CLINICAL STUDY

Pro- and mature IGF-II during diet-induced weight loss in obese subjects

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Abstract

Background: In normal subjects up to 10% of circulating insulin-like growth factor II (IGF-II) consists of pro-IGF-II. However, its regulation and biological impact remains unknown. In obese subjects, serum free and total IGF-II are increased, and we therefore investigated the impact of obesity and diet on serum pro-IGF-II.

Design: Non-diabetic, obese subjects ($n = 34$) with a body mass index (BMI) of 38.9 ± 0.5 kg/m² were subjected to 8 weeks with very low calorie diet (800 kcal/day) followed by 12 weeks with a weight-stabilizing diet. Fasting serum was collected before the study, and after 8 and 20 weeks. Pro-IGF-II was determined after acid-gel chromatography using a novel, highly specific in-house assay, free and total IGFs were measured after ultrafiltration and acid-ethanol extraction, respectively, and IGF-binding proteins (IGFBPs) were measured with specific immunoassays.

Results: Diet reduced BMI and fasting levels of insulin and glucose ($P < 0.001$). Serum pro-IGF-II was markedly reduced in obese subjects as compared with matched normal-weight controls (means and 95% confidence intervals: 93 µg/l (82–104 µg/l) versus 171 µg/l (152–192 µg/l), respectively; $P < 0.001$), and levels remained unchanged after the weight loss. In contrast, during the study period total and free IGF-II decreased ($P < 0.05$), whereas total IGF-I, IGFBP-1 and IGFBP-2 increased ($P < 0.001$). Serum free IGF-I remained unaltered. Cross-sectional and longitudinal correlation analyses showed that pro-IGF-II was closer and more consistently associated with IGF-I than IGF-II.

Conclusion: This study demonstrates that pro-IGF-II is reduced in obesity, in contrast to mature IGF-II. This indicates a hitherto unrecognized link between nutrition and pro-IGF-II. In addition, our data indicate that pro-IGF-II is regulated independently of mature IGF-II.

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Introduction

The insulin-like growth factor (IGF) system plays a central role in the processes that control cell proliferation, differentiation and apoptosis (1, 2). IGF-I and IGF-II are the primary growth-promoting members of the IGF system, and in adult life they mediate their anabolic effects through interaction with the IGF-I receptor (IGF-IR), which is a transmembrane tyrosine kinase receptor distributed throughout the body (3). There are, however, other receptors which may be activated by the IGFs. The IGF-II/mannose 6-phosphate receptor (IGF-II/Man-6-PR) is a single-chain receptor with two different binding sites, one for IGFs and one for mannose 6-phosphate-containing proteins (4). The IGF-II/Man-6-PR recognizes IGF-II as well as IGF-I, the latter with lower affinity, while insulin does not bind (5). After ligand binding to the IGF-II/Man-6-PR, the entire complex is internalized and degraded, for which reason it has been suggested to serve as a

scavenger receptor, reducing peri-cellular levels of IGF-II (1, 6). Some controversy exists as to whether binding of IGF-II to the IGF-II/Man-6-PR elicits biological signals (4). Moreover, IGF-II binds with low affinity to insulin receptor (IR) subtype B (2% compared with insulin) and with high affinity to IR subtype A (15% compared with insulin) (7). The latter receptor is able to exert metabolic as well as mitogenic effects depending on whether it is activated by insulin or IGF-II (8).

The *Igf2* gene encodes a 180-amino acid protein known as pre-pro-IGF-II, which consists of a 24-amino acid signal peptide at the N-terminus, the 67-amino acid mature IGF-II peptide and an 89-amino acid extension (the E-peptide) at the C-terminus (9). After cleavage of the signal peptide, the 156-amino acid pro-IGF-II becomes glycosylated and cleaved in the Golgi apparatus, resulting in a 104-amino acid peptide (10). Two additional cleavages takes place, probably at the time of secretion, finally yielding mature 7.5 kDa IGF-II (11). A fraction of the

synthesized pro-IGF-II is not cleaved and exits the cell as larger peptides of 10–18 kDa, generally referred to as big IGF-II.

