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The production and regulation of IGF and IGFBPs in human adipose tissue cultures

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ABSTRACT

Background: Adipocytes express and secrete IGFs and IGFBPs; proteins with important effects on adipocyte homeostasis. However, the factors that control adipocyte generation of IGFs and IGFBPs are not clarified.
Aim: To identify regulators of the synthesis of IGFs and IGFBs in adipose tissue.
Methods: Subcutaneous adipose tissue fragments (500 mg) from 7 healthy lean women were incubated for 48 h following addition of GH (50 μg/l), dexamethasone (DXM, 20 nM), insulin (100 nM), interleukin (IL)-1β (50 ng/l), IL-6 (50 ng/l) and tumor-necrosis factor (TNF)-α (10 ng/l). Outcome parameters included tissue mRNA and culture media IGF and IGFBP levels.
Results: Adipose tissue cultures secreted more IGF-II than IGF-I protein (1.14 ± 0.41 vs. 0.26 ± 0.09 μg/l [mean ± SEM]; $P<0.02$). IGF-I mRNA and protein levels were stimulated by GH (to 340% [153; 477] (median [interquartiles]) and 270 ± 26%, respectively; $P<0.003$), and inhibited by IL-1β (to 28% [21; 77] and 68 ± 11%, respectively; $P<0.003$). TNF-α reduced IGF-I and IGF-II protein levels to 51 ± 8% and 69 ± 8%, respectively ($P\leq0.002$), without affecting mRNA levels. IGF protein levels were unaffected by DXM, insulin and IL-6. All IGFBPs IGFBP-1 were expressed. IGFBP-4 was by far the most predominant IGFBP by immunoassay and WLB revealed two bands at 28 and 24 kDa, most likely representing glycosylated and non-glycosylated IGFBP-4.
Conclusion: Adipose tissue cultures secrete more IGF-II than IGF-I, and predominantly IGFBP-4. The secretion of IGF-I is affected by GH, IL-1β and TNF-α, whereas IGF-II is affected by TNF-α only. Hence, cytokines may control adipocyte homeostasis by affecting local IGF-generation.

1. Introduction

Adipose tissue has traditionally been regarded exclusively as a place for storing excess energy in the form of triglycerides. However, the awareness that adipose tissue is able to produce a variety of biologically active substances such as sex hormones, cytokines, adipokines and growth factors has changed this perception completely and nowadays adipose tissue is considered to be a genuine endocrine organ [1,2]. As virtually all adipocyte-derived substances have been linked to the development of obesity-associated diseases such as insulin resistance, atherosclerosis and cancer, there has been a growing interest in exploring the factors that control the endocrine activity of adipose tissue [3–6].

IGF-I is one of the adipocyte-derived substances which appears to be important for adipocyte homeostasis. IGF-I has been shown to stimulate adipocyte growth and lipogenesis through differentiation of mesenchymal stem cells into mature adipocytes [7], to stimulate differentiation of pre-adipocytes into mature adipocytes and to inhibit death-receptor induced apoptosis [7–10]. These findings indicate that locally produced IGF-I may exert important paracrine/autocrine effects in adipose tissue *in vivo*. Adipocytes also express and secrete IGFBPs [11]. Although the role of the IGFBPs in sequestering IGF-I and thereby affecting IGF-I receptor (IGF-IR) phosphorylation is well known [12,13], recent experiments in 3T3-L1 adipocytes have shown that IGFBP-3 is able to induce insulin resistance independent of IGF-I signaling [8].

Interestingly, recent data indicate that the interaction between IGF-I and adipocytes may have effects, which reach beyond the boundaries of adipose tissue. Thus, mice specifically lacking the IGF-IR gene in adipocytes showed elevated plasma IGF-I levels and an increased somatic growth, concomitant with an increased IGF-I expression in liver and adipose tissue. Accordingly, the study indicated that IGF-I signaling in adipose tissue either directly or indirectly via the liver controls circulating IGF-I levels and body growth; however, the exact mechanism remains to be clarified [14].

