that might affect the results, only one gender was selected for investigation. The reason for choosing females over males was that the proportion of females in our Obesity and Diabetes Department is higher than males, which made patient recruitment easier.

Five out of thirteen T2DM patients had been treated with oral antidiabetic drugs (metformin, sulphonylurea derivatives or thiazolidinediones), 4 were treated with insulin and the rest by diet only. The antidiabetic treatment remained unchanged for at least three months prior to the start of the study.

Twelve out of thirteen diabetic patients were on antihypertensive treatment, 5 patients were treated by statins, one by fibrate. 3 patients received thyroid hormone substitution therapy. None of the patients had acute decompensation of diabetes**,** malignant tumor or acute infectious disease. In the obese non-diabetic group, 4 patients were treated with antihypertensives and one of them was on lipid-lowering medication with a statin. Control subjects had no history of obesity and/or diabetes mellitus, arterial hypertension or lipid metabolism disturbances and received no medication. Blood tests confirmed normal blood count, biochemical and hormonal parameters.

Both T2DM subjects and non-diabetic OB subjects were consecutively hospitalized in the 3rd Department of Medicine, General University Hospital, Prague for planned intensive in-hospital intervention aimed at body weight reduction using very-low-calorie diet and complex reeducation of the patients. All T2DM patients underwent a 2-week very-low-calorie diet (VLCD) with energy intake of 2500 kJ/day (600 kcal/day). The diet consisted of 50 g (33%) of saccharides (15–20 g of mono- and disaccharides, 30–35 g of polysaccharides), 20 g (31%) of lipids (6–8 g of saturated fatty acids, 12–14 g of unsaturated fatty acids) and 55 g of proteins (36%) per day. The diet contained 20 g of fiber per day.

The body weight of all study participants remained stable for at least 3 months before the beginning of the study. Written informed consent was signed by all participants before being enrolled in the study. The study was approved by Human Ethical Review Committee, 1st Faculty of Medicine and General University Hospital, Prague, Czech Republic, and was performed in accordance with the guidelines proposed in Declaration of Helsinki.

2.2. Anthropometric examination, blood and adipose tissue sampling

All patients with type 2 diabetes mellitus were examined twice; at basal state before the beginning of any intervention and after two weeks of VLCD, while obese non-diabetic and normal-weight healthy women were examined only once. All subjects were measured and weighted and their body mass index (BMI) was calculated. Percentage of total body fat was examined by bioimpedance analysis (Multi-frequency Bodystat QuadScan 4000, Douglas, UK) at body current flow of 5, 50, 100 and 200 kHz, respectively. Blood samples for IGF-1, IGFBPs, insulin, and biochemical parameters measurement were withdrawn between 07:00 and 08:00 h after 12 h of overnight fasting. Blood samples were separated by centrifugation for 10 min at 1000*g* within 30 min from blood collection. Serum was subsequently stored in aliquots at −80 °C until further analysis.

Blood samples for monocyte isolation were collected in Na-EDTA anticoagulant from all subjects and processed within 1–2 h.

Samples of subcutaneous adipose tissue for mRNA expression analysis were obtained from the abdominal region as described previously elsewhere (Dolezalova et al., 2007). Approximately 100 mg of tissue was collected to 1 ml of RNA stabilization Reagent (RNA later, Qiagen, Germany) and stored at –80 °C until further analysis.

2.3. Hormonal and biochemical assays

Serum levels of total insulin-like growth factor-1 (IGF-1) were measured by IRMA kit (Immunotech, Prague, Czech Republic). LOD (Limit of detection) and LOQ (Limit of quantitation) were 12.0 ng/ml. Plasma levels of free insulin-like growth factor-1 (Free IGF-1) were measured by ELISA kit (Diagnostic Systems Laboratories, Inc., Webster, USA). LOD and LOQ was 0.015 ng/ml. Serum IGFBP-1, IGFBP-2 and IGFBP-3 levels were measured by ELISA kits (DiaSource ImmunoAssays S.A., Nivelles, Belgium). LOD was 0.4 ng/ml for IGFBP-1, 0.2 ng/ml for IGFBP-2 and 10 ng/ml for IGFBP-3. LOQ was 6.4 ng/ml for IGFBP-1, 4.2 ng/ml for IGFBP-2 and 10 ng/ml for IGFBP-3. Growth hormone (GH) levels were measured by IRMA kits (Immunotech, Prague, Czech Republic). LOD and LOQ were 0.10 mIU/l. Insulin concentrations were measured by RIA kit (Cis Bio International, Gif-sur-Yvette, France). LOD and LOQ were 2.0 μIU/ml. Serum adiponectin levels were measured by commercial ELISA kit (Linco Research, St. Charles, Missouri, USA). LOD was 0.78 ng/ml and LOQ was 390 ng/ml. Serum leptin concentrations were measured by commercial ELISA kit (Biovendor, Brno, Czech Republic). LOD and LOQ were 0.2 ng/ml. Serum C-reactive protein (CRP) levels were measured by high sensitive ELISA (Bender Medsystems, Vienna, Austria) with a LOD of 3 pg/ml and LOQ 1.5 ng/ml. Serum estradiol concentrations were measured by chemiluminescent microparticle immunoassay (CMIA) technology, referred to as ChemiflexTM (ABBOTT Laboratories, IL 60064 USA). LOD and LOQ were 0.04 nmol/l.

The intra- and interassay variabilities for all methods were less than 5.0 and 10.0%, respectively.

Biochemical parameters (glucose, total and HDL-cholesterol and triglycerides) were measured in the Department of Biochemistry of General University Hospital, Prague by standard laboratory methods. The value of LDL-cholesterol was calculated according to Friedewald formula.

Glycated hemoglobin was analyzed by high performance liquid chromatography (HPLC) on Variant II BioRad analyzer (BioRad).

2.4. Peripheral monocyte separation and total RNA isolation from monocytes and adipose tissue

Peripheral blood leukocytes were obtained from blood sample using Ficoll-Paque™ Plus (Amersham Biosciences AB, Sweden).

Monocytes were isolated from cell pellet with magnetic activated cell sorting technique (MiniMacs Miltenyi Biotec, Germany) using microbeads coated with CD14 antibody (MACS CD14 MicroBeads; Miltenyi Biotec, Germany). Total RNA was extracted from CD14 + monocyte samples on MagNA Pure instrument using MagNA Pure Compact RNA Isolation kit (Roche Diagnostics GmbH, Germany) as described in detail previously elsewhere (Mraz et al., 2011).

Samples of subcutaneous adipose tissue were homogenized on a MagNA Lyser Instrument using MagNA Lyser Green Beads (Roche Diagnostics GmbH, Germany). Total RNA was extracted from the homogenized sample using RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Hilden, Germany) as described in detail previously (Lacinova et al., 2008). The RNA concentration was determined from absorbance at 260 nm on a BioPhotometer (Eppendorf AG, Hamburg, Germany). The integrity of the RNA was checked by visualization of 18S and 28S ribosomal bands on 1% agarose gel with ethidium bromide.

2.5. Determination of mRNA expression by quantitative real-time PCR (qRT-PCR)

Total RNA was used for reverse transcription to synthesize the first strand cDNA. Reverse transcription was performed using 0.25 μg of total RNA to synthesize the first strand cDNA using the random primers as per the instructions of the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Further cDNA was used for determination of relative gene expression of IGF-1, IGF-2, IGF-1R, IGFBP-1, IGFBP-2 and IGFBP-3 by qRT-PCR using TaqMan®Universal PCR MasterMix, NO AmpErase® UNG and specific TaqMan Gene expression Assays (Applied Biosystems, Foster City, CA). The catalogue numbers for the assays were as follows: IGF-1 (Hs01547656_m1), IGF-2 (Hs01005963_m1), IGF-1R (Hs99999020_m1), IGFBP-1 (Hs00426285_m1), IGFBP-2 (Hs01040719_m1) and IGFBP-3 (Hs00426289_m1). Measurements of gene expression were performed on an ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA). Forty-five cycles were run with the following parameters: 2 min at 50 °C, 10 min at 95 °C and for each cycle 15 s at 95 °C for denaturation and 1 min at 60 °C for elongation. Controls with no template cDNA were performed with each assay and all samples were run in duplicate at a minimum. The increase in fluorescence was measured in real time and threshold cycle (Ct) values were obtained. To compensate for variations in the amount of RNA used and the variable efficiency of reverse transcription, the target gene Ct number was normalized to the endogenous reference beta-2-microglobulin (B2M) and the formula $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{Target} - Ct_{B2M}$) was used to calculate relative gene expression. Quantification of mRNA was performed as described in detail previously elsewhere (Dolezalova et al., 2007).