The pro-forms of IGF-II have been detected in serum as well as in cerebrospinal and amniotic fluids (12). In serum from healthy subjects the concentration of pro-IGF-II constitutes roughly 10–15% of circulating IGF-II (13, 14). The regulation and biological role of pro-IGF-II is unknown, but its insulin-like bioactivity has been shown to be 3-fold increased in the rat fat-cell bioassay as compared with mature IGF-II (14). Increased serum pro-IGF-II has been a key finding in non-islet cell tumour hypoglycaemia (NICTH), where it is thought to play a pathogenetic role in the development of hypoglycaemia (15, 16).

The aim of the present study was 2-fold: first, to learn more about the regulation of pro-IGF-II, we wanted to develop a specific immunoassay for measurement of levels in serum; second, we aimed to investigate the possible nutritional regulation of pro-IGF-II. For this purpose we studied lean and obese subjects. Obesity was chosen because this is a condition where serum levels of free as well as total IGF-II appear to be upregulated (17).

Materials and methods

Pro-IGF-II assay buffers

The pro-IGF-II assay was performed using the following buffers: coating buffer (40 mmol/l phosphate, pH 8.0, 9 g/l NaCl, 2 g/l human serum albumin (HSA), 2 ml/l Tween 20 and 1.6 g/l diethylenetriaminepenta-acetate (DTPA)), wash solution (50 mmol/l Tris/HCl, pH 8.0, 9 g/l NaCl, 5 ml/l Tween 20 and 0.5 g/l NaN₃) and assay buffer (400 mmol/l Tris buffer, 9 g/l NaCl, 2 g/l HSA, 2 ml/l Tween 20 and 1.6 g/l DTPA). The pH of the assay buffer was 8.50 before and 7.80 after mixing with an equal volume of 200 mmol/l acetic acid. The buffer used for acid-gel chromatography was 200 mmol/l acetic acid with 2 g/l BSA and 2 ml/l Tween 20. All buffer reagents were from Sigma-Aldrich (Brøndby, Denmark) with the exception of HSA, which was obtained from ICN Biomedical (Aurora, OH, USA).

Pro-IGF-II antigen and pro-IGF-II antibodies

Pro-IGF-II was detected by a novel, in-house, time-resolved immunofluorometric assay (TR-IFMA) based on commercial reagents. A polyclonal rabbit antibody (GroPep, Adelaide, Australia) directed against amino acids 78–88 of the E-peptide of recombinant human pro-IGF-II was used for coating. Since mature IGF-II consists of 67 amino acids, this coating antibody was highly specific for pro-IGF-II. Recombinant human pro-IGF-II containing amino acids 1–104 (GroPep) served as assay standard. A recombinant monoclonal rat IGF-II antibody (clone S1F2; Upstate Biotechnology, Charlottesville, VA, USA) was used for detection after

being labelled with europium using an Eu³⁺ labelling kit (PerkinElmer Life Sciences, Turku, Finland) according to the manufacturer's instructions.

Assay principle

Ninety-six-well microtitre plates pre-coated with polyclonal goat anti-rabbit IgG (PerkinElmer Life Sciences) were washed once with an automated plate washer and added 200 µl per well of pro-IGF-II antibody diluted 1:900 in coating buffer. After overnight incubation at 5 °C all wells were washed once, and then 100 µl assay buffer and 100 µl of either standard (a serial dilution of recombinant human pro-IGF-II ranging from 0.125 to 4.0 µg/l) or unknown sample was added. After mixing for 5 min, the microtitre plates were incubated overnight at 5 °C. The following day all wells were washed once and incubated at ambient temperature for 3.5 h with 200 µl Eu³⁺-labelled IGF-II antibody dissolved in coating buffer. Afterwards, all wells were washed six times, and 200 µl Enhancement Solution (PerkinElmer Life Sciences) added before time-resolved fluorometry was performed according to the manufacturer (PerkinElmer Life Sciences).

Assay validation

IGF-I and -II cross-reactivity was estimated by addition of serial dilutions up to 10 000 µg/l recombinant human IGF-I and -II (both from Austral Biologicals, San Ramon, CA, USA). The within-assay coefficients of variation (CVs) for standards and samples were calculated as the respective means. The between-assay CV was estimated from repetitive measurement of a pro-IGF-II standard (1 µg/l).

