FEBS Letters 348 (1994) 185–191

FEBS 14216

Free insulin-like growth factors (IGF-I and IGF-II) in human serum

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Received 25 May 1994

Abstract

Using ultrafiltration by centrifugation we have isolated the free, unbound fractions of insulin-like growth factor I and II (free IGF-I and IGF-II) in human serum. In this way near in vivo conditions could be maintained before and during isolation. The recovery was 80 to 100% in the ultrafiltrates, which contained no detectable amounts of IGF-binding proteins (IGFBPs) as measured by Western ligand blotting and IGFBP-1 and IGFBP-3 immunoassays. The concentration of free peptides was measured in two ultrasensitive non-competitive IGF-I and IGF-II time-resolved fluoroimmunoassays. We found that (i) equilibrium between free and protein-complexed IGF was strongly dependent on re-establishment of in vivo conditions (temperature, pH, ionic milieu and dilution); (ii) metabolic events (glucose load and fasting) caused significant changes in free IGF-I and IGF-II levels *without* concomitant changes in total circulating levels of IGFs; (iii) in 49 healthy adult subjects (20 to above 60 years) free IGF-I was inversely related to age and ranged from 950 ± 150 ng/l (mean ± S.E.M.) (20–30 years) to 410 ± 70 ng/l (>60 years). The relative percentage was, however, unchanged, being 0.38 ± 0.02% of total IGF-I. In contrast, free IGF-II was independent of age, being 1,480 ± 80 ng/l (~0.20 ± 0.01% of total IGF-II).

Key words: IGF-I; IGF-II; Ultrafiltration; IGFBP; Immunoassay

1. Introduction

The insulin-like growth factors (IGF-I and IGF-II) exist in human serum complexed to specific proteins, the insulin-like growth factor binding proteins, of which 6 are known (IGFBP-1 to IGFBP-6). These proteins are believed to modulate IGF-actions by inhibiting or possibly in some situations enhancing the effects of IGFs on target cells [1]. However, by analogy with sex and adrenal steroids and thyroid hormones, the unbound moiety may be the dominating fraction for the biological activity [2]. The methods which have been used to obtain an estimate of the concentration of free IGF-I and IGF-II include ultracentrifugation [2], size-exclusion gel chromatography (SEC) [3–7], high pressure liquid chromatography (HPLC) [8–11] and reverse phase chromatography [12]. Recently, immunochemical analyses based on direct determination of free IGF-I in serum using antibodies directed against the unbound peptide have been described [13,14]. However, the fraction of unbound IGF-I determined with these methods has varied considerably: from undetectable levels [2,7,9] to 19% of total IGF-I [4]. Free IGF-II has been estimated to be about 1% of total IGF-II [5]. This diversity is probably due to varying degrees of disturbance of equilibrium between free and IGFBP-complexed IGF. Maintenance or re-establishment of in vivo conditions (i.e. temperature, pressure and ionic milieu) is impossible using chromatographic separation, and the influence of sample dilution was unknown. Assay-incubation conditions approximating in vivo milieu are possible in immunochemical analyses. However, the interference of antibodies on the equilibrium between the IGFBPs and IGF-I and IGF-II is unpredictable and will vary in different samples.

The aim of the present study was to develop a method that under physiological circumstances (temperature, pH and ionic composition) isolates the free fractions of IGF-I and IGF-II in serum and leaves the equilibrium unchanged: this was attained using ultrafiltrates obtained by centrifugation at 37°C. The concentrations of total and free IGF-I and IGF-II were determined using two newly developed ultrasensitive non-competitive time-resolved fluoroimmunoassays (TR-FIAs) [15]. We (i) investigated the importance of re-establishing in vivo conditions before and during separation of free from bound peptide; (ii) compared the results with those obtained using HPLC separation; (iii) measured the concentrations of free IGF-I and IGF-II in (patho-) physiological situations, when endogenous IGFBP-1 concentrations had been manipulated; and (iv) measured free IGFs in healthy volunteers and subjects with abnormal growth hormone production (acromegaly and growth hormone deficiency (GHD)).