2.6. Statistical analysis

Statistical analysis was performed on SigmaStat software (SPSS Inc., Chicago, IL). Anthropometric, biochemical, hormonal and gene expression results are expressed as means ± SEM (standard error of the means). Differences of serum parameters and gene expression between obese with/without T2DM and non-obese were evaluated using One-Way ANOVA followed by Holm-Sidak method or ANOVA on Ranks followed by Dunn's test as appropriate. Differences between obese women with T2DM before and after VLCD were evaluated using paired *t*-test or Wilcoxon Signed-Rank test as appropriate. Statistical significance was assigned to $p < 0.05$.

Spearman correlation test was used to calculate the relationships between serum IGF-1, IGFBP-1, IGFBP-2 levels, relative gene expression of IGF-1, IGFBP-3, IGF-1R in adipose tissue or relative gene expression of IGF-1R, IGFBP-3 in peripheral monocytes and other parameters. A p value < 0.05 denoted statistical significance.

3. Results

3.1. Clinical, hormonal and metabolic characteristics of study subjects

Anthropometric, biochemical and hormonal characteristics of all study groups are summarized in Table 1. As expected, body weight, BMI, waist circumference and % of total body fat, circulating CRP and leptin levels were increased in both OB and T2DM patients relative to control group. On the contrary, circulating adiponectin levels were decreased in both OB and T2DM subjects compared to control group. Serum concentrations of insulin, glucose, HbA1c, triglycerides and HOMA-IR index were significantly increased and serum HDL-cholesterol levels were decreased in T2DM patients compared with control group. Serum levels of total and LDL-cholesterol did not differ among the groups (data not shown). Estradiol levels did not significantly differ among the groups. Serum levels of total IGF-1, IGFBP-1 and GH were significantly lower in obese women both with and without T2DM compared to controls. IGFBP-2 level was significantly decreased in obese women without diabetes, while in obese diabetic women IGFBP-2 level did not significantly differ from the control group. Similarly, levels of IGFBP-3 and free IGF-1 did not significantly differ among the groups.

3.2. Clinical, hormonal and metabolic characteristics of T2DM patients after VLCD

The influence of VLCD on anthropometric, biochemical and hormonal parameters is summarized in Table 1. Two weeks of VLCD in T2DM subjects significantly reduced BMI, body weight, waist circumference, HOMA-IR, levels of glucose, CRP, total cholesterol (data not shown), leptin and further reduced serum total IGF-1 concentrations. On the contrary, IGFBP-2 levels significantly increased after VLCD. Serum insulin, HDL-cholesterol, LDL-cholesterol, triglycerides, adiponectin as well as free IGF-1, IGFBP-1 and-3 levels were not significantly affected by VLCD. HbA1c was not assessed after VLCD.

3.3. mRNA expression of IGF-axis components in peripheral monocytes and subcutaneous adipose tissue

In peripheral monocytes, mRNA expression of IGF-1R was significantly increased in T2DM women compared to control group (Fig. 1A) and did not differ between OB and control group. IGFBP-3 and IGFBP-2 mRNA expression in PM did not significantly differ among the groups (data for IGFBP-2 not shown). mRNA expression of IGF-1, IGF-2 and IGFBP-1 was not detected in PM in any of the studied groups. Two weeks of VLCD did not change the IGF-1R and IGFBP-2 mRNA expression. After VLCD the IGFBP-3 mRNA expression in T2DM group became significantly higher compared to control and obese non-diabetic group (Fig. 1B).

In subcutaneous adipose tissue, IGF-1 and IGFBP-2 mRNA expression was decreased in T2DM women compared to control group (Fig. 2A and C) and IGF-1R and IGFBP-3 mRNA expression was significantly decreased in T2DM women compared to control and obese non-diabetic group (Fig. 2B and D). After VLCD, IGF-1, IGF-1R and IGFBP-3 expression did not change, whereas IGFBP-2 mRNA expression increased (Fig. 2C). mRNA expression of IGFBP-1 was not detected in SCAT. IGF-2 expression did not differ among the groups and was not affected by VLCD (data not shown).

3.4. Relationship of circulating IGF-axis components and its expression in PM and SCAT to other parameters

The relationship of circulating IGF-axis components and its mRNA expression in PM and SCAT to other parameters was

Table 1
Clinical, hormonal and metabolic characteristics of study subjects.

	Controls	Obese non-diabetic	Obese T2DM	
			Before VLCD	After VLCD
Number (n)	18	11	13	13
Age (years)	49.5 ± 2.3	48.7 ± 3.0	54.2 ± 2.0	54.2 ± 2.0
Body mass index (kg/m^2)	23.8 ± 0.4	45.0 ± 3.5*	51.8 ± 2.5*	49.1 ± 2.3*°
Body weight (kg)	67.9 ± 1.6	124.8 ± 7.7*	138.3 ± 6.3*	128.8 ± 5.9*°
Waist circumference (cm)	81 ± 2	126 ± 5*	137 ± 5*	133 ± 5*°
CRP (mg/l)	0.27 ± 0.10	1.57 ± 0.30*	2.44 ± 0.59*	1.55 ± 0.44*°
Fasting insulin (mIU/l)	18.7 ± 1.1	28.3 ± 4.0	30.8 ± 3.4*	27.3 ± 3.9
Fasting blood glucose (mmol/l)	4.77 ± 0.09	5.08 ± 0.27	8.35 ± 1.16*	6.45 ± 0.68*°
HbA1c (% IFCC)	3.58 ± 0.13	4.43 ± 0.39	6.58 ± 0.53*	Not assessed
HOMA-IR index	3.97 ± 0.26	6.45 ± 1.02	12.07 ± 1.93*	8.75 ± 1.43*°
HDL cholesterol (mmol/l)	1.67 ± 0.09	1.29 ± 0.15	1.04 ± 0.04*	0.95 ± 0.06*
Triglycerides (mmol/l)	1.19 ± 0.14	1.73 ± 0.36	1.91 ± 0.18*	1.68 ± 0.16
Leptin (ng/ml)	16.4 ± 2.6	56.1 ± 3.9*	60.8 ± 10.1*	52.4 ± 10.4*°
Adiponectin (mg/l)	13.2 ± 1.3	8.4 ± 1.1*	7.9 ± 1.3*	7.5 ± 0.8*
Estradiol (nmol/l)	0.24 ± 0.07	0.09 ± 0.01	0.22 ± 0.07	Not assessed
Free IGF-I (ng/ml)	1.06 ± 0.22	0.72 ± 0.21	1.04 ± 0.12	0.89 ± 0.12
Total IGF-I (μg/l)	200 ± 26	111 ± 23*	143 ± 14*	116 ± 13*°
IGFBP-1 (μg/l)	7.03 ± 1.18	2.13 ± 0.52*	2.25 ± 0.50*	2.50 ± 0.79
IGFBP-3 (mg/l)	2.95 ± 0.12	3.14 ± 0.21	2.86 ± 0.18	2.78 ± 0.15
IGFBP-2 (ng/ml)	344 ± 44	178 ± 36*	232 ± 30	259 ± 23°
GH (mIU/l)	14.18 ± 3.73	1.00 ± 0.33*	0.99 ± 0.23*	0.99 ± 0.20*

T2DM: type 2 diabetes mellitus, VLCD, very-low-calorie diet, IFCC, International Federation of Clinical Chemistry; IGF-1, insulin-like growth factor-1; IGFBP, insulin-like growth factor binding protein; GH, growth hormone.
Values are means ± SEM. Statistical significance is from One Way ANOVA or ANOVA on ranks as appropriate. Differences between obese women with T2DM before and after VLCD were evaluated using paired *t*-test or Wilcoxon Signed-Rank test as appropriate. Statistical significance was assigned to $p < 0.05$.
*$p < 0.05$ vs. non-obese controls, °$p < 0.05$ vs. T2DM before VLCD.
NS, non-significant.

assessed in a combined population of healthy controls and obese subjects both with and without T2DM. Serum concentrations of IGF-1, IGFBP-1 and IGFBP-2 correlated inversely with BMI, serum insulin levels and HOMA index ($p < 0.014$). IGF-1, IGF-1R and IGFBP-3 mRNA expression in SCAT correlated inversely with BMI and glycaemia ($p < 0.04$). Moreover, IGF-1 and IGF-1R mRNA expression in SCAT negatively correlated with serum insulin levels and HOMA index ($p < 0.04$). IGF-1R and IGFBP-3 mRNA expression in PM positively correlated with BMI, glycaemia and HOMA index ($p < 0.045$). Serum insulin concentrations correlated positively with IGF-1R mRNA expression in PM ($p < 0.045$).