Serum extraction of pro-IGF-II

Pilot studies proved the necessity of extracting pro-IGF-II from the IGF-binding proteins (IGFBPs) prior to assay. Initially, we tried an acid-ethanol extraction method similar to the one used in our laboratory for mature IGF-I and -II (18), but this method yielded an unsatisfactory recovery (<80%) due to co-precipitation of pro-IGF-II. It was therefore chosen to separate pro-IGF-II from the IGFBPs by acid size-exclusion gel chromatography, using 20 ml open columns (1.5 × 12 cm; BioRad Laboratories, Hercules, CA, USA) packed with Sephadex G-50 Superfine (Amersham Biosciences, Uppsala, Sweden). These columns allowed acidified serum to be eluted within 1 h, enabling the processing of several serum samples within one working day. The elution protocol was as follows: 75 µl serum were added 325 µl 1 mol/l acetic acid and incubated for approximately 20 min at ambient temperature. Then 300 µl were loaded onto the column and eluted with chromatography buffer. Fractions of 1 ml were collected manually and assayed

for IGFBP-2, pro-IGF-II, and IGF-I and -II. The separation of pro-IGF-II from the IGFBPs was investigated by measurement of IGFBP-2 using a highly specific and sensitive in-house TR-IFMA as previously described (19). Mature IGF-I and -II were measured using previously described in-house TR-IFMAs (18). In all assays the acidified fractions were neutralized with 400 mmol/l Tris buffer, and assay standards contained the same amount of acetic acid as the unknown samples. Column recovery was assessed by acid-gel filtration of exogenous pro-IGF-II (100 µg/l dissolved in 0.2 mol/l acetic acid with 0.2% HSA) according to the protocol described above.

Other assays

Glucose was determined using the glucose oxidation method (Beckman Instruments, Fullerton, CA, USA). Serum insulin was determined with a commercially available kit (DakoCytomation A/S, Glostrup, Denmark). Total IGF-I and -II, free IGF-I and -II and IGFBP-1 and IGFBP-1-bound IGF-I were determined by validated in-house assays (18–21).

Clinical study

To study the possible nutritional regulation of pro-IGF-II, we compared fasting serum samples from age- and gender-matched groups of lean (11 males and 13 females, age 40.5 ± 2.2 years, body mass index (BMI) 23.6 ± 0.5 kg/m²) and non-diabetic obese subjects (13 males and 21 females, age 44.3 ± 1.6 years, BMI 38.9 ± 0.5 kg/m²). We only had access to a limited serum sample volume from the healthy controls, and therefore only pro-IGF-II was measured in this group. In contrast, we were able to perform a more detailed study of the changes in the circulating IGF system in the obese subjects before and after a diet-induced weight loss. During the diet program, the obese subjects received a very low calorie diet (VLCD; approximately 800 kcal/day) for 8 weeks followed by 12 weeks on a weight-stabilizing diet (a 600 kcal reduction in the total energy need per day; Fig. 1). The latter was estimated by calculations based on height, sex, level of physical activity and others. Fasting blood samples were obtained from the vein before commencement of the diet

Figure 1 Study design: obese subjects participated in a 20-week weight-loss program with 8 weeks on a VLCD followed by 12 weeks on a weight-stabilizing diet.

(baseline), after the 8 weeks of VLCD and again at the end of the study after a total of 20 weeks of diet. Serum was separated and frozen at -20°C until analysis. The diet study was performed at Medical Department C, Aarhus University Hospital, Denmark. Metabolic data from the diet study have been published previously (22). All patients gave their informed written consents, and the study was approved by the local ethics committee.

Statistics

All data were natural-log-transformed prior to statistical analysis to improve the normal distribution, which was subsequently confirmed by normality plots for each variable. Changes over time were assessed with one-way analysis of variance (ANOVA) for repeated measurements. A significant ANOVA was followed by the Student–Newman–Keuls method for pairwise multiple comparisons. For comparison of two independent groups, Student's unpaired *t*-test was used. Correlation coefficients were made to relate changes in the IGF system to changes in BMI/insulin, and to look for possible modifiers of pro-IGF-II levels. A *P* value of less than 0.05 was considered statistically significant. Data are means $\pm$ S.E.M.

Results

Pro-IGF-II assay

A representative standard curve is shown in Fig. 2. The non-specific background signal averaged ~ 600 c.p.s., and the lowest pro-IGF-II standard was ~ 1300 c.p.s. The assay detection limit (non-specific background signal + 3 S.D.) was estimated at 0.08 µg/l. The within-assay CVs for standards and samples averaged 2.5 and 3.7%, respectively (seven set-ups). The between-assay CV was 10%. The pro-IGF-II TR-IFMA was highly

Figure 2 A representative standard curve of the pro-IGF-II TR-IFMA. Data are means $\pm$ S.E.M. CPS, counts per second.