Despite substantial evidence pointing to IGF-I as an important regulator of adipose tissue, there are only limited data on factors that may regulate the secretion of IGF-I and IGFBPs in adipose tissues. However, it is clear from literature that adipocytes are able to respond to hormones that may stimulate IGF-I generation, for instance GH and insulin [15], as well as to actively secrete pro-inflammatory proteins

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that may induce IGF-I resistance, for instance tumor-necrosis factor (TNF)-α as well as interleukin (IL)-1β and IL-6 [1,2]. Consequently, the aim of the present study was to examine the expression and production of IGF-I, IGF-II and the IGFBPs in human adipose tissue cultures treated with pro-inflammatory proteins, steroids, GH and insulin.

2. Materials and methods

2.1. Subjects and adipose tissue handling

Adipose tissue was obtained from 7 women (age 34.1 ± 2.8 years, BMI 22.9 ± 0.7 kg/m^2) who have had subcutaneous liposuction performed from the abdominal region for cosmetic reasons. All the subjects included were healthy and no one took any medication that could influence adipose tissue metabolism. The project was approved by the local Ethical Committee and performed in accordance with the Helsinki Declaration.

Immediately after collection, the adipose tissue was rinsed in sterile NaCl and incubated in fragments of 500 mg, freely floating in 5 ml Medium 199 (Sigma, Brøndby, Denmark; consisting of 25 mM Hepes, pH 7.4 and 1% bovine serum albumin) at 37 °C, 5% CO_2. After an equilibration period of 24 h, various agents were added, and incubated for further 48 h before harvest of cell media. The following reagents were tested: GH (Novo Nordisk A/S, Bagsværd, Denmark; 50 μg/l), Dexamethasone (DXM, Sigma, Brøndby, Denmark; 20 nM), insulin (Actrapid, Novo Nordisk A/S, Bagsværd, Denmark; 100 nmol/l), IL-1β (Sigma, Brøndby, Denmark; 50 ng/l), IL-6 (R&D Systems, Abingdon, UK; 50 ng/l) and TNF-α (Sigma, Brøndby, Denmark; 10 ng/l). After addition, all tissues were incubated for 48 h before the media were collected and frozen at −20 °C for later analysis of IGFs and IGFBPs. Concomitantly, all adipose tissue fragments were quickly frozen in liquid nitrogen and thereafter kept at −80 °C for later extraction of mRNA. The chosen concentrations of the tested cytokines were based on previous experiences, based on similar tissue fragments [16]. The chosen concentrations of GH were based on pilot studies comparing GH doses ranging from 0 to 100 nM (i.e. 0 to 2200 μg/l), and tissues incubated for 24 and 48 h after the equilibration period of 24 h. These data showed that already at the lowest dose of GH (22 μg/l), IGF-I levels were maximally increased. Thus, we chose 50 μg/l for further experiments. Regarding the time experiments, levels of IGF-I and IGF-II were approximately 2 to 3 fold higher after 48 h of incubation as compared to 24 h. Thus, there was no indication that incubation time *per se* affected the GH responsiveness of the tissue fragments (pilot data not shown).

2.2. Determination of IGF-I and IGF-II protein levels in the cell media

IGF-I and IGF-II were extracted from the IGFBPs using the IGFBP-blocking principle originally described by Blum et al. [17]. In brief, the media assayed for IGF-I (250 μl) were added 40 μl 1.0 M acetic acid and 35 μl 1.0 M HCl. This reduced the pH to less than 2.5, sufficient to dissociate the IGFs from their IGFBPs. For determination of IGF-I, the acidic acid was added an excess of IGF-II (100 μg/l, obtained from Austral Biologicals, San Ramon, CA, USA). After incubation for 1 h at room temperature, the media were neutralized with 600 μl Wallac assay buffer (Perkin Elmer Life Sciences, Turku, Finland) and 75 μl 1.0 M NaOH, and assayed by an in-house IGF-I time-resolved immunofluorometric assay (TR-IFMA) as previously described [18]. The IGF-I assay calibrators were added the same amount of IGF-II, HCl and NaOH as the samples. IGF-II was extracted in a similar way as IGF-I, using acidic acid with an excess of IGF-I (100 μg/l obtained from Austral Biologicals, San Ramon, CA, USA), and analyzed by in-house IGF-II TR-IFMA [18]. At the chosen concentrations neither IGF-I nor IGF-II (100 μg/l) caused any significant cross reactivity when added in the heterologous IGF-immunoassays (data not shown). The recovery of the IGFBP-blocking procedure was investigated by the addition of increasing concentrations (from 1.0 to 100 μg/l) of IGF-I and IGF-II, respectively to the cell media prior to assay as described above. For both IGF-peptides the recoveries averaged 96 ± 2% (mean ± SEM). All IGF-samples were analyzed in one assay run in duplicates with a within assay coefficient of variation (CV) averaging 15% and 10% for IGF-I and IGF-II, respectively.