4. Discussion

Previous studies documented the complex interactions between circulating monocytes and adipose tissue in the development of systemic subclinical inflammation, type 2 diabetes mellitus and its atherosclerotic complications (Mraz et al., 2011; Bourlier et Bouloumie, 2009; Suganami et al., 2005). Significantly increased IGF-1R mRNA expression in monocytes in T2DM patients relative to both OB and control groups documented in this study suggests a possible contribution of IGF-1 axis components to the regulation of peripheral monocytes inflammatory status in T2DM patients. Previously it has been reported that IGF-1 was involved in inflammation-induced angiogenic processes in the porcine model of sterile heart inflammation (Kluge et al., 1997) and in the development of endothelial cell dysfunction by enhancing pro-inflammatory cytokine signal transduction (Che et al., 2002). Positive effects of IGF-1 have been described on the stimulation of natural killer cell cytotoxicity and proliferation of human peripheral blood mononucelar cells (Koojiman et al., 1992a) as well as on the maturation and inhibition of apoptosis of cord blood monocyte-derived dendritic cells (Law et al., 2008). Moreover, in the presence of IGF-1 monocytes isolated from GH-deficient patients differentiated into macrophages (Serri et al., 2004). These findings suggest that IGF-1 has a more general role in the regulation of inflammatory and

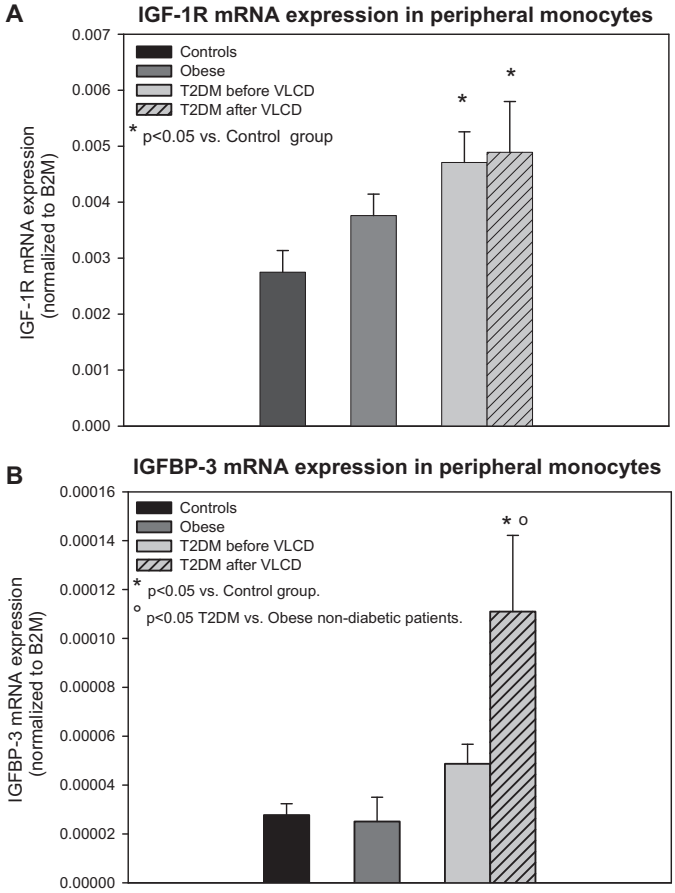

Fig. 1. mRNA expression of IGF-1R (A) and IGFBP-3 (B) in peripheral monocytes of control lean women (black bars; $n = 18$), obese non-diabetic women (dark gray bars; $n = 11$) and obese women with T2DM before VLCD (gray bars) and obese women with T2DM after VLCD (shaded gray bars; $n = 13$). Values are means ± SEM. *$p < 0.05$ vs. control group. °$p < 0.05$ T2DM vs. obese non-diabetic patients.

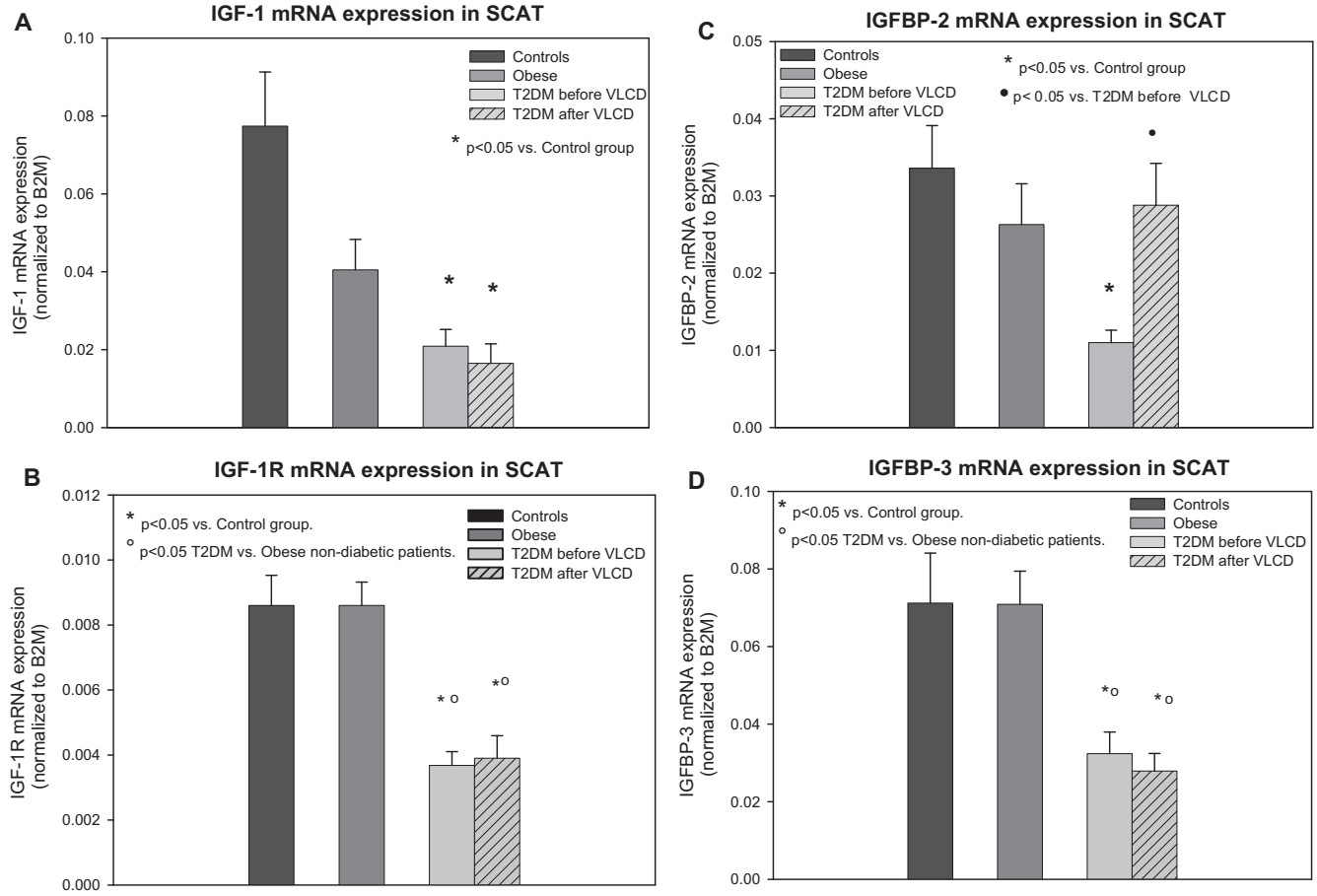

Fig. 2. mRNA expression of IGF-1 (A), IGF-1R (B), IGFBP-2 (C) and IGFBP-3 (D) in subcutaneous adipose tissue (SCAT) of control lean women (black bars; $n = 18$), obese non-diabetic women (dark gray bars; $n = 11$) and obese women with T2DM before very-low-calorie diet (VLCD) – (gray bars) and obese women with T2DM after VLCD (shaded gray bars; $n = 13$). Values are means ± SEM. $^{*}p < 0.05$ vs. control group. $^{\circ}p < 0.05$ T2DM vs. obese non-diabetic patients, $^{\bullet}p < 0.05$ vs. T2DM before VLCD.

immunological processes. Therefore, increased binding capacity for IGF-1 due to elevated IGF-1R expression in peripheral monocytes of obese and T2DM patients documented in this study may suggest a novel regulatory role of IGF-1 in the development of subclinical inflammation through the proinflammatory activation of peripheral monocytes.