Figure 3 The average elution profile of four normal serum samples. Serum (75 µl) was acidified with 350 µl acetic acid (1 mol/l), incubated for 20 min at ambient temperature, whereafter 300 µl was loaded onto a 20 ml open column containing Sephadex G-50 superfine gel. The column was eluted with 200 mmol/l acetic acid containing BSA and Tween 20. Fractions of 1 ml were collected and assayed as described in the Materials and methods section.

specific: IGF-I showed no detectable cross-reactivity and IGF-II yielded a signal of less than 0.2 µg/l when added at a concentration of 10 000 µg/l. Conversely, pro-IGF-II (up to 10 000 µg/l) did not cross-react in the IGF-I assay, but cross-reacted with 20% in the IGF-II assay.

An acid-gel chromatography profile based on the mean of four normal serum samples is shown in Fig. 3. The 20 ml columns provided an almost complete separation between pro-IGF-II and the IGFBPs as estimated by measurement of IGFBP-2, and based on these profiles we decided to pool the fractions eluting between 12 and 19 ml for later analysis of pro-IGF-II (Fig. 3). As compared with the overall amount, this 7 ml pool contained more than 97% of the total eluted amount of IGF-I, IGF-II and pro-IGF-II. After acid-gel chromatography, the recovery of exogenous pro-IGF-II was estimated to be 105%. We assessed NICTH serum for pro-IGF-II levels after acid-gel chromatography and found that levels were four or five times higher than in control serum obtained from healthy blood donors (data not shown). The column-to-column CV was 4%. The overall CV of unknown samples including column-to-column and assay-to-assay variation was estimated to be 14%

(one serum sample gel-filtrated 18 times on different days and different columns).

Clinical study

Twenty weeks of diet reduced the body weight, BMI and fasting levels of insulin and glucose (Table 1). Serum pro-IGF-II was markedly reduced in obese subjects compared with matched normal-weight controls (mean and 95% confidence interval: 93 µg/l (82–104 µg/l) versus 171 µg/l (152–192 µg/l), respectively; $P < 0.001$), and levels remained unchanged after weight loss (Table 1, Fig. 4). In contrast, both total and free IGF-II decreased after diet (Table 1, Fig. 5). Total IGF-I showed a gradual increase over the period, whereas free IGF-I remained constant (Table 1, Fig. 5). IGFBP-1 and -2 both increased during the diet (Table 1, Fig. 6). The binary complex consisting of IGF-I bound to IGFBP-1 increased (Table 1, Fig. 6).

Changes in BMI and insulin were compared with changes in IGF parameters by correlation analysis (Table 2). Interestingly, longitudinal changes in pro-IGF-II levels from baseline to weeks 8 and 20, respectively, correlated with changes in total IGF-I levels (Table 2). Furthermore, a cross-sectional analysis

Table 1 Metabolic and IGF-related parameters during the diet study. Data are given as means $\pm$ S.E.M.

	Baseline	Week 8	Week 12	ANOVA <i>P</i> -value
BMI (kg/m ²)	38.9 $\pm$ 0.5	34.5 $\pm$ 0.6	33.8 $\pm$ 0.7	<0.001
Weight (kg)	114.3 $\pm$ 2.6	101.6 $\pm$ 2.4	99.3 $\pm$ 2.6	<0.001
Insulin (pmol/l)	101 $\pm$ 7	74 $\pm$ 5	81 $\pm$ 6	<0.001
Glucose (mmol/l)	6.3 $\pm$ 0.1	5.2 $\pm$ 0.2	5.3 $\pm$ 0.2	<0.001
Pro-IGF-II (µg/l)	93 $\pm$ 5	96 $\pm$ 7	100 $\pm$ 8	NS
Total IGF-II (µg/l)	1124 $\pm$ 48	1018 $\pm$ 43	1043 $\pm$ 44	0.049
Free IGF-II (µg/l)	4.16 $\pm$ 0.25	3.75 $\pm$ 0.19	3.51 $\pm$ 0.23	0.02
Total IGF-I (µg/l)	111 $\pm$ 5.5	127 $\pm$ 8.6	141 $\pm$ 9.2	<0.001
Free IGF-I (µg/l)	1.32 $\pm$ 0.09	1.48 $\pm$ 0.10	1.33 $\pm$ 0.09	NS
IGFBP-1 (µg/l)	13.5 $\pm$ 1.8	21.5 $\pm$ 3.8	23.8 $\pm$ 4.0	<0.001
IGFBP-2 (µg/l)	347 $\pm$ 22	505 $\pm$ 38	433 $\pm$ 29	<0.001
IGFBP1 bound IGF-I (µg/l)	2.4 $\pm$ 0.6	4.3 $\pm$ 0.5	5.8 $\pm$ 1.4	<0.001