2. Experimental and results

All chemicals were of analytical grade, and if not otherwise stated, from Merck (Darmstadt, Germany). Human serum albumin (HSA) was obtained from Behring AG, Marburg, Germany.

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SSDI 0014-5793(94)00602-R

2.1. IGF-I and IGF-II assays

Measurement of IGF-I and IGF-II was performed by two sensitive non-competitive time-resolved fluorometric immunoassays (TR-FIAs) based on a modified FIA-technique: dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) [15]. Two sets of monoclonal IGF-I and IGF-II antibodies were used: the first was immobilised on microtest-plate wells (IGF-I: MAB 41, Novo Nordisk A/S, Bagsvaerd, Denmark; IGF-II: anti-rat IGF-II IgG, Upstate Biotechnology Inc., NY, USA), the second labelled with europium (IGF-I: clone 021, Diagnostic System Laboratories Inc., Webster, TX, USA; IGF-II: MAB 73, Novo Nordisk A/S, Bagsvaerd, Denmark). Biosynthetic hIGF-I (Amgen Biologicals, CA, USA purchased from Amersham Int., Amersham, Bucks, UK) and recombinant human IGF-II (Austral Biologicals, San Ramon, CA, USA) served as standards. IGF-I and IGF-II cross-reactivity in heterologous assays was below 0.0002%. The detection limits were 0.0025 μg/l and 0.010 μg/l for the IGF-I and IGF-II assay. The operating range was 0.005 μg/l to 2.5 μg/l (IGF-I) and 0.020 μg/l to 10.0 μg/l (IGF-II), and both calibration curves were linear in these intervals. Intra- and inter-assay coefficient of variation (CV) were less than 5% and 10%. *Total* IGF-I and IGF-II were determined in duplicates after acid ethanol extraction [16] in a final dilution of 1:1,000 (IGF-I) and 1:2,000 (IGF-II). *Free* IGF-I and IGF-II were determined in ultrafiltrates diluted 1:11 or 1:21; ultrafiltrates (2 or 3 per sample) were analyzed in single determinations.

2.2. Centrifugation procedure

The method of separating free from bound IGF was based on ultrafiltration by centrifugation. Amicon YMT 30 membranes and MPS-1 supporting devices were used (Amicon Division, W.R. Grace and Co. Beverly, MA, USA). Since batch to batch variation (rarely) occurred, is was necessary to check each batch for IGF-I and -II recovery and IGFBP-1 retention.

To remove particles, samples were prefiltered through a 0.22 μm filter (Millex-GV, Millipore S.A., Molsheim, France) before centrifugation at 300 × *g* (1,500 rpm) (Hettich Zentrifugen, Tuttlingen, Germany, model Rotixa/RP (swinging bucket rotor)). Ultrafiltrates were collected in 5 ml polyethylene (PE) tubes, which prior to centrifugation were pretreated in order to minimize peptide adsorption: the tubes were filled with 2 g/l HSA dissolved in purified water and left for 2 h at room temperature. After emptying 0.250 ml HSA solution was added to each tube and the content lyophilized.

2.2.1. Sample collection. Collection of ultrafiltrate must be initiated at a time when the IGF flux across the membrane is constant. However, some early non-specific adsorption of peptide to the YMT membrane and supporting device was observed: the concentration of IGF-I in the ultrafiltrate increased during the first 40 min, followed by a constant level in the intervals from 40 to 70, 40 to 100 and 40 to 130 min of centrifugation. Two typical profiles are shown in Fig. 1A. Pretreatment of the filters with insulin and/or Tween-20 did not further change this profile.

2.2.2. Sample volume. The relation between the applied sample volume, ultrafiltrate volume, and the resulting IGF-I content was investigated. For two sera 4 volumes (0.4, 0.6, 0.8 and 1.0 ml) were centrifuged and the ultrafiltrate corresponding to the interval 40 to 100 min collected. Fig. 1B shows the results from one of the sera. For both sera tested, sample volumes between 0.4 and