In our previous study (Mraz et al., 2011) T2DM subjects had a more pronounced expression profile of several proinflammatory chemokine, cytokine and other proinflammatory receptors in peripheral monocytes than obese subjects without glucose metabolism disturbances suggesting important role of monocytes in the progression of metabolic disturbances from simple obesity and insulin resistance to overt disturbances of glucose metabolism. Similar results were found in our present study as far as mRNA expression of IGF axis components is concerned. In contrast to significantly increased IGF-1R mRNA expression in peripheral monocytes and decreased IGF-1 and IGFBP-2 mRNA expression in subcutaneous adipose tissue of obese T2DM patients only a non-significant trend towards these changes was found in OB subjects without diabetes. These findings indicate a possible role of the changes in mRNA expression of local IGF axis components in gradual progression of metabolic disturbances. Nevertheless, the cross-sectional design of our study does not allow us to exclude the alternative possibility i.e., that the differences in insulin and glucose levels between T2DM and OB group contributed to the alterations in IGF-1 axis componentś mRNA expression.

While the changes in IGF-1 axis components in peripheral monocytes were mostly subtle, obesity and T2DM were associated with more pronounced alterations of IGF-1 axis components in subcutaneous adipose tissue. We observed decreased IGF-1, IGFBP-2, IGFBP-3 and, in contrast to peripheral monocytes, also decreased IGF-1R mRNA expression in subcutaneous adipose tissue of T2DM patients suggesting different regulation of these molecules in fat relative to both peripheral monocytes and its circulating levels. Previous studies have documented an important role of IGF-axis components in the stimulation of differentiation of preadipocytes (Blüher et al., 2005; Peter et al., 1993; Sato et al., 2008; Wabitsch et al., 2000), in the regulation of adipocyte differentiation (Mur et al., 2003) and lipid accumulation in vitro (Grohmann et al., 2005) and in modulation of the expansion of white adipose tissue mass (Kloting et al., 2008). Decreased IGF-1, IGF-1R and IGFBP-3 mRNA expression in subcutaneous fat found in our study thus could have contributed to the reduced differentiation capacity of adipose tissue in obese T2DM patients. This defect may in turn lead to excessive lipid accumulation in non-adipose tissues and promote the development of insulin resistance and other accompanying metabolic pathologies.

Two weeks of stringent caloric restriction significantly decreased body weight, improved metabolic parameters and reduced CRP levels documenting the reduction of low-grade inflammation in our T2DM patients. In our previous studies (Dolinkova et al., 2008; Mraz et al. 2011) on similar study groups of obese females with and without T2DM VLCD induced a consistent decrease in mRNA expression of almost all up-regulated chemokine and cytokine receptors in peripheral monocytes suggesting its reduced response to chemotactic signals. This decrease could subsequently reduce monocyte recruitment into adipose tissue and partially explain positive metabolic effects of short-term VLCD. Interestingly, we did not observe

any effect of VLCD on IGF-1R mRNA expression in peripheral monocytes in this study. On the other hand, we detected an increase in IGFBP-3 mRNA expression in peripheral monocytes after VLCD reaching even higher levels than in control group. This finding could be important since IGFBP-3 was reported to be involved in the induction of growth arrest and apoptosis and to exert antiproliferative activity against myeloid leukemia cells (Ikezoe et al., 2004) and to be induced by LPS (lipopolysaccharide) stimulated apoptosis of human monocytic cells (Agnese et al., 2002). We therefore suggest that the increase of IGFBP-3 expression in peripheral monocytes after VLCD found in our study could have contributed to the down-regulation of proinflammatory monocytic activity associated with VLCD-induced metabolic improvement.

Another profound effect of VLCD found in our study was the normalization of markedly decreased IGFBP-2 mRNA expression in subcutaneous adipose tissue of obese T2DM subjects. It has been suggested that IGFBP-2 is an important modulator of IGF-1 action on adipogenesis (Boney et al., 1994). IGFBP-2 has been shown to inhibit IGF-dependent cell proliferation in a number of in vitro studies (Hoeflich et al., 1999). Significantly lower IGFBP-2 mRNA expression in visceral, but not in subcutaneous adipose tissue was found in genetically obese *ob/ob, db/db* mice and mice with high-fat-diet-induced obesity relative to lean animals (Li and Picard, 2010). Based on these data, it was suggested that IGFBP-2 secreted from white adipose tissue contributed to the prevention of diet-induced obesity and age-related insulin resistance in mice (Li and Picard, 2010). Markedly increased IGFBP-2 expression in subcutaneous adipose tissue found in our study after VLCD is in line with its suggested role as a modulator of positive metabolic effects on both local and systemic level possibly through the regulation of adipogenic capacity of adipose tissue, the limitation of further weight gain (Claudio et al., 2010) and the regulation of IGF-1 bioavailability during short-term calorie restriction in obese subjects (Clemmons et al., 1991).

We are aware that our study has several limitations such as relatively low number of subjects and its cross-sectional design that is not sufficient for a clear dissection of the cause and the consequence of the changes. The results could have also been affected by the fact that we have studied only female population. The interpretation of our results is rather difficult and sometimes speculative also due to the fact that only a very limited number of studies on the same topic have been published so far.

In summary, we have shown that obese T2DM subjects have decreased IGF-1, IGF-1R and IGFBP-3 expression in subcutaneous adipose tissue and increased IGF-1R expression in peripheral monocytes, which might contribute to changes in fat differentiation capacity and to regulation of subclinical inflammation by peripheral monocytes, respectively. We have also detected a significant increase in subcutaneous adipose tissue and circulating IGFBP-2 and peripheral monocyte IGFBP-3 after VLCD, that could play a role in metabolic improvements after VLCD. The precise mechanisms of regulation of IGF-1 axis components expression in subcutaneous adipose tissue or peripheral monocytes, its clinical significance and causal relationship to regulation of metabolic disarrangements and subclinical inflammation need to be addressed in further studies.

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The possible role of mRNA expression changes of GH/IGF-1/insulin axis components in subcutaneous adipose tissue in metabolic disturbances of patients with acromegaly

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Abstract

We explored the effect of chronically elevated circulating levels of growth hormone (GH)/insulin-like-growth-factor-1 (IGF-1) on mRNA expression of GH/IGF-1/insulin axis

components and p85alpha subunit of phosphoinositide-3-kinase (p85alpha) in subcutaneous adipose tissue (SCAT) of patients with active acromegaly and compared these findings with healthy control subjects in order to find its possible relationships with insulin resistance and body composition changes. Acromegaly group had significantly decreased percentage of truncal and whole body fat and increased homeostasis model assessment-insulin resistance (HOMA-IR). In SCAT, patients with acromegaly had significantly increased IGF-1 and IGF-binding protein-3 (IGFBP-3) expression that both positively correlated with serum GH. P85alpha expression in SCAT did not differ from control group. IGF-1 and IGFBP-3 expression in SCAT were not independently associated with percentage of truncal and whole body fat or with HOMA-IR while IGFBP-3 expression in SCAT was an independent predictor of insulin receptor as well as of p85alpha expression in SCAT. Our data suggest that GH overproduction in acromegaly group increases IGF-1 and IGFBP-3 expression in SCAT while it does not affect SCAT p85alpha expression. Increased IGF-1 or IGFBP-3 in SCAT of acromegaly group do not appear to contribute to systemic differences in insulin sensitivity but may have local regulatory effects in SCAT of patients with acromegaly.

