Responses of insulin-like growth factor binding protein-1 (IGFBP-1) and the IGFBP-3 complex to administration of insulin-like growth factor-I

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The recent availability of recombinant DNA-derived insulin-like growth factor-I (IGF-I) for use in human trials has raised the possibility of a variety of therapeutic applications; for example as a growth stimulant in GH-insensitivity syndrome (Laron dwarfism) and an insulin substitute in insulin-resistant subjects with type II diabetes mellitus. Endogenous serum IGFs circulate predominantly in a GH-dependent ternary complex with IGF binding protein-3 (IGFBP-3) and a non-IGF-binding glycoprotein, the acid-labile subunit or α-subunit (1, 2). Studies in rats suggest that IGFBP-3 levels are inducible by IGF-I, whereas the α-subunit is GH-dependent (3). In the complexed form, IGFs are greatly stabilized (4), and probably biologically inactive. Several other circulating IGFBPs are also thought to contribute to serum binding of IGFs; these include IGFBP-1, IGFBP-2 and IGFBP-4. IGFBP-1, which binds IGF-I and IGF-II with similar affinity (5), differs from other well-characterized IGFBPs in that its serum concentration is regulated dynamically under the influence of insulin and metabolic status (6–9).

Since circulating IGFBPs have the potential to influence the biopotency of administered IGF-I, it is important to determine the IGFBP responses to IGF injection and infusion. In this study we examine the responses of IGFBP-1 and the IGFBP-3 complex to a single subcutaneous injection of IGF-I in fasted subjects, and to daily IGF-I injections for 7 days in fed subjects.

Materials and methods

Recombinant human IGF-I

Recombinant human IGF-I for administration was kindly provided by Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. It was dissolved in physiological saline at a concentration of 6 g/l just before use.

Experimental procedures

All subjects entering the study had normal blood glucose levels and glycohemoglobin A1 values, and none showed glycosuria. All studies were performed with the approval of the Human Subjects Investigation Committee, Tokyo Women’s Medical College.

Study 1: 4 healthy adults aged 39–46 years received 0.1 mg/kg recombinant human IGF-I, and four others aged 31–44 years received a dose of 0.125 mg/kg. Subjects were fasted overnight, and subcutaneous injections made at 08.00 (time 0). Standard meals were taken via free access
6, 10, 24, 28 and 34 h after injection. Venous blood samples were drawn before and at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 30, 36 and 48 h after the injection.

Study 2: Details of the design of this study have been reported previously (10). In brief, healthy subjects aged 26–48 years were given 0.1 mg/kg IGF-I, or saline for the control group, by sc injection each day between 08.30 and 09.00 for 7 days. Breakfast was taken 30 min before each injection, and lunch, a snack and dinner were taken 4, 6 and 10 h after each injection. In the original study (10), six subjects received IGF-I and three received saline. In the present report, samples from only four subjects treated with IGF-I, and the three controls, have been analysed, due to insufficient serum from the remaining IGF-treated subjects. Blood samples were taken at 0, 1, 2, 3, 4, 6, 12 and 24 h after the first and seventh IGF-I administration. For all studies, serum or plasma samples were stored at −20°C before assay.

**Analytical methods**

Plasma IGF-I was measured by RIA (11) after extraction with acid-ethanol; free IGF-I was determined as previously described (10). Blood glucose was measured by autoanalyzer. Serum levels of IGFBP-1, IGFBP-3 and α-subunit were measured using previously described RIA methods (5, 12, 13). Serum insulin was measured by commercially available RIA kit with a detection limit of 2.5 mU/l, and urinary GH was measured as previously reported (14).

**Ligand blotting**

Five microliter aliquots of serum were fractionated, non-reduced, on 12% SDS-polyacrylamide gels overnight at 100 V, then proteins were transferred to Hybond-C Extra nitrocellulose membrane (Amersham, Bucks, UK) as previously described (15). IGFBPs were detected by ligand blotting with 125I-labeled IGF-II (15), followed by autoradiography using Hyperfilm MP film (Amersham) for 1–3 days at −70°C. Densitometry was performed on a Model 620 Video Densitometer (Bio-Rad, Richmond, CA). Since the IGFBP-3 doublet appeared as a single peak on densitometric analysis, no attempt was made to analyse the two bands separately. Due to the marked difference between the density of bands identified as IGFBP-3 and the other IGFBPs, autoradiographs exposed for different times were used for densitometry of the various proteins; therefore the arbitrary density units for IGFBP-3 are different from those for the other bands.