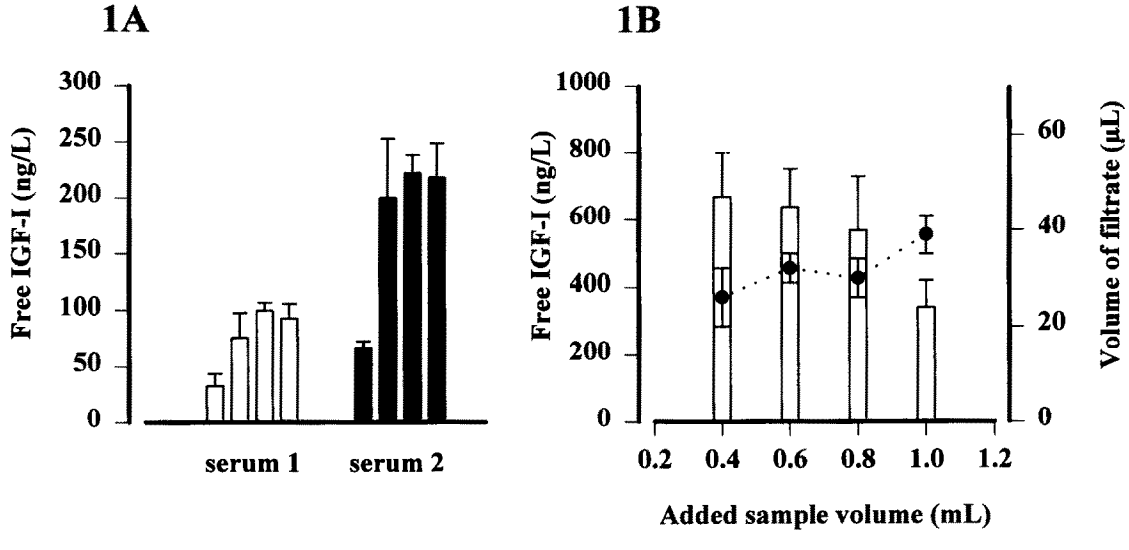

Fig. 1. (A) Sample collection: for each of the two profiles the 4 columns represent the IGF-I concentration obtained in the ultrafiltrates after 0 to 40, 40 to 70, 40 to 100 and 40 to 130 min of centrifugation, respectively. (B) Influence of sample volume on ultrafiltrate volume (circles; right Y axis) and IGF-I concentration (columns; left Y axis). Values are means ± S.E.M..