Keywords:

acromegaly, GH/IGF-1/insulin axis components, adipose tissue, insulin resistance, p85alpha subunit of PI3K

Short title: IGF-1 and IGFBP-3 in acromegalic adipose tissue

Abbreviations:

AC, acromegaly group;

C, control group;

CRP, C-reactive protein;

DEXA, dual-energy X-ray absorbtiometry;

GH, growth hormone;

GH-R, growth hormone receptor;

HDL, high-density lipoprotein;

HOMA-IR – homeostasis model assessment – insulin resistance;

IFCC, International Federation of Clinical Chemistry;

IGF-1, insulin-like growth factor-1;

IGF-2, insulin-like growth factor-2;

IGFBP-1-3, insulin-like growth factor binding protein-1-3;

IGF-1R, insulin-like growth factor-1 receptor;

IGF-2R, insulin-like growth factor-2 receptor;

INS-R, insulin receptor;

LBM, lean body mass;

LDL, low-density lipoprotein;

p85alpha, p85alpha subunit of phosphoinositide-3-kinase;

PI3K, phosphoinositide-3-kinase;

qRT-PCR, quantitative real-time PCR;

SCAT, subcutaneous adipose tissue.

1. Introduction

Acromegaly is associated with an increased prevalence of glucose metabolism disorders. Clinically confirmed diabetes mellitus is observed in approximately one quarter of all patients with acromegaly (Droste et al. 2014). The degree of glucose intolerance has been reported to correlate with serum GH levels, age, duration of the disease and family history of diabetes (Resmini et al. 2009). Importantly, patients with acromegaly-associated diabetes display exponentially increased mortality rates, since untreated acromegaly and increased plasma insulin levels/insulin resistance in diabetes are both associated with higher rate of cardiovascular mortality and malignancies (Droste et al. 2014). GH-induced insulin resistance, increased hepatic glucose production and accentuated lipolysis contribute to the presence of diabetes and impaired glucose tolerance in acromegaly (Rodrigues et al. 2011). IGF-1 has rather opposing effects on insulin sensitivity and lipolysis than GH; however, in acromegaly, increased IGF-1 levels are unable to counteract the negative metabolic actions of GH excess (Resmini et al. 2009).

We have previously shown that type 2 diabetes and obesity are accompanied by differences not only in serum concentrations but also in mRNA expression of some GH/IGF-1/insulin axis components in subcutaneous adipose tissue and we suggested that these differences might contribute to allover metabolic and adipose tissue metabolism disturbances in type 2 diabetes and to their improvement after dietary intervention (Touskova et al. 2012).

Adipose tissue is the major target of growth hormone action (Garten et al. 2012) and increased lipolysis and reduced triglyceride accumulation together with inhibited preadipocyte differentiation due to elevated GH levels in acromegaly contribute to the reduction of adipose tissue mass (Plockinger et Reuter 2008; Richelsen 1997). GH may mediate its actions on adipose tissue via specific GH receptors on both preadipocytes and mature adipocytes, but some effects are mediated indirectly through the GH-regulated secretion of IGF-1 (Richelsen 1997). Locally produced IGFBP-1-3 have been suggested to play a protective role against obesity among other mechanisms also via inhibiting the stimulatory effects of IGF-1 on adipogenesis (Nguyen et al. 2015; Wheatcroft et al. 2007; Ueda et Ashida 2012), and to also have specific regulatory roles in glucose metabolism (Rajpathak et al. 2009; Claudio et al. 2010; Chan et al. 2005). Subcutaneous adipose tissue appears to be an interesting target tissue in terms of exploring GH effects, since several previous studies demonstrated differences in the amount of subcutanoues adipose tissue in accordance with differences of GH action (Berryman et al. 2004; Lin et al. 2012; Ibáñez et al. 2010).

Numerous studies showed several different mechanisms by which GH affects insulin sensitivity in adipose tissue, including interferences with insulin signalling cascade (Castro et al. 2004; Smith et al. 1997). Among others, an up-regulation of p85alpha regulatory subunit of PI3K by GH has been considered as a potential explanation for the insulin resistance in white adipose tissue of mice with GH excess (del Rincon et al. 2007).

To our knowledge, the local expression of GH/IGF-1/insulin axis components in subcutaneous adipose tissue of patients with acromegaly has been very scarcely studied so far. In the current study, we hypothesized that chronically elevated GH/IGF-1/insulin serum levels in acromegalic patients might induce differences in mRNA expression of GH/IGF-1/insulin axis components in subcutaneous adipose tissue that may in turn contribute to dysregulation

of glucose metabolism and reduction of adipose tissue mass. To this end, we measured serum concentrations and mRNA expression of selected components of GH/IGF-1/insulin axis in subcutaneous adipose tissue together with metabolic and anthropometric parameters in acromegalic patients and compared them with the findings in healthy age-matched subjects. To gain further insight into the mechanism of GH-induced insulin resistance in subcutaneous adipose tissue in acromegaly we also explored the mRNA expression of p85alpha regulatory subunit of PI3K.

2.1. Methods

Study subjects

Twelve acromegalic patients (AC group, 8 men and 4 women, aged 49.6 ± 8.1 years) and twelve lean healthy subjects (C group, 4 men and 8 women, aged 50.7 ± 5.2 years) were included in the study. Acromegalic patients were examined at the moment of establishing the diagnosis of acromegaly, therefore they had no prior treatment for acromegaly. The diagnostic criteria for acromegaly were increased IGF-1 serum levels above the upper limit of the normal range (according to age) and the non-supresibility of GH serum levels below 0.4 ng/ml in the oral glucose tolerance test. Exclusion criteria were age <18 years old, malignancy, inflammatory disease, type 1 diabetes, current treatment with glucocorticoids. Five out of twelve acromegalic patients were on antihypertensive treatment, three used oral antidiabetic treatment and one was treated with insulin. The treatment remained unchanged for at least three months prior to the start of the study. Control subjects had no history of acromegaly, obesity and/or diabetes mellitus, arterial hypertension, or lipid metabolism disturbances and received no medication. Blood tests confirmed normal blood count, biochemical and hormonal parameters.

During the program all acromegalic patients were hospitalized at the Third Department of Medicine, General University Hospital in Prague. Written informed consent was signed by all participants before the beginning of the study. The study was approved by Human Ethics Review Board, First Faculty of Medicine and General University Hospital, Prague, Czech Republic and was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

2.2. Anthropometric examination, blood and adipose tissue sampling

All participants included in the study were examined only once. All subjects were measured and weighted, and their BMI was calculated. Blood samples for biochemical and hormonal parameters measurement were taken after overnight fasting. Blood samples were separated by centrifugation for 10 min at 1000 x g within 30 min from blood collection. Serum or plasma was subsequently stored in aliquots at -80 °C until further analysis.

Samples of subcutaneous adipose tissue for mRNA expression analysis were obtained from abdominal region with subcutaneous needle aspiration biopsy from all participants. Approximately 100 mg of adipose tissue was collected to 1 ml of RNA stabilization Reagent (RNAlater, Qiagen, Hilden, Germany) and stored at -80 °C until further analysis.

The amount and percentage of whole body fat, truncal body fat and lean body mass was assessed by body composition measurement using Dual-Energy X-Ray Absorptiometry (DEXA, Hologic Discovery, USA).

2.3. Hormonal and biochemical assays

Serum levels of total insulin-like growth factor-1 (IGF-1) were measured by IRMA kit (Immunotech, Prague, Czech Republic). LOD (Limit of detection) and LOQ (Limit of quantitation) were 12.0 ng/ml. Serum IGFBP-1, IGFBP-2 and IGFBP-3 levels were measured

by ELISA kits (DiaSource ImmunoAssays S.A., Nivelles, Belgium). LOD was 0.4 ng/ml for IGFBP-1, 0.2 ng/ml for IGFBP-2 and 10 ng/ml for IGFBP-3. LOQ was 6.4 ng/ml for IGFBP-1, 4.2 ng/ml for IGFBP-2 and 10 ng/ml for IGFBP-3. Growth hormone (GH) levels were measured by IRMA kits (Immunotech, Prague, Czech Republic). LOD and LOQ were 0.10 mIU/l. Serum C-reactive protein (CRP) levels were measured by high sensitive ELISA (Bender Medsystems, Vienna, Austria) with a LOD of 3 pg/ml and LOQ 1.5 ng/ml. The intra- and interassay variabilities for all methods were less than 5.0 and 10.0 %, respectively. Biochemical parameters (fasting insulin, fasting blood glucose, HbA1c, total and HDL-cholesterol and triglycerides) were measured in the Department of Biochemistry of General University Hospital, Prague by standard laboratory methods. The value of LDL-cholesterol was calculated according to Friedewald formula.