**Statistics**

For each study, treatment effects were first analysed by analysis of variance using a repeated measures design. In study 1, where the two IGF-I doses were tested with different groups of four subjects, sampling time was treated as a repeated measure, and IGF-I dose as a between factor. Initial analysis in this way showed no difference in IGFBP-1 response for the two IGF-I doses (F = 0.001, p = 0.980); therefore data for all eight subjects were combined for further analyses. Differences between time points were determined by Fisher’s Protected LSD test after an analysis of combined data, treating time as a between factor. In study 2, day of treatment and sampling time were treated as repeated measures in the initial analysis, with IGF-I dose (0 or 0.1 mg/kg) as a between factor. Subsequently, integrated 24-h IGFBP-3 and α-subunit values for each subject were calculated by averaging the eight values recorded over each 24-h period. Where indicated, group means were compared using two-tailed t-tests. Analyses were performed using Statview and SuperANOVA, Abacus Concepts, Berkeley, CA.

**Results**

Fig. 1 shows the serum IGFBP-1 and glucose responses over the 48-h period following IGF-I administration to eight fasted subjects at 0.1 or 0.125 mg/kg. As illustrated in Fig. 1a, a rapid response in IGFBP-1 was seen, with values peaking at 6 h at levels 10-fold higher than the zero-time fasting value. IGFBP-1 fell rapidly after 6 h, following food intake at that time. In a control study (not shown), five fasted subjects given saline alone showed no IGFBP-1 response.

Serum insulin levels, determined only in the four subjects receiving IGF-I at 0.125 mg/kg, fell rapidly in response to IGF-I from a fasting value of 9.7 ± 0.9 mU/l to mean levels of 4.6 ± 0.9, 4.3 ± 0.8, 4.6 ± 0.7, 4.7 ± 0.7 and 4.1 ± 0.6 mU/l at 2, 3, 4, 5 and 6 h, respectively. No insulin values fell below the detection limit of the assay (2.5 mU/l). Although 6-h IGFBP-1 peak values were strongly associated with initial insulin values (r = 0.979, p = 0.021), no significant correlations were seen with insulin values between 1 and 6 h after IGF-I administration.

Glucose levels declined by about 20% in response to IGF-I administration (Fig. 1b), with values from 2 to 6 h significantly lower than time 0 (p < 0.05). Following food ingestion at 6 h, a marked rise at 8 h was seen (p < 0.01 vs time 0). At subsequent sampling times, glucose levels did not differ significantly from the values recorded before IGF-I administration. We have previously hypothesized that serum IGFBP-1 might have a role in regulating insulin-like activity of IGFs (9); peak IGFBP-1 values might then influence subsequent blood glucose values. Fig. 2 shows the relationship between peak IGFBP-1 values, measured at 6 h, and post-prandial peak glucose values, measured at 8 h. The strong positive association (r = 0.941, p = 0.0005) suggests that the blood glucose response to a meal taken at 6 h may indeed be influenced by ambient IGFBP-1 levels.

Fig. 3 shows plasma IGF-I and IGFBP-3 values in this study. IGF-I rose rapidly after administration, remaining at peak levels for 6 h before gradually declining over the
next two days (Fig. 3a). In contrast to the rapid increase in IGFBP-1 values after IGF-I administration, IGFBP-3 showed only a slight change (Fig. 3b). After an apparent initial rise (not significant), IGFBP-3 levels declined between 6 and 36 h, with the 36-h value slightly lower than those determined from 0 to 10 h (p < 0.05).