NS, not significant.

Figure 4 Pro-IGF-II levels in obese and normal-weight subjects. Columns represent (left to right) levels in obese subjects at baseline, 8 weeks and 20 weeks, and levels in normal-weight controls (means $\pm$ S.E.M.). $\diamond P < 0.05$ compared with baseline, 8 weeks and 20 weeks.

unmasked a correlation between pro-IGF-II and both total and free IGF-I levels at all three sample times (baseline, 8 weeks and 20 weeks; Table 3).

Discussion

The present study describes the development of a highly specific and sensitive sandwich assay for detection of

human pro-IGF-II, and its application in serum samples from lean and obese subjects. The assay is based on an immobilized polyclonal antibody directed against the E-peptide of pro-IGF-II, and a monoclonal detection antibody directed against mature IGF-II. This principle ensures that intact pro-IGF-II is fully detectable, whereas any free E-peptide is ignored. Using this assay we have been able to demonstrate that pro-IGF-II is reduced in obesity, indicating a hitherto unrecognized link between nutrition and pro-IGF-II.

A number of methods have been described for measurement of pro-IGF-II. The first assessment of pro-IGF-II levels was made by Daughaday *et al.* (23) using acidic size-exclusion gel chromatography and subsequent IGF-II measurement of the collected fractions by RIA based on a polyclonal antibody against IGF-II. This yielded an elution profile of the IGF-II immunoreactivity, and by calculating the peak sizes the ratio of pro- to mature IGF-II was determined (23). Using a similar technique, Zapf *et al.* (14) found three peaks of IGF-II immunoreactivity: one for pro-IGF-II, one for IGF-II dimers and one for mature, monomeric IGF-II. The fractions corresponding to the first peak was assessed in the IGF-II assay and the result interpreted as pro-IGF-II levels (14). These methods gave important information on the molecular size of pro-IGF-II and eliminated interference from the

Figure 5 Free and total IGF levels in obese subjects. Columns represent (left to right) levels in obese subjects at baseline, 8 weeks and 20 weeks (means $\pm$ S.E.M.). $\square P < 0.05$ compared with baseline; $\Delta P < 0.05$ compared with baseline and 8 weeks.

Figure 6 IGFBP-1, IGFBP-2 and IGFBP-1-bound IGF-I levels in obese subjects. Columns represent (left to right) levels in obese subjects at baseline, 8 weeks and 20 weeks (means $\pm$ S.E.M.). $\square P < 0.05$ compared with baseline; $\triangle P < 0.05$ compared with baseline and 8 weeks.

IGFBPs. Major drawbacks are the multiple IGF-II determinations required per patient sample and the time-consuming methodology. In addition, it has been speculated that the use of an antibody directed against mature IGF-II due to a lower affinity for pro-IGF-II may result in an underestimation of pro-IGF-II levels (13).

The development of specific RIAs based on antibodies recognizing various parts of the E-peptide has facilitated the measurement of pro-IGF-II (12, 24–26). Apparently, these RIAs do not require extraction of the IGFBPs prior to analysis (24). By use of RIAs directed against the E-peptide of pro-IGF-II, a variety of biological fluids and patient categories have been assessed. Compared with normal serum, elevated levels were found in patients with NICTH, chronic renal failure and acromegaly, as well as in amniotic