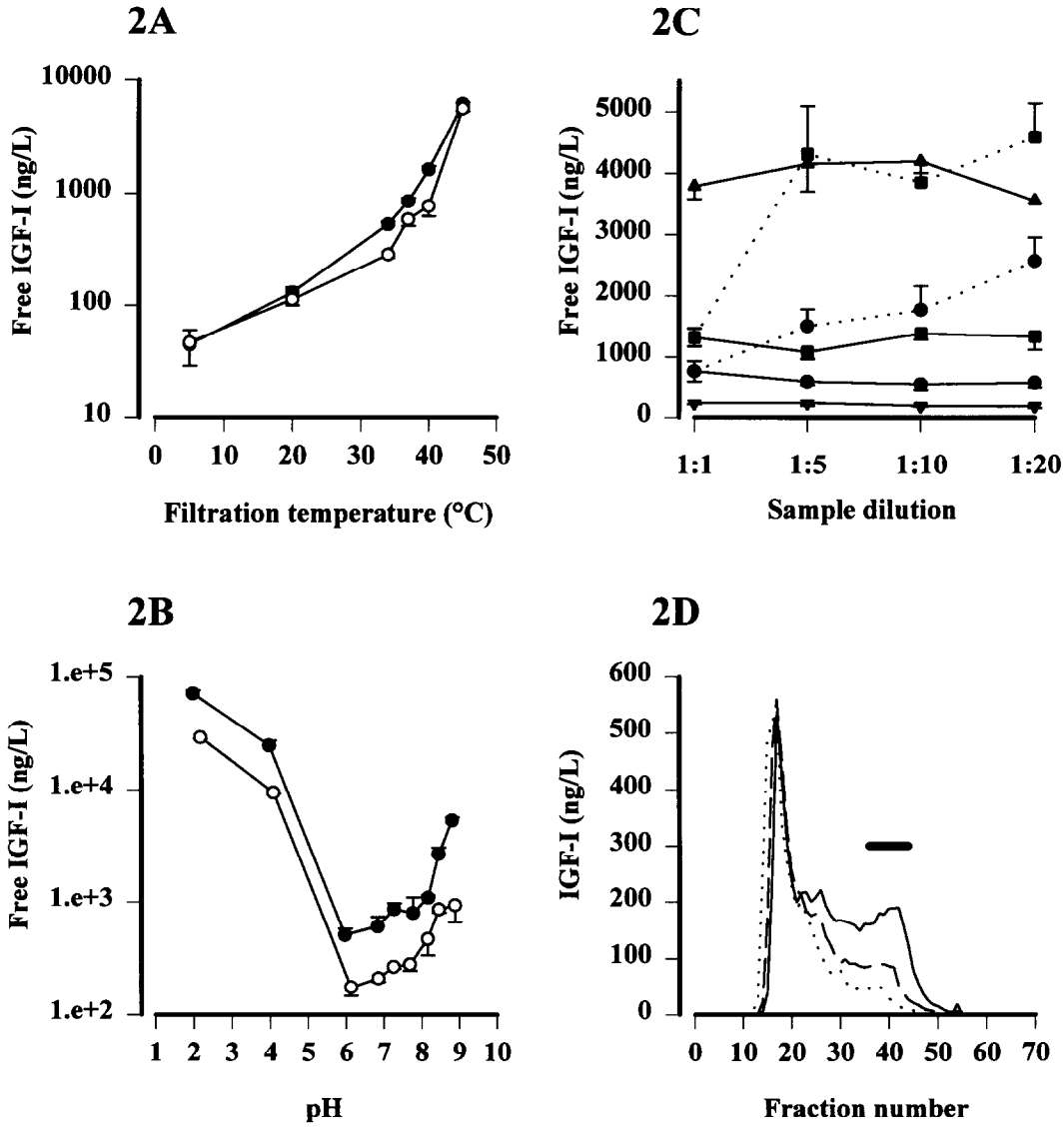

Fig. 2. Influence of (A) temperature and (B) sample pH on the concentration of free IGF-I in the ultrafiltrates for two sera obtained from healthy volunteers. (C) The influence of dilution in KRB (solid lines) in serum from two healthy volunteers (■ and ●), one acromegalic patient (▲) and one growth hormone deficient subject (▼). For comparison of the influence of ionic milieu on the concentration of free IGF-I, the two sera from the healthy volunteers were also diluted in Tris buffer (broken lines). All values are means ± S.E.M., where S.E.M. exceeds the size of the symbol. (D) Free IGF-I was separated from bound using HPLC. Three different flows were chosen: 0.25 ml per min (solid line), 0.5 ml per min (broken line) and 1.0 ml per min (dotted line). The IGF-I standard eluted in fraction 36 to 44 (solid bar).

0.8 ml resulted in similar ultrafiltrate volumes and IGF-I concentrations. Hence a sample volume of 0.5 ml was chosen for further experiments.

2.2.3. Analytical recovery. Recovery studies of exogenous IGF were performed in newborn calf serum containing only small amounts of endogenous IGF (total IGF-I ~30 μg/l and total IGF-II <2 μg/l). IGF-I (940 μg/l) and IGF-II (600 μg/l) were added to serum, centrifuged and the ultrafiltrate and concentrate (above the filter) analyzed for IGF-I and IGF-II. The volume of the ultrafiltrate (determined by weight) averaged 121 ± 8 μl (0 to 40 min, $n = 9$), 20 ± 1 μl (40 to 70 min, $n = 3$), 32 ± 4 μl (40 to 100 min, $n = 3$), and 46 ± 7 μl (40 to 130 min, $n = 3$). In these intervals, the recovery of IGF-I in the *ultrafiltrates* was 37 ± 3%, 89 ± 7%, 105 ± 2%, and 105 ± 9%, respectively; and of IGF-II 33 ± 2%, 82 ± 6%, 83 ± 5%, and 76 ± 15%, respectively. In the *concentrates*, which were collected after 70, 100 and 130 min of centrifugation, the recovery of IGF-I averaged 143 ± 2% and of IGF-II 116 ± 2%. Based on these findings all further experiments were performed on ultrafiltrates collected in the interval from 40 to 100 min of centrifugation.

