A five day treatment with daily subcutaneous injections of growth hormone-releasing peptide-2 causes response attenuation and does not stimulate insulin-like growth factor-I secretion in healthy young men

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Abstract

The synthetic hexapeptide growth hormone-releasing peptide (GHRP)-2 specifically stimulates GH release in man. To determine the effects of prolonged treatment and whether response attenuation occurs in man, we administered to nine healthy subjects a daily s.c. injection of $100\,\mu$g GHRP-2 over 5 days.

Every day blood samples were taken to determine GH, IGF-I, IGF-binding protein (IGFBP)-3 and osteocalcin levels. On days 1, 3 and 5, GH was measured at -20, 0, 20, 40, 60, 90, 120 and 180 min using an immunometric and an immunofunctional assay. Mean ± s.d. peak GH concentrations were 83 ± 31, 59 ± 22 and $51 \pm 13\,\mu$g/l on days 1, 3 and 5 respectively.

Mean ± s.d. areas under the curve for days 1, 3 and 5 were 6366 ± 2514, 3987 ± 1418 and 3392 ± 1215 mU/l per min. Despite the maintained GH release, analysis of variance revealed that significant response attenuation occurred ($P < 0.01$). Mean serum IGF-I concentration did not increase after a 5 day treatment with GHRP-2. Mean basal levels were 22, 25, 23, 25, 23, 24 nmol/l measured on days 1 to 6. However, osteocalcin, another serum marker of GH activity in tissue, increased significantly from 3.2 ± 1.0 to $4.2 \pm 0.4\,\mu$g/l (mean ± s.d.) ($P < 0.01$).

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Introduction

Various growth hormone (GH)-releasing factor analogues (growth hormone secretagogues) have been developed, of which the GH-releasing peptides (GHRPs) form the major group (1, 2). This class of small synthetic peptides release GH in various animal species as well as in humans. These secretagogues could perhaps replace recombinant GH (rhGH) treatment in those forms of GH deficiency in which endogenous GH secretion can be stimulated, e.g. the hyposomatotropism of aging, obesity or acute catabolic conditions (1, 3).

The hexapeptide GHRP-2, like the other GH-releasing peptides, is active when administered orally (1). Release of GH in response to this and other GH secretagogues is short in duration, and mimics the physiological release of GH *in vivo* (1, 2, 4). A possible advantage of GH secretagogues over rhGH is the maintenance of spontaneous physiological GH secretion (5, 6). The potential utility of a GH-releasing factor superanalogue in a long acting form has been suggested (7). The use of GH secretagogues in place of the natural GH-releasing hormone (GHRH) has the advantages of inducing a larger GH release than a similar dose of GHRH and the possibility of oral administration (8, 9). Moreover, GHRPs act at receptors distinct from GHRH receptors, making GH release possible in subjects in which the response or sensitivity to GHRH is diminished (e.g. the elderly) (10, 11, 12).

Most studies carried out so far have evaluated the response to a GH secretagogue after a single bolus injection or a single oral administration (4, 13, 14). Information on insulin-like growth factor-I (IGF-I) generation and response attenuation after repeated or prolonged administration of GHRPs in healthy subjects is limited. Huhn *et al.* (15) and Jaffe *et al.* (16) investigated the effects of GHRP-6 infusion for 24 and 34 h respectively, and reported conflicting data on both effects. Ghigo *et al.* (9) reported an even higher GH response after 4 days of twice daily GHRP-2, but observed no significant rise in IGF-I in the seven elderly healthy women studied. The same authors found a trend towards an increased GH response after 8 days of intranasal hexarelin and no change after 15 days of oral

administration of the same peptide. IGF-I did not change in the first experiment in elderly subjects, but increased slightly but significantly in the second (17). Partial response attenuation was reported after a 6 h infusion of GHRP in healthy subjects (18). In a recent study by Copinschi et al.(19), a 7 day oral treatment with MK-677, a non-peptide GHRP receptor agonist, was shown to significantly increase IGF-I levels.

We studied the effect of repetitive GHRP-2 administration on GH response and IGF-I generation as well as on prolactin, cortisol and the GH-sensitive marker of bone turnover, osteocalcin. GHRP-2 is the most potent member of the family of GHRPs. A single s.c. dose of $1\,\mu$g/kg induces a GH peak response of approximately $50\,\mu$g/l in normal young men (8). To our knowledge no significant increases in serum prolactin, cortisol, luteinizing hormone, follicle-stimulating hormone or testosterone have been reported after GHRP-2 administration.