and seminal fluids. Serum and plasma samples from patients with growth hormone (GH) deficiency and type 1 diabetes, pregnant women and foetal chords contained levels fully comparable to those observed in normal sera. Low levels of E-peptide immunoreactivity were found in cerebral spinal fluid (12, 26). However, it is important to recognize that these E-peptide-specific RIAs provide no distinction between free E-peptide and intact pro-IGF-II. Therefore, van Doorn *et al.* (9) developed a sandwich assay similar to ours, with a coating antibody specific for the first part of the E-peptide (amino acids 68–88) and a detection antibody against mature IGF-II (amino acids 1–67). Using this assay, the authors were able to compare measurements of E-peptide by RIA and ELISA and they found a substantial discrepancy between the two methods (9). Thus, the demonstration of elevated E-peptide serum levels by the RIA needs to be accompanied by a more thorough analysis of the molecular size of the immunoreactive signal to differentiate between free E-peptide and intact pro-IGF-II. The assay developed in our laboratory detects only E-peptide when it is part of pro-IGF-II and is therefore highly specific for pro-IGF-II. Furthermore, the serum levels of pro-IGF-II comprised approximately 10% of total IGF-II in healthy subjects, but much higher levels in patients with NICTH, in line with findings by other groups (9, 12, 14, 26).

The obese subjects investigated in this study experienced a significant diet-induced weight loss followed by changes in the circulating IGF system: total and free IGF-II declined, whereas pro-IGF-II remained unaltered. As a novel finding, pro-IGF-II levels were suppressed in obese subjects when compared with age-matched controls. Total IGF-I levels increased, whereas free IGF-I remained constant. Both IGFBP-1, IGFBP-2 and IGFBP-1-bound IGF-I increased during the weight loss.

Previous studies have indicated that serum levels of free and total IGF-II are elevated in obese subjects. In this study it was not possible to directly compare IGF-II concentrations in lean and obese subjects, but nevertheless the present obese cohort showed markedly higher levels of free and total IGF-II than previously observed in lean subjects with a similar age, thereby confirming earlier findings (17, 20, 27). We now show that a diet-induced weight loss is accompanied by a reduction in free as well as total IGF-II levels, which thereby approach values in normal-weight subjects. This suggests a significant influence of nutrition on IGF-II levels. In favour of this hypothesis, significant positive correlations were observed between changes from baseline in fasting levels of serum insulin and those of total IGF-II. However, the present data do not allow us to conclude whether the correlation between IGF-II and insulin is causal or simply illustrates a biologically unrelated covariation.

To our knowledge, the nutritional regulation of pro-IGF-II levels has not previously been investigated.

Table 2 Linear regression analysis of longitudinal data. Only significant correlations have been included.

		Change from baseline to week 8		Change from baseline to week 20	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
BMI vs	Total IGF-I			−0.49	0.004
	Total IGF-II	0.39	0.02		
	IGFBP-2	−0.65	<0.001	−0.60	<0.001
	IGFBP-1-bound IGF-I	0.46	0.02		
Pro-IGF-II vs	Insulin			0.51	0.02
	Total IGF-I	0.51	0.003	0.67	<0.001
	Total IGF-II	0.43	0.01		
	Insulin	0.37	0.04		
Total IGF-II vs	Glucose	0.43	0.01		
	Insulin	0.39	0.02	0.39	0.02
	IGFBP-2	0.37	0.03		
IGFBP-1 vs	Free IGF-I	−0.39	0.048		
	IGFBP-1-bound IGF-I	0.56	0.02		
IGFBP-2 vs	Free IGF-I	−0.44	0.03		
	Total IGF-II	0.37	0.03		
	IGFBP-1-bound IGF-I	0.55	0.003		

Our data indicate that pro-IGF-II is affected by the presence of obesity and that levels are less responsive to weight changes than the rest of the IGF system. Contrary to expectations, pro-IGF-II levels were markedly reduced in obese subjects as compared with matched controls. Thus, although pro-IGF-II did cross-react with 20% in our IGF-II assay, this is unlikely to explain the upregulation of mature IGF-II levels in obesity. It could be speculated that there is an increased enzymatic conversion of pro-IGF-II to mature peptide in obese subjects, but this remains to be clarified. On the other hand, during the study longitudinal changes in pro-IGF-II correlated positively with those of total IGF-I, and a cross-sectional analysis of data also showed positive correlations between pro-IGF-II and total IGF-I, as well as free IGF-I. Similar correlations between pro- and mature IGF-II were not found. These findings indicate that pro-IGF-II may be regulated independently of IGF-II. Supportive of this notion, Tally *et al.* (26) found normal pro-IGF-II levels in GH deficiency patients ($n = 9$) despite low IGF-II levels.