2.2.4. Insulin-like growth factor binding proteins. The possible presence of IGFBPs in the *ultrafiltrates* was examined performing Western ligand blotting as described [17,18] on sera from 4 healthy volunteers: 2 μl serum and 10 μl ultrafiltrate were analyzed. Serum samples yielded five bands of IGFBPs with apparent molecular weights of 38–47 kDa (doublet) (~IGFBP-3), 32 kDa (~IGFBP-2), 28 kDa (~IGFBP-1), and 24 kDa (~IGFBP-4). Binding of ^{125}I-IGF-II to these species in ligand blotting experiments was specific, since co-incubation of radioligand with unlabelled IGF-II led to disappearance of binding. In contrast, neither simple inspection nor scanning (Shimadzu laser densitometer, model CS-9001PC) of autoradiographed ultrafiltrates revealed any trace of IGFBPs. Immunoassays for IGFBP-1 (IGFBP-1 ELISA, Medix Biochemica, Kainiainen, Finland: detecting limit 0.4 μg/l) and IGFBP-3 (IGFBP-3 RIA, Diagnostic System Laboratories Inc., Webster, TX, USA: detecting limit 1.0 μg/l) were also negative (data not shown).

In addition, membrane retention of rhIGFBP-1 (Medix Biochemica, Kainiainen, Finland) dissolved in newborn calf serum (130 μg/l) was examined. The concentration of IGFBP-1 in the ultrafiltrates were read as 0.3, 0.2 and 0.2 μg/l, respectively. Although below the detection limit given by the manufacturer of the assay (0.4 μg/l) these results suggested a minimum retention of 99.8%.

2.3. Importance of re-establishing in vivo conditions before and during separation of free IGF

2.3.1. Temperature. Two sera were centrifuged at 5, 20, 34, 37, 40 and 45°C. Before ultrafiltration sera were incubated for 30 min at the centrifugation temperature, which was checked at 0, 40 and 100 min of centrifugation. The result (Fig. 2A) demonstrates a strong, positive temperature dependency of free IGF-I and that maintenance of 37°C is critical. When compared to 37°C, the relative concentration of free IGF-I in the 2 sera was only 48% and 62% at 34°C and as much as 132% and 176% at 40°C. At 5 and 20°C, where separation of free and bound IGF takes places in most other techniques, the relative concentration was about 5–8% and 15–19%, respectively.

2.3.2. Hydrogen ion concentration. Two sera were adjusted to a range of pH-levels (2.0 to 9.0) using HCl or NaOH followed by incubation at 37°C for 30 min before centrifugation. After centrifugation, pH was measured again, and the final pH calculated as the mean of the two determinations (Fig. 2B). In both sera the concentration of free IGF-I followed a curve with nadir at pH 6, and with increasing concentrations at acid as well as alkaline pH levels. However, in the vicinity of pH 7.4 (from 7.27 to 8.17) a fairly constant level of free peptide was observed. Hence, prior to further ultrafiltrations samples were adjusted to pH 7.4 with CO_2.

2.3.3. Ionic milieu and sample-dilution. Two normal sera (total IGF-I was 70 μg/l and 270 μg/l, respectively) were diluted 1:1, 1:5, 1:10 and 1:20 in Krebs–Ringer bicarbonate buffer (KRB) (50 g/l HSA, pH was adjusted to 7.4 with CO_2) and Tris-buffer (300 mmol/l, pH 7.4, 50 g/l HSA). After dilution the samples were equilibrated for 30 min at 37°C. Within the investigated range, dilution in KRB was virtually without any effect on the estimate of free IGF-I, whereas dilution in Tris-buffer increased the concentration several-fold (Fig. 2C). Also results in serum obtained from one GHD and one acromegalic patient (total IGF-I was 40 μg/l and 1140 μg/l, respectively) were unaffected by dilution (Fig. 2C).