In the second study, IGF-I was administered to fed subjects daily for seven days, and blood samples were drawn over 24-h periods following the first and seventh injections. Free IGF-I levels from this study have previously been reported (10). There was a significant difference between the pattern of free IGF-I on days 1 and 7 (p = 0.020), with the mean area under the curve slightly higher on day 7 than on day 1 (260 ± 11 μg/l vs 222 ± 22 μg/l, p = 0.019 by paired two-tail t-test). Fig. 4 illustrates the IGFBP-1 levels in these samples. Significant effects were seen for IGF treatment (p = 0.003), sampling day (p = 0.002) and time after injection (p = 0.0001). In these subjects who ate a meal 30 min before each injection the mean IGFBP-1 level fell rapidly over the first 2 h of sampling, and in the saline-treated subjects, did not exceed 30 μg/l at any time over the 24-h sampling periods on either the first or seventh days (Fig. 4a). In the subjects receiving IGF-I, however, a small increase in IGFBP-1, to a mean value (±SE) of 48.6 ± 15.4 μg/l, was seen between 4 and 6 h after the day 1 injection (Fig. 4b). Although this IGFBP-1 response is significantly different from that seen on day 1 in control subjects (p = 0.007), it is very minor compared to the dramatic IGFBP-1 rise seen in fasted subjects receiving a similar IGF-I dose, in whom peak values over 500 μg/l were observed (Fig. 1). This blunted response in fed subjects is consistent with the previously reported suppressive effect of food intake on IGFBP-1 levels (9).

In contrast to the minimal response to IGF-I seen on the first day of treatment, by the seventh day, the same IGF-I dose elicited a marked increase in IGFBP-1, with values reaching a mean 6-h peak of 174.5 ± 23.2 μg/l (Fig. 4b). This occurred despite food intake 30 min before, and 4 h after, receiving IGF-I. For IGF-I treated subjects, both the sampling time (p = 0.0001) and the duration of treatment (p = 0.006) were significant influences on IGFBP-1 levels. This suggests that the suppressive effect of food intake on the IGFBP-1 response to IGF-I injection, seen on the first day, is significantly overcome by repeated daily IGF-I injection.

IGFBP-3 responses were also studied in these subjects. Analysis of all IGFBP-3 data indicated that daily IGF-I injections for seven days had a significant effect on IGFBP-3 levels compared to saline injections (treat-
Fig. 3. Changes in (a) serum IGF-I levels and (b) serum IGFBP-3 levels in the eight subjects described in Fig. 1. Values are means±SEM.

Fig. 4. Serum immunoreactive IGFBP-1 levels in healthy subjects treated with (a) saline (N = 3) or (b) rhIGF-I, 0.1 mg/kg (N = 4) by daily sc injection for seven days. Blood was sampled over 24-h periods after the first (○) and seventh (●) injections. Subjects were fed 30 min before, and 4, 6 and 10 h after, each injection. Values are means ± SEM.

ment × days of treatment, p = 0.005). Different sampling times from 0 to 24 h after IGF-I or saline administration did not influence IGFBP-3 levels (p = 0.832). As shown in Fig. 5a, 24-h IGFBP-3 values in saline-treated subjects did not differ between day 1 and day 7. Integrated 24-h IGFBP-3 levels (i.e. the average of the eight recorded values) were 3.96 ± 0.75 mg/l on day 1 and 3.84 ± 0.80 mg/l on day 7 (mean ± sd). In contrast, in subjects treated with IGF-I for seven days (Fig. 5b), integrated 24-h IGFBP-3 levels fell by 20% from a mean (±sd) of 4.01 ± 0.65 mg/l on day 1 to 3.18 ± 0.43 mg/l on day 7 (p = 0.007, paired two-tail t-test). When samples were fractionated on a Superose-12 gel permeation column, there was no alteration in the apparent molecular weight distribution of immunoreactive IGFBP-3 after seven days of IGF-I treatment (not shown).

The α-subunit of the circulating ternary IGFBP complex appears to remain in a relatively constant two- to threefold molar excess over IGFBP-3 (13). To determine whether this excess is maintained even when IGFBP-3 levels decline, α-subunit levels were measured on the same serum samples. As shown in Fig. 6, daily injection with saline for seven days had little effect on α-subunit profiles. Integrated 24-h levels changed less than 10% between days 1 and 7 (24.3 ± 0.9 mg/l on day 1 vs 22.9 ± 0.2 mg/l on day 7).
As seen for IGFBP-3 levels, treatment for seven days with IGF-I caused a significant decline in z-subunit levels (Fig. 6b). Integrated 24-h values fell by 30% from a mean (±sd) of 26.6 ± 3.2 mg/l on day 1 to 19.0 ± 2.9 mg/l on day 7 (p = 0.003, paired two-tail t-test). Suppression of GH secretion by IGF-I administration was not apparent: the mean daily urinary GH excretion for the seven days of treatment ranged from 1.49 to 4.11 ng/g creatinine for IGF-I treated subjects, and from 1.67 to 2.52 ng/g creatinine for controls, with no difference between the means of the groups (p = 0.545).