2.3. Total RNA isolation from adipose tissue

Samples of subcutaneous adipose tissue were homogenized on a MagNA Lyser Instrument using MagNA Lyser Green Beads (Roche Diagnostics GmbH, Germany). Total RNA from homogenized tissue was extracted on MagNA Pure instrument using Magna Pure Compact RNA Isolation kit (tissue) (Roche Diagnostics GmbH, Germany). The integrity of the RNA was checked by visualization of 18S and 28S ribosomal bands on 1 % agarose gel with ethidium bromide. The RNA concentration was determined from absorbance at 260 nm on a NanoPhotometer (Implen, Munchen, Germany).

2.4. Determination of mRNA expression by quantitative real-time PCR

Total RNA was used for reverse transcription to synthesize the first strand cDNA. Reverse transcription was performed using 0.25 μg of total RNA to synthesize the first strand cDNA

using the random primers as per the instructions of the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA).

Measurements of mRNA expression were performed on an ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix, NO AmpErase® UNG and specific TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). All PCRs for each gene were amplified separately. Controls with no template cDNA were performed with each assay and all samples were run at least in duplicates. The increase in fluorescence was measured in real time and threshold cycle (Ct) values were obtained. To compensate for variations in RNA amount and efficiency of reverse transcription, beta-2-microglobulin was used as endogenous reference and results were normalized to the mean of these values. The formula $2^{-\Delta Ct}$ was used to calculate relative gene expression.

2.6. Statistical analysis

Statistical analysis was performed on SigmaStat software Version 3.0 and the graphs were created in Sigma Plot software Version 8.0 (SPSS Inc., Chicago, IL, USA). Prior to analysis, all continuous variables were assessed for normality (Kolmogorov-Smirnov test). Anthropometric, biochemical and hormonal data are expressed as mean ± standard deviation (SD) or median (interquartile range), according to the normality of data. Comparisons of anthropometric, biochemical, hormonal and other parameters among the two groups studied (AC, C) were evaluated by Unpaired t-test or Mann-Whitney Rank Sum test as appropriate. The associations between serum and mRNA expression of GH/IGF-1/insulin axis components and p85alpha in SCAT and other variables in a combined group of AC subjects and age-matched controls were estimated by Spearman´s rank order correlation. Further backward stepwise regression analysis calculations were performed to show the independent

relationships of GH/IGF-1/insulin axis components, p85alpha in SCAT and other biochemical or anthropometric characteristics. Only the parameters with significant correlation from Spearman correlation test ($p < 0.05$) were used for these analyses. In all statistical tests p values <0.05 were considered statistically significant.

3. Results

3.1. Anthropometric, metabolic and hormonal characteristics of study subjects

Anthropometric, metabolic and hormonal characteristics of the study subjects (AC and C groups) are summarized in Table 1.

BMI and percentage of lean body mass were significantly increased in AC group compared with C group, while percentage of whole body fat and truncal fat were decreased in AC group relative to C group.

Serum levels of GH, total IGF-1 and IGFBP-3, fasting glucose, insulin, HbA1c, HOMA-IR and triglycerides were significantly increased in AC group compared with C group.

On the contrary, IGFBP-1, IGFBP-2, HDL and CRP serum levels were decreased in acromegalic patients. Total and LDL cholesterol did not significantly differ from control group.

3.2. mRNA expression of GH/IGF-1/insulin axis components and p85alpha in subcutaneous adipose tissue

The summary of mRNA expressions of GH/IGF-1/insulin axis components and p85alpha in subcutaneous adipose tissue of AC and C group is shown in Figure 1.

IGF-1 and IGFBP-3 mRNA expression in subcutaneous adipose tissue were significantly increased in patients with acromegaly compared with control group. GH-R, IGF1-R, IGF-2,

IGF-2R, IGFBP-2, INS-R, p85alpha mRNA expression did not significantly differ from control group. IGFBP-1 was not detected in adipose tissue.

3.3 Relationships of IGF-1 and IGFBP-3 mRNA expression in SCAT with other parameters

The relationships between IGF-1 and IGFBP-3 mRNA expression in SCAT (the only significantly increased parameters in SCAT in AC group compared to C group) with the other studied parameters were explored using Spearman correlation test. The significant associations are summarized in Table 2. Backward stepwise regression analysis was performed in order to explore the independent predictors of IGF-1 and IGFBP-3 mRNA expression in SCAT. The significant relationships are summarized in Table 3. In addition, we explored whether these two parameters might be independent predictors of selected metabolic (HOMA-IR, INS-R, p85alpha mRNA expression in SCAT) and body composition parameters (the percentage of truncal and whole body fat). The significant relationships are summarized in Table 4. The parameters used as independent variables for these selected metabolic and body composition parameters were the parameters with significant correlation in Spearman correlation test (data not reported).

IGF-1 mRNA expression in SCAT was inversely associated with percentage of truncal fat and positively with percentage of LBM. IGF-1 in SCAT correlated positively with serum GH, INS-R, GH-R, p85alpha and IGFBP-3 mRNA expression in SCAT and inversely with serum CRP levels (Table 2). IGF-1 mRNA expression in SCAT could be independently predicted only from **IGFBP-3 mRNA expression in SCAT** (Table 3).

IGFBP-3 mRNA expression in SCAT was inversely associated with percentage of truncal fat, whole body fat and positively with percentage of LBM. IGFBP-3 mRNA expression in SCAT correlated positively with fasting blood glucose, insulin, HOMA-IR, serum GH, mRNA

expression of IGF-1, INS-R, GH-R, p85alpha in SCAT and was inversely associated with serum CRP (Table 2). In Model 1 (including: fasting glucose, fasting insulin, HOMA-IR, serum CRP, serum GH, IGF-1 mRNA expression in SCAT as independent variables), IGFBP-3 mRNA expression in SCAT could be predicted from a linear combination of five independent variables: **fasting glucose, fasting insulin, serum GH, serum CRP levels** and **IGF-1 mRNA expression in SCAT** (Table 3). In Model 2 (including: percentage of truncal fat, whole body fat and LBM, IGF-1 and p85alpha mRNA expression in SCAT as independent variables), IGFBP-3 mRNA expression in SCAT could be predicted only from **IGF-1 mRNA expression in SCAT** (Table 3)**.**

HOMA-IR could be independently predicted only from **serum IGF-1 levels** (Table 4).
INS-R mRNA expression in SCAT could be predicted from a linear combination of three independent variables: **IGFBP-3 in SCAT, serum HDL and IGF-1R in SCAT** (Table 4).
P85alpha mRNA expression in SCAT could be predicted from a linear combination of five independent variables: **SCAT mRNA expression of IGFBP-3, IGFBP-2, INS-R, GH-R and percentage of LBM** (Table 4).
HbA1c was found to be the independent predictor of percentage of truncal and whole body fat (Table 4).
IGF-1 and IGFBP-3 in SCAT were not found to be among the independent predictors of HOMA-IR and percentage of truncal and whole body fat, while IGFBP-3 in SCAT was found to be an independent predictor of INS-R and p85alpha mRNA expression in SCAT.
The other non-significant relationships are not reported.

Discussion

The most important finding of our study is significantly increased mRNA expression of *IGF-1* and *IGFBP-3* in SCAT in patients with acromegaly relative to healthy lean subjects (Fig. 1). Both *IGF-1* and *IGFBP-3* mRNA expression in SCAT positively correlated with serum GH levels (Table 2) suggesting its direct stimulatory effect on local IGF-1 and IGFBP-3 production in subcutaneous fat. Previous studies have reported adipose tissue production of IGF-1 in response to GH stimulation in experimental conditions and in healthy individuals (Vikman et al. 1991; Peter et al. 1993; Wabitsch et al. 1996; Jørgensen et al. 2006). Peter et al. (Peter et al. 1993) reported that IGFBPs, including IGFBP-3 mRNA expression, in rat white adipose tissue were all regulated by GH. Other experimental studies showed a stimulatory effect of GH on IGFBP-3 expression in the liver, muscle and skin in GH deficient rats (Lemmey et al. 1997), on serum IGFBP-3 (Wester et al. 1998) or on its secretion from porcine adipose tissue (Chen et al. 1996) or human preadipocytes (Wabitsch et al. 2000). Recently, increased IGF-1 and IGFBP-3 mRNA expression was found also in SCAT of subjects with acromegaly (Hochberg et al. 2015). In our study, serum GH was one of the independent predictors of *IGFBP-3* mRNA expression in SCAT confirming its direct regulatory role (Table 3).