2.3.4. Comparison of HPLC-separation and ultrafiltration. HPLC was performed at room temperature on a Pharmacia Superdex 75–10/30 column (Pharmacia Biotechnology, Sweden) eluted with 200 mmol/l Tris, pH 7.4, 6 g/l NaCl, 2 g/l HSA, 0.5% (w/v) NaN_3, 0.1% (v/v) Tween-20 and 1.6 mg/l Titriplex V. Two sera from young healthy subjects (25 μl per sample) were chromatographed using 3 different flow rates: 0.25, 0.5 and 1.0 ml per min. Fractions of 250 μl corresponding to the elution of an IGF-I standard were collected and analyzed. For both sera immunoactive 'free' IGF-I eluted in strongly flow-dependent profiles; the result from one of the sera is shown in Fig. 2D. Hence, corresponding to a flow of 0.25, 0.5 and 1.0 ml per min the estimated 'free' IGF-I was 6.6, 3.1 and 2.2 μg/l (serum 1) and 8.7, 4.0 and 3.3 μg/l (serum 2). In contrast, free IGF-I as measured after ultrafiltration was 0.90 ± 0.09 μg/l (serum 1) and 1.18 ± 0.04 μg/l (serum 2).

2.4. Free IGF-I and IGF-II in different physiological and pathophysiological conditions

The ultrafiltration method was evaluated by measuring free IGFs under 'metabolic' conditions.

2.4.1. Oral glucose tolerance test (OGTT). After an overnight fast, an OGTT (75 g glucose dissolved in 150 ml) was performed on one healthy subject. Venous blood samples (10 ml) were collected at every 15 min for the first 3 h, and every 30 min for the next 3 h. Samples were analyzed for free and total IGF-I and IGF-II, glucose (glucose oxidase method), insulin (an inhouse DELFIA assay performed as previously described [19,20]), growth hormone (hGH) (DELFIA hGH kit, Wallac Oy, Turku, Finland) and IGFBP-1 (Fig. 3A–C). The observed changes in serum glucose, insulin and hGH were as expected. *Total* IGF-I and IGF-II were unchanged, being 170 ± 10 μg/l and 1,010 ± 10 μg/l, respectively. *Free* IGF-I and IGF-II exhibited almost parallel changes, both in an inverse manner of IGFBP-1: from a basal