To examine other IGFBP responses to IGF-I injection, the 24-h serum samples taken on day 7 from control and IGF-I treated subjects were fractionated by SDS-PAGE and probed with $^{125}$I-labeled IGF-II. Fig. 7 shows that there was a slight decrease in the density of the IGFBP-3 doublet band from the IGF-I treated subjects. When analysed by densitometry, a 10% decrease was apparent by this technique, from 67 ± 2 to 60 ± 5 arbitrary units (p = 0.088). In contrast, an IGFBP of approximately 35 kDa appeared markedly increased by this treatment (Fig. 7). A protein of similar size, inducible by infusion of IGF-I
in healthy subjects, has been identified as IGFBP-2 (16). When analysed by densitometry after autoradiography of ligand blots, the increase appeared to be threefold, from 7 ± 1 to 23 ± 3 arbitrary units (p = 0.0002). Another IGFBP, of approximately 25 kDa, resembled IGFBP-3 in showing a decrease after IGF-I administration, from 32 ± 6 to 17 ± 4 arbitrary units (p = 0.010). It is likely that this protein is IGFBP-4, although a definitive identification was not made. Other IGF-binding bands, of approximately 30 kDa, showed variable changes with IGF-I. Since it was not possible to identify these proteins, and they could not be resolved by densitometry, no quantitation was attempted.

Discussion

The hepatic production of IGFBP-1 is thought to be regulated by a cyclic nucleotide-dependent mechanism which can be activated by depriving the liver of metabolic substrate, and is potently inhibited by insulin (17, 18). Consistent with the concept of stimulation by substrate deprivation, insulin-induced hypoglycemia (9), fasting (9) and prolonged vigorous exercise (19) all greatly increase serum IGFBP-1 levels. However, since insulin, a potent suppressor of IGFBP-1 in vivo (6, 8), falls under these conditions, the stimulatory effect of limited metabolic substrate is presumably complemented by a decrease in the inhibitory effect of insulin.

In this study we have shown that a single sc injection of human IGF-I in fasting subjects causes a rapid rise in IGFBP-levels, with little effect on IGFBP-3. Although IGF-I caused blood glucose levels to fall, as demonstrated previously in fasted humans (20), hypoglycemia does not appear to be the major stimulus to IGFBP-1 secretion in these subjects, since the decline in glucose levels was minimal. Falling insulin levels after IGF-I administration may be an important stimulus to IGFBP-1 production. However, for the four fasting patients for whom insulin levels were measured, no significant association between insulin levels and the 6-h IGFBP-1 peak was seen at any time between 1 and 6 h after IGF-I administration. Owing to the small data set analysed in

![Fig. 7. IGF-II ligand blots on the 24-h samples taken on the seventh day from control (N = 3) and IGF-I treated (N = 4) subjects. Samples were probed with 125I-labeled IGF-II after SDS-PAGE. Upper panel: three-day exposure of autoradiograph. Positions of molecular weight markers, in kDa, are indicated by arrows. Lower panel: one-day exposure of the IGFBP-3 bands (i.e. bands at approximately 43 kDa) to show detail.](image-url)
this way, confirmation of the role of insulin will require further experimentation. It is also possible that free IGF-I itself directly stimulates IGFBP-1 production, as suggested by in vitro studies using fetal fibroblasts (21). However, this contrasts with the observation in fetal liver explant cultures, which do not increase IGFBP-1 production in response to IGF-I (22). Thus the mechanism by which IGFBP-1 production is stimulated by IGF-I injection remains unclear.