Previous studies demonstrated stimulatory effect of IGF-1 on skin (Lemmey et al. 1997) or liver (Gosteli-Peter et al. 1994) IGFBP-3 mRNA expression, but to our best knowledge no such data exist to date for the presence of this effect in adipose tissue. Importantly, in our study *IGF-1* mRNA expression in SCAT was an independent predictor of *IGFBP-3* mRNA expression in SCAT and vice versa (Table 3), suggesting their possible local mutual regulatory interactions.

As expected, we found significantly decreased percentage of whole body as well as truncal fat and increased percentage of lean body mass in acromegalic patients compared to control group (Table 1), which is in agreement with previous studies (Katznelson 2009). Furthemore,

we observed an inverse relationship of *IGFBP-3* expression in SCAT with percentage of truncal and whole body fat and of *IGF-1* expression in SCAT with percentage of truncal fat (Table 2), suggesting a possible causal relationship. In previous studies, IGF-1 and IGFBP-3 expression have been shown to increase during human preadipocyte differentiation (Baxter et al. 2009). While IGF-1 stimulates this process and adipogenesis (Peter et al. 1993; Chen et al. 1995) IGFBP-3 has the opposite effects (Baxter et al. 2009; Chan et al. 2009). In our previous study obese diabetic women had decreased *IGFBP-3* mRNA expression in subcutaneous fat (Touskova et al. 2012). While the concept of local regulatory role of IGF-1 and IGFBP-3 in SCAT is tempting, we did not confirm *IGF-1* or *IGFBP-3* mRNA expression in SCAT as independent predictors of percentage of truncal and whole body fat in a backward stepwise regression analysis (Table 4), however this concept certainly warrants further investigation. To gain further insight into its possible regulatory role in systemic metabolic changes in acromegaly we explored the relationships between *IGF-1* and *IGFBP-3* mRNA expression in SCAT with selected markers of glucose metabolism and insulin resistance (Table 2). In previous studies, the IGF-1 expression in adipose tissue transplants was shown to be associated with anti-inflammatory and favourable metabolic effects in diabetic mice (Gunawardana et Piston 2015). Both, IGF-1 (Kubota et al. 2008; Neascu et al. 2013) and IGFBP-3 (Mohanraj et al. 2013) exerted anti-inflammatory effects and insulin-sensitizing effects in adipocytes. On the other hand, several studies indicated that IGFBP-3 may decrease insulin sensitivity in adipocytes by various mechanisms (Kim et al. 2007; Chan et al. 2005). In our current study we did not find an independent association of *IGF-1* or *IGFBP-3* in SCAT with HOMA-IR. Interestingly, *IGFBP-3* in SCAT was an independent predictor of *INS-R* as well as of *p85alpha* mRNA expression in SCAT (Table 4), suggesting possible local role of IGFBP-3 in the regulation of insulin sensitivity in adipose tissue of AC.

In acromegaly, the increased insulin resistance is paradoxically often present despite the decreased amount of adipose tissue. For all the studies documenting different local mechanisms of GH-induced insulin resistance in adipose tissue (Castro et al. 2004; Smith et al. 1997), some experimental studies suggested that GH action in adipose tissue is not crucial in deterioration of the overall insulin resistance (List et al. 2013; Johansen et al. 2005). In our study, increased HOMA-IR was not significantly associated with the decreased percentage of whole body or truncal fat. These findings may support the hypothesis that in acromegaly, adipose tissue may not be the main site contributing to the whole body insulin resistance and point to the liver or the skeletal muscle as other important contributors.

Numerous previous experimental studies have suggested an important role of GH excess in the stimulation of p85alpha regulatory subunit of PI3K tissue expression and subsequent development of GH-induced insulin resistance (Del Rincon et al. 2007; de Castro Barbosa et al. 2009; Barbour et al. 2005). On the other hand, an attenuation of p85alpha expression has been proposed as one of the mechanisms for the treatment of type 2 diabetes (Mauvais-Jarvis et al. 2002). Insulin has been shown as another important regulator of p85alpha regulatory subunit. Insulin resistant conditions were previously associated with contrasting results showing increased (Adochio et al. 2009; Cornier et al. 2006) but also decreased (Anai et al. 1998) p85alpha tissue (liver, muscle) expression and also with blunted stimulatory effect of acute hyperinsulinemia on p85alpha tissue expression (Lefai et al. 2001). In our study, elevated serum GH/insulin levels in patients with acromegaly failed to increase *p85alpha* mRNA expression in subcutaneous adipose tissue where its mRNA expression was comparable to that of the control group (Fig. 1), which confirms the result of the recent study on patients with acromegaly (Hochberg et al. 2015). No significant relationships were found between serum GH/IGF-1/insulin and *p85alpha* in SCAT or between *p85alpha* in SCAT and metabolic parameters (HOMA-IR, fasting blood glucose, insulin, HbA1c) arguing against an

involvement of p85alpha regulatory subunit in SCAT in mediating the metabolic effects of systemic GH excess in acromegaly. P85alpha was positively associated with INS-R mRNA expression in SCAT, suggesting a possible parallel regulation of their expressions.
The limitations of our study include the relatively low number of study subjects and the cross-sectional design of the study. We are aware that the correlations found in our study do not necessarily establish a causal connection and that the mRNA expression differences in subcutaneous adipose tissue might be secondary to insulin resistance. In addition, for the assessment of insulin resistance we have only measured HOMA-IR and did not perform hyperinsulinemic euglycemic clamp that would be more precise for the whole body insulin sensitivity evaluation.

In conclusion, in our study we found increased *IGF-1* and *IGFBP-3* mRNA expression in subcutaneous adipose tissue of patients with acromegaly. None of these factors independently predicted the changes in body composition or systemic insulin sensitivity. Nevertheless, their local effect on adipogenesis and insulin sensitivity in subcutaneous adipose tissue could be present and further investigation of this possibility is needed. On the contrary, our data do not support an up-regulation of *p85alpha* subunit of PI3K expression as a mechanism of GH-induced insulin resistance in subcutaneous adipose tissue of acromegalic patients.

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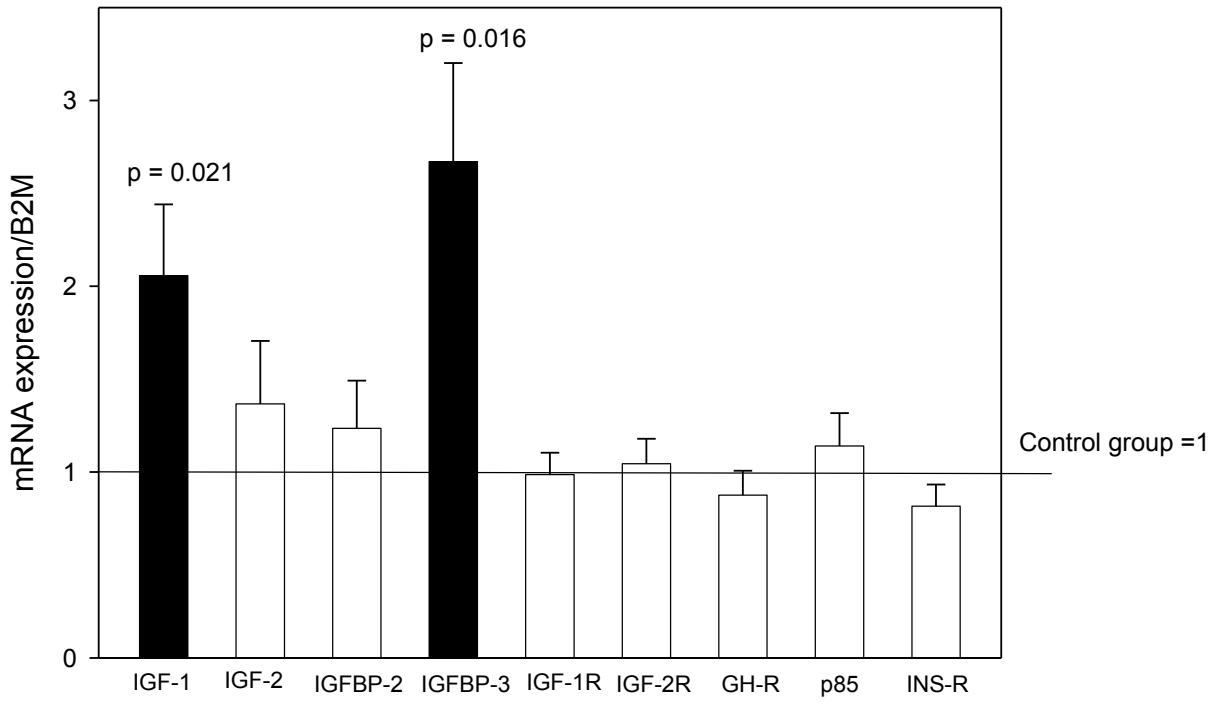
Table 1. Clinical, anthropometric, metabolic and hormonal characteristics of the study groups