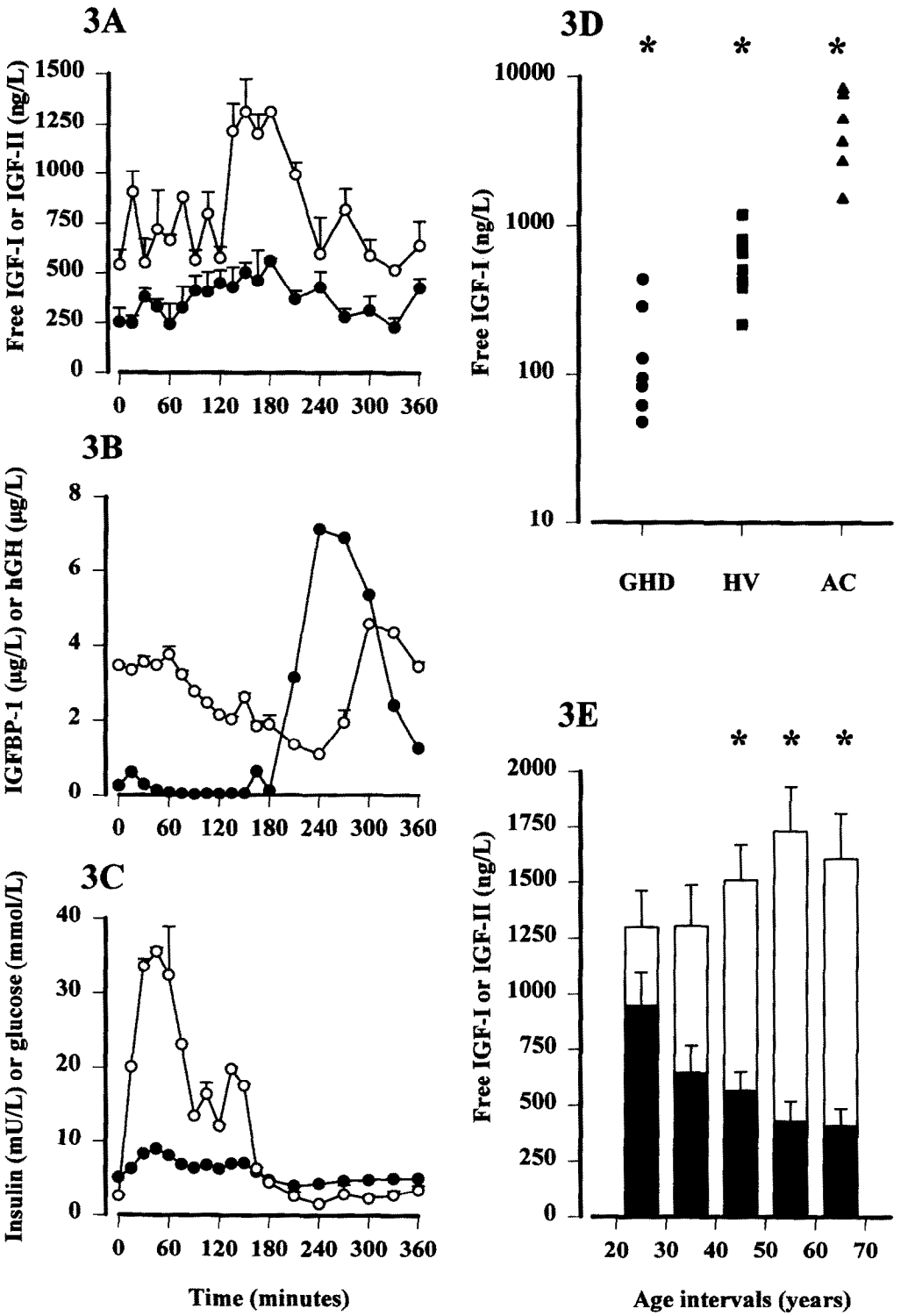

Fig. 3. The results from an oral glucose tolerance test are shown (A–C). (A) The changes in free IGF-I (●) and free IGF-II (○); (B) IGFBP-1 (○) and hGH (●); (C) glucose (●) and insulin (○). Values are means ± S.E.M., where S.E.M. exceeds the size of the symbol. The CV averaged 32% for free IGF-I and 23% for free IGF-II. (D) Illustrates free IGF-I levels from growth hormone deficient subjects (GHD (●); $n = 7$), healthy volunteers (HV (■); $n = 8$) and 6 acromegalic patients (AC (▲); $n = 6$). *$P < 0.01$ when compared to the other groups. (E) Shows the levels of free IGF-I (solid columns) and free IGF-II (open columns) in 49 healthy volunteers. *$P < 0.05$ when comparing free IGF-I levels in these groups to the youngest group (aged 20–29 years). Values are means ± S.E.M.

level of about 250 ng/l (IGF-I) and 550 ng/l (IGF-II) both peptides increased about 2-fold to maximum values of 550 ng/l (IGF-I) and 1,320 ng/l (IGF-II). Levels of free IGF-II were significantly higher than time-matched free IGF-I levels (Wilcoxon paired non-parametric test; $P < 0.001$). Concomitant with the increase in free IGF a 75% decrease in IGFBP-1 level was observed. Linear correlations were obtained between IGFBP-1 (independent variable) and free IGF-I (equation: $Y = 533-58X$ ($P < 0.01$); $r^2 = 0.36$) and free IGF-II (equation: $Y = 1174-135X$ ($P < 0.05$); $r^2 = 0.28$). In addition, levels of free IGF-I (independent variable) and -II correlated (equation: $Y = 292 + 1.35X$ ($P < 0.05$); $r^2 = 0.26$).

2.4.2. Fasting in healthy subjects. Free and total IGF-I and IGF-II were determined in 8 healthy subjects after 12 (overnight) and 60 h of fasting. During the extended fasting, IGFBP-1 levels increased from 2.6 ± 0.3 μg/l (mean ± S.E.M.) to 13.9 ± 2.4 μg/l ($P < 0.005$). *Total* IGF-levels were unchanged (IGF-I: 160 ± 20 μg/l (12 h) vs. 150 ± 20 μg/l (60 h); IGF-II: 770 ± 50 μg/l (12 h) vs. 810 ± 60 μg/l (60 h). In contrast, a significant decrease in levels of *free* IGF-I (620 ± 110 ng/l (12 h) vs. 290 ± 60 ng/l (60 h); $P < 0.05$) and IGF-II (1,410 ± 80 ng/l (12 h) vs. 860 ± 70 ng/l (60 h); $P < 0.01$) was observed. The CV of the free IGF-I and IGF-II determinations was 23%.