In contrast to the studies in which IGF-I was administered to fasting subjects, when the subjects were fed before receiving IGF-I, the IGFBP-1 response on day 1 was blunted by over 90%, with a mean peak value below 50 μg/l. This emphasizes the very prominent role that nutritional status plays in IGFBP-1 regulation. Unexpectedly, after seven days of IGF-I administration, the suppressive effect of food intake on the IGFBP-1 response to IGF-I appeared largely to have been overcome, with a marked IGFBP-1 response to IGF-I despite recent food intake. This altered responsiveness to IGF-I could not be attributed to different insulin or glucose responses to IGF-I administration, as these have been shown to be similar after the first or seventh IGF-I dose (10).

One role of IGFBP-1 might be to inhibit the insulin-like activity of circulating IGFs that are not sequestered in the ternary IGFBP-3 complex (23). In this way it might exert a counter-regulatory role in the maintenance of blood glucose levels, blocking available IGF activity during conditions, such as fasting, when excess insulin-like activity would be deleterious. The failure of IGF-I to cause a marked increase in IGFBP-1 in fed subjects, in contrast to the response in fasted subjects, is consistent with this role, since following food intake, insulin-like IGF activity might provide a useful supplement to the glucose-lowering effect of insulin. If this theory is correct, the observation that, after seven days of IGF-I administration, IGFBP-1 shows a strong response to IGF-I despite food intake, suggests that at this time the IGF-I dose might lead to increased available IGF activity compared to that resulting from the same dose on day 1. This increased IGF availability might result from a decrease in the concentration of the ternary complex, which sequesters the IGFs in an inactive form.

The concept that IGF-I, rather than GH, is the physiological regulator of IGFBP-3, is suggested by in vivo studies in rodents (3) as well as in cell culture (24). It was therefore surprising to find that administration of IGF-I to healthy subjects for seven days caused a significant decrease in immunoreactive IGFBP-3 levels. A similar decrease was observed in the acid-labile component of the high molecular weight IGF complex, the α-subunit. This concordance between changes in immunoreactive IGFBP-3 and α-subunit levels is also seen over the lifespan and in a variety of conditions including pregnancy and GH disorders (12, 13). If IGFBP-3 and the α-subunit are indeed both GH-dependent in man, then the observed decrease in their concentrations could result from a suppression of GH secretion by IGF-I. While such suppression might be expected, the lack of change in urinary GH excretion over the treatment period did not support this explanation.

In this study, the 24-h integrated level of free IGF-I seen after the seventh daily IGF-I injection was slightly greater than that seen after the first. Overall, however, there were minimal changes in 24-h IGF-I levels between the first and seventh days, as previously reported (25). In contrast, 24-h IGF-II levels were significantly lower after seven days than on the first day (25). Since steady-state IGF-II levels may simply reflect binding sites on IGFBP-3 that are unfilled by IGF-I (12), the low IGF-II probably results from a decrease in IGFBP-3 in the face of continued high IGF-I levels.

The cause of the decline in IGFBP-3 levels, however, remains unexplained. This finding contrasts with that reported by Zapf et al. (16), who measured IGFBP-3 levels by ligand blotting in two subjects infused with IGF-I for six days. In that study, a marginal, transient increase in IGFBP-3 was seen, which was enhanced when GH was co-infused with IGF-I, and it was also noted that IGF-I caused some of the IGFBP-3 to shift from the 150-kDa region to a lower apparent size. No change in size distribution was seen in the present study, when analysed by RIA after gel permeation chromatography. Whether the differences between the two studies result from the different mode of IGF-I administration, or have some other cause, remains to be determined.

In addition to the alterations in IGFBP-1 and IGFBP-3 detected by RIA, repeated IGF-I administration also caused changes in other IGFBPs, the identity of which was inferred from their mobility on SDS-PAGE: an increase in IGFBP-2, as previously demonstrated after IGF-I infusion (16), and a decrease in IGFBP-4. Thus we have demonstrated several distinct adaptive changes in the secretion and/or turnover of IGFBPs after IGF-I injection for seven days. While the consequences of these changes on the metabolic and other actions of IGF-I are still speculative, it is clear from this study that, if IGF-I is to be introduced as a therapeutic agent, its adaptive effects on IGFBPs will have to be taken into consideration.

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