In lean subjects, dietary restriction is accompanied by a gradual decline in serum total IGF-I, which becomes apparent within a few days of dieting (28). In contrast, obese subjects appear to be more resistant to dietary restriction: thus, two studies have reported unchanged levels of serum total IGF-I after 3 weeks of diet (resulting in a weight loss of 5 kg) (29) as well as after 13 weeks of diet (resulting in a weight loss of 15 kg) (30), whereas others have observed a 40% increase in serum total IGF-I after a massive diet-induced weight loss of 30 kg, resulting in a decline in mean BMI from 38.5 to 27.8 kg/m² (31). In the present study the diet resulted in an average weight loss of 15 kg (an approximate reduction in mean BMI from 38.9 to 33.8 kg/m²) and a 27% increase in serum total IGF-I. The reason for the different responses in serum total IGF-I in lean and obese subjects remains unknown. However, insulin sensitivity increases after weight loss and this may be of importance, because insulin appears to affect IGF-I production directly as well as indirectly. Studies in cultured hepatocytes have shown that insulin stimulates IGF-I mRNA and

Table 3 Linear regression analysis of cross-sectional data.

		Baseline		At 8 weeks		At 29 weeks	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Pro-IGF-II vs	Total IGF-I	0.57	<0.001	0.50	0.003	0.68	<0.001
	Free IGF-I	0.60	0.001	0.40	0.02	0.41	0.02
	Total IGF-II	0.27	NS	0.32	NS	0.33	NS
	Free IGF-II	0.28	NS	0.28	NS	0.59	<0.001
	IGFBP-1	−0.45	0.008	−0.19	NS	0.05	NS
	IGFBP-2	0.04	NS	0.05	NS	0.11	NS
	Insulin	0.14	NS	−0.19	NS	−0.17	NS
Age vs	Total IGF-I	−0.43	0.01	−0.63	<0.001	−0.50	0.003

NS, non-significant.

peptide synthesis in the absence of GH, and that the effects of insulin are additive to those of GH (32). In addition, insulin appears to be essential for GH-stimulated hepatic IGF-I production through an upregulation of hepatic GH binding at the receptor level (33). Thus we speculate that the increase in serum total IGF-I is caused primarily by a diet-induced increase in the hepatic insulin sensitivity.

Obese subjects are characterized by a marked GH hyposecretion, but they are not GH-deficient since serum total IGF-I remains within the normal range (34, 35). This paradoxical finding has been explained by concomitant hyperinsulinaemia. Increased portal levels of insulin may at the same time upregulate the hepatic GH receptor density and hence GH sensitivity, reduce serum levels of IGFBP-1 as well as IGFBP-2 and increase free IGF-I levels (33, 36, 37). These changes are likely to reinforce the feedback inhibition of circulating IGF-I on the pituitary GH secretion, thereby contributing to the obesity-related hyposomatropinaemia (34). Weight loss, on the other hand, is known to normalize the secretion of GH, and in some obese cohorts it also increases serum total IGF-I (31). In the present study the diet-induced weight loss caused a significant increase in serum total IGF-I, IGFBP-1-bound IGF-I, IGFBP-1 and IGFBP-2, while free IGF-I remained constant and above the levels previously reported in lean subjects (17, 20, 34). Normally, overnight fasting levels of free IGF-I are correlated positively with total IGF-I and inversely with IGFBP-1 and -2 (34). Thus we speculate that the positive effect of an increase in serum total IGF-I on free IGF-I is outbalanced by the increase in serum IGFBP-1 and IGFBP-2. Supportive of this hypothesis we observed an increased formation of IGFBP-1-bound IGF-I after the diet.

In conclusion, we describe a highly specific as well as sensitive and precise TR-IFMA for measurement of pro-IGF-II in human serum. Using this assay we found that serum pro-IGF-II was markedly reduced in obese subjects, despite elevated serum levels of free and total IGF-II. Theoretically, this finding may be explained by an increased conversion of pro-IGF-II to mature peptide, but this remains to be proven. On the other hand, pro-IGF-II appeared to be less influenced by weight loss than the other components of the circulating IGF system, and cross-sectional as well as longitudinal correlation analyses indicated that pro-IGF-II was more closely associated with IGF-I than IGF-II. Thus we speculate that pro-IGF-II is regulated independently of mature IGF-II. However, further studies are needed to explore this hypothesis.

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