2.4.3. Free IGF-I in growth hormone disorders. After an overnight fast the concentration of free IGF-I was measured in 6 acromegalic patients (total IGF-I: 490 to 1,250 μg/l; free IGF-I: 1,530 to 8,490 ng/l) and 7 GHDs (total IGF-I: 20 to 90 μg/l; free IGF-I: 50 to 440 ng/l) and compared to the 8 healthy controls (total IGF-I: 110 to 290 μg/l; free IGF-I: 220 to 1,200 ng/l) (Fig. 3D). Despite some overlap between GHDs and healthy volunteers, the results were statistically different ($P < 0.01$ (non-parametric analysis of variance (Kruskal–Wallis) followed by Mann–Whitney)).

2.4.4. Normal material. Finally, total and free IGF-I and IGF-II were measured (in duplicates) in 49 healthy subjects. They were grouped in 5 age intervals each containing between 8 and 10 subjects (Fig. 3E). Free IGF-I was 950 ± 150 ng/l (20–29 years), 650 ± 120 ng/l (30–39 years), 560 ± 90 ng/l (40–49 years), 430 ± 90 ng/l (50–59 years) and 410 ± 70 ng/l (>60 years). Free IGF-I was significantly higher in the youngest group when compared to the 3 oldest (one way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons). However, when related to the total concentration, the percentage of free IGF-I was similar in the 5 groups: 0.38 ± 0.02%. In contrast, free IGF-II was independent of age, being 1,480 ± 80 ng/l (~0.20 ± 0.01% of total IGF-II). The CV of the single filtrations made on two different days was 33%. No correlations were found between free and total IGF levels, and between free IGF-I and free IGF-II.

3. Discussion

The method described is the first to isolate the free, unbound fractions of IGF-I and IGF-II under circumstances close to in vivo conditions. As shown it is of crucial importance to maintain physiological temperature, pH and ionic milieu; a finding in accordance with previous reports on determinations of free thyroid hormones [21,22]. The recovery in the ultrafiltrate of IGFs was between 80 and 100%, and the ultrafiltrate contained no detectable IGFBPs. Furthermore, changes in sample constitution (above filter) were minor: less than one third of the sample volume was removed during centrifugation, accompanying a modest increase in protein-content (16–43%). This appears to have little or no impact on equilibrium as seen in Fig. 1A. It is noteworthy that equilibria and ultrafiltrate concentrations have been shown to be unaltered during ultrafiltration by centrifugation for other protein-bound substances, even if the protein content is increased almost 2-fold [23].

The fraction of free IGFs has never unequivocally been demonstrated, but most studies find that the percentage of free IGF-I in healthy subjects accounts for 1–5% of total, extractable IGF-I [8,12,24]. Our results are considerably lower, being 0.38 ± 0.02% (range 0.14–0. 76%). However, all previous studies were based on separation procedures precluding in vivo conditions and hence in vivo-like equilibria.

The YMT membrane was selected among several candidates because of its resulting high recovery and IGFBP-free ultrafiltrate. Despite a CV of 23 to 33% this method is capable of detecting changes in free IGFs during physiological metabolic challenges such as glucose loads and fasting and the observed inverse relation between free IGF-I (and IGF-II) and IGFBP-1 is in accordance with suggestions put forward by others on theoretical basis [1,25]. Interestingly, the free peptide concentration seems to be a very sensitive indicator of early catabolism and growth hormone dysfunction and the obtained results suggest that the concentrations of free IGF-I and IGF-II may have greater physiological and clinical relevance than the total concentrations.

Acknowledgements: This study was supported by grants from the Danish Medical Research Council and Institute of Experimental Clinical Research, University of Aarhus. We are indebted to Mrs. K. Mathiassen for skilled technical assistance.

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