2.3. Determination of IGFBPs by Western ligand blotting and specific immunoassays

The cell media were assessed for IGFBPs by sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE) and Western ligand blotting (WLB) as originally described by Hossenlopp et al. [19]. The cell media were subjected to SDS-PAGE (10% polyacrylamide) under non-reducing conditions, using ^{125}I- IGF-I as tracer. The iodination was performed in-house using the chloramine-T method (^{125}I was obtained from Amersham, Buckinghamshire, UK). Approximately 100,000 CPM was added per sample. The resulting autoradiograms were scanned by laser densitometer (Shimadzu model CS 90001 PC, Shimadzu Europe GmbH, Duisburg, Germany). The resultant relative densities of the individual bands were expressed in arbitrary pixel density units (absorbency per mm^2). In addition, the IGFBPs were assessed by specific immunoassays directed against IGFBP-2 [20], IGFBP-3 (DiaSource Inc., Nivelle, Belgium) and IGFBP-4, -5 and -6 (R and D Systems, Abingdon, UK). The latter three assays were performed essentially as described by the manufacturer. Due to a limited sample volume, we had to collect new samples for determination of immunoreactive IGFBP-levels. However, the second cohort was comparable to the first, consisting of women (n = 9, age 42.3 ± 4.2 years, BMI 24.0 ± 1.2 kg/m^2) undergoing cosmetic abdominal liposuction. Immunoreactive IGFBP-levels were determined in the baseline condition only.

2.4. Real time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 250 mg frozen adipose tissue using Trizol reagents (Gibco BRL, Life Technologies, Roskilde, Denmark). RNA concentration was determined by absorbance at 260 nm, and purity was checked by a 260/280 ratio and by gel electrophoresis. RT-qPCR was performed with random hexamer primer as described by GeneAmp PCR kit (Perkin Elmer Cetus, Norwalk CT, USA). Real-time quantification of target gene expression to β-actin was made with a SYBER-Green real time PCR assay using an ICycler PCR machine from Bio-Rad Laboratories (Copenhagen, Denmark). The primers for target gene and β-actin are shown in Table 1. Quantification of results obtained by RT-qPCR was expressed as a cycle threshold (C_T). The relative gene expression of target gene to β-actin was calculated by the formulae "$2^{(CT\ target - CT\ \beta\text{-actin})}$" as previously described [21].

2.5. Statistics

All data are stated as mean ± SEM with the exception of mRNA levels which showed a skewed distribution and therefore are stated as median [25 and 75 interquartiles]. The 7 different treatments were compared by Friedman repeated measures analysis of variance on ranks, which if significant was followed by the Student–Newman–Keul's method for an all pair-wise multiple comparison. Comparisons of two groups were performed using the Wilcoxon signed rank test (paired observations) or the Mann–Whitney Signed rank sum test (unpaired observations) as appropriate. *P*-values below 0.05 were considered to be statistically significant.

3. Results

3.1. Baseline gene expression and protein release of IGFs

Both IGF-I and IGF-II mRMA were expressed and the protein secreted in readily detectable concentrations. In the RT-qPCR analysis of gene expression, the CT-values of IGF-I and IGF-II were 25.95 and 21.89, respectively. In comparison, the CT-value of beta-actin was