	Control group	AC group	P - value
No. of subjects	12	12	NA
Sex (male/female)	4/8	8/4	NA
Age (years)	50.7 ± 5.2	49.6 ± 8.1	0.701
Body mass index (kg/m^2)	23.5 (22.0-25.2)	31.0 (28.5-33.5)	**<0.001**
Whole body fat (%)	29.3 ± 5.1	21.4 ± 5.7	**0.021**
Truncal fat (%)	27.7 ± 4.9	20.3 ± 5.1	**0.019**
Lean body mass (%)	69.0 ± 5.1	77.1 ± 6.2	**0.023**
Fasting blood glucose (mmol/l)	4.97 ± 0.37	6.06 ± 0.85	**<0.001**
Fasting insulin (mIU/l)	19.2 ± 6.8	45.3 ± 25.1	**0.006**
HbA1c (% IFCC)	3.72 ± 0.84	4.70 ± 0.76	**0.014**
HOMA-IR index	1.88 (1.35-2.81)	10.01 (8.03-13.69)	**0.006**
Triglycerides (mmol/l)	1.17 ± 0.41	2.02 ± 0.76	**0.004**
Total cholesterol (mmol/l)	5.25 ± 0.72	4.73 ± 0.81	0.107
LDL cholesterol (mmol/l)	3.30 ± 0.72	2.77 ±0.74	0.087
HDL cholesterol (mmol/l)	1.42 ± 0.31	1.04 ± 0.22	**0.009**
CRP (mg/l)	0.49 (0.23-2.00)	0.09 (0.07-0.22)	**0.004**
GH (mIU/l)	1.5 (0.6-2.3)	61.1 (9.7 -96.1)	**0.003**
Total IGF-1 (ug/l)	137 (127-154)	1028 (655-1429)	**<0.001**
IGFBP-1 (ug/l)	6.53 (2.64-10.03)	0.12 (0.05-0.49)	**<0.001**
IGFBP-2 (ug/l)	293 (232-332)	141 (89-163)	**0.006**
IGFBP-3 (mg/l)	2.97 ± 0.58	6.89 ± 1.27	**<0.001**

Normally distributed data are shown as mean ± SD, non-parametric data as median (interquartile range). Statistical significance is from Unpaired t-test or Mann-Whitney Rank Sum test as appropriate. P value <0.05 indicated statistical significance. Values were adjusted for sex in both AC and C group and for the presence of diabetes in AC group.
AC, acromegalic group; C, control group; CRP, C-reactive protein; GH, growth hormone; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR – homeostasis model assessment – insulin resistance; IFCC, International Federation of Clinical Chemistry;

IGF-1, insulin-like growth factor-1; IGFBP, insulin-like growth factor binding protein; LDL, low-density lipoprotein; NA, not applicable.

Figure 1. GH/IGF-1/insulin axis components and p85alpha mRNA expression differences in SCAT of AC group (n=12) relative to control group (n=12)



The mean relative mRNA expressions for the parameters of acromegalic group are expressed as relative ratio to the mean mRNA expression of control group that is taken as 1.0 (line-Control group) for every gene separately.
Statistical significance is from Unpaired t-test or Mann-Whitney Rank Sum test as appropriate. P value <0.05 indicated statistical significance. Values were adjusted for sex in both AC and C group and for the presence of diabetes in AC group.
GH-R, growth hormone receptor; IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2; IGFBP, insulin-like growth factor binding protein; IGF-1R, insulin-like growth factor-1 receptor; IGF-2R, insulin-like growth factor-2 receptor; INS-R, insulin receptor; p85, p85alpha subunit of phoshoinositide-3-kinase.

Table 2. The significant relationships of mRNA expression of IGF-1 and IGFBP-3 in SCAT with anthropometric, metabolic parameters, serum and SCAT GH/IGF-1/insulin axis components and p85alpha in combined population of acromegalic patients and normal-weight healthy subjects

	SCAT (n=24)			
	IGF-1		IGFBP-3	
	R	p	R	p
Truncal fat (%)	-0.552	**0.039**	-0.574	**0.031**
Whole body fat (%)	-0.446	0.105	-0.543	**0.043**
LBM (%)	0.615	**0.024**	0.613	**0.019**
Fasting blood glucose	0.292	0.174	0.453	**0.030**
Fasting insulin	0.332	0.162	0.511	**0.021**
HOMA-IR index	0.330	0.151	0.541	**0.009**
CRP	-0.699	**<0.001**	-0.449	**0.041**
Serum GH	0.555	**0.009**	0.552	**0.013**
IGF-1 in SCAT	x	x	0.837	**<0.001**
IGFBP-3 in SCAT	0.837	**<0.001**	x	x
INS-R in SCAT	0.516	**0.014**	0.486	**0.022**
GH-R in SCAT	0.463	**0.023**	0.437	**0.033**
p85alpha in SCAT	0,841	**<0.001**	0,717	**<0.001**

Statistical significance is from Spearman correlation test. Statistical significance was assigned to $p < 0.05$.
CRP, C-reactive protein; GH, growth hormone; GH-R, growth hormone receptor; HOMA-IR – homeostasis model assessment – insulin resistance; IGF-1, insulin-like growth factor-1; IGFBP, insulin-like growth factor binding protein; INS-R, insulin receptor; LBM, lean body mass; p85alpha, p85alpha subunit of phoshoinositide-3-kinase; SCAT, subcutaneous adipose tissue.
The non-significant correlations are not reported.

Table 3. The independent predictors of IGF-1 and IGFBP-3 mRNA expression in SCAT

Dependent	Independent	p	Standardized coefficients beta	AdjR²

IGF-1 in SCAT	IGFBP-3 in SCAT	0.010	0.569	0.839
IGFBP-3 in SCAT (Model 1)	Fasting glucose	<0.001	0.0539	0.971
	Fasting insulin	0.024	$-6.18*10^{-4}$	
	Serum GH	0.002	$7.71*10^{-4}$	
	Serum CRP	0.016	0.0106	
	IGF-1 in SCAT	<0.001	0.568	
IGFBP-3 in SCAT (Model 2)	IGF-1 in SCAT	<0.001	0.541	0.833

Statistical significance is from backward stepwise regression analysis. Statistical significance was assigned to $p < 0.05$.
CRP, C-reactive protein; GH, growth hormone; IGF-1, insulin-like growth factor-1; IGFBP, insulin-like growth factor binding protein; SCAT, subcutaneous adipose tissue.
The non-significant correlations are not reported.

Table 4. The independent predictors of selected metabolic and body composition parameters

Dependent	**Independent**	**p**	**Standardized coefficients beta**	**AdjR²**
HOMA-IR	Serum IGF-1	<0.001	0.0162	0.682
INS-R in SCAT	**IGFBP-3 in SCAT**	<0.001	0.109	0.809
	HDL	<0.001	0.0351	
	IGF-1R in SCAT	0.027	4.479	
P85alpha in SCAT	**IGFBP-3 in SCAT**	<0.001	0.166	0.981
	IGFBP-2 in SCAT	<0.001	3.144	
	INS-R in SCAT	0.013	0.318	
	GH-R in SCAT	<0.001	-0.0451	
	LBM (%)	0.006	$-9.72*10^{-4}$	

Truncal fat (%)	HbA1c	0.009	-4.866	0.505
Whole body fat (%)	HbA1c	0.011	-4.896	0.479

Statistical significance is from backward stepwise regression analysis. Statistical significance was assigned to $p < 0.05$.

GH, growth hormone; GH-R, growth hormone receptor; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR – homeostasis model assessment – insulin resistance; IGF-1, insulin-like growth factor-1; IGFBP, insulin-like growth factor binding protein; IGF-1R, insulin-like growth factor-1 receptor; INS-R, insulin receptor; LBM, lean body mass; p85alpha, p85alpha subunit of phoshoinositide-3-kinase; SCAT, subcutaneous adipose tissue. The non-significant correlations are not reported.