Materials and methods

Subjects and study design

Nine healthy male volunteers, aged 20–25 years, height 1.72–1.95 m, body mass index 20–25 kg/m^2, were recruited. The protocol was approved by the local ethical committee, and informed written consent was obtained from each subject before participation in the study. All studies took place within a period of 3 weeks in the clinical research centre of the Department of Endocrinology of the Free University Hospital, Amsterdam.

On 5 succeeding days, all subjects received $100\,\mu$g GHRP-2 by a single s.c. injection into the abdominal wall ($t = 0$ min). After an overnight fast, baseline blood samples were withdrawn from an antecubital vein from 0800 h ($t = -15$ min) onwards. On day 1 blood samples for measurement of serum GH were obtained at $t = -15, 0, 20, 40, 60, 90, 120$ and 180 min. This was repeated on days 3 and 5. Sleep was not permitted during the sampling period. Blood pressure and pulse rate were measured 30 min before and after the injection. Fasting blood samples for measuring basal serum IGF-I concentrations, osteocalcin, GH and IGF-binding protein (IGFBP)-3 were taken on all study days and 1 day after the last s.c. injection of GHRP-2 (day 6). On the 1st and 5th days, additional blood samples were taken for prolactin and cortisol determinations at $t = -15, 0, 20, 40$ and 60 min.

To confirm the bioactivity of the secreted GH, an immunofunctional GH assay was carried out by one of us (CJS).

GHRP-2 used in the study was a gift from Professor CY Bowers, Tulane University, New Orleans, LA, USA.

Assays

Serum GH was measured with a commercially available immunometric assay: HGHK-2 (Sorin Biomedics, Saluggia, Italy). IGF-I was measured with an IRMA after acid–alcohol extraction (DSL, Webster, TX, USA). The detection limits for GH and IGF-I are 0.3 and $5\,\mu$g/l respectively. For both GH and IGF-I the intra- and inter-assay coefficients of variation are 4 and 7%. In our laboratory the normal range of IGF-I in adult males aged 20–40 years is found to be 18–38 nmol/l.

Other routine assays used were an RIA for osteocalcin (Incstar Corporation, Stillwater, MN, USA), an IRMA for IGFBP-3 (DSL), an IRMA for prolactin (Medgenix Diagnostics, Fleurus, Belgium) and an RIA for cortisol (Coat-A-Count; DPC, Los Angeles, CA, USA).

To investigate the structural integrity of GH secreted after GHRP-2 stimulation, basal and peak GH levels detected by the Sorin hGH assay were further analysed by an immunofunctional GH assay. In this assay, a monoclonal antibody directed to binding site 2 of the hGH molecule (code 7B11) is immobilized and captures all molecules exhibiting the receptor interaction site 2. After a wash step, biotin-labelled recombinant GH-binding protein, corresponding to the full-length hGH receptor ectodomain, is added and the mixture incubated overnight. After a second wash step, the signal is detected after incubation with an excess of Europium-labelled streptavidin in a time-resolved fluorimeter (DELFIA; Wallac, Turku, Finland). In the immunofunctional assay, the rhGH reference preparation NIBSC 88/264 was used, which is more potent than pituitary-derived reference preparations and accounts for the lower readings of samples. The lower detection limit in this assay is $0.05\,\mu$g/l, the intra-assay coefficient of variation is below 9% and the interassay coefficient of variation below 13% for concentrations between 0.5 and $20\,\mu$g/l (20).

Statistical analysis

Data were analysed using the SPSS Statistical Software Package version 6.0. ANOVA, with Bonferoni multiple comparison test, was used to test for a significant trend in peak GH levels on days 1, 3 and 5 and the area under the curve (AUC) on days 1, 3 and 5. Student's paired t-test was used to test for significant differences between days 1 and 3, 1 and 5, and 3 and 5. Non-normally distributed data were log-transformed before subsequent analysis. Analysis of variance for repeated measurements (MANOVA) was used to test for significant differences (trend) beween the IGF-I levels. AUC was calculated using the trapezoidal rule. Statistical significance was assumed at $P < 0.05$.

Results

Basal GH levels were below the detection limit ($<0.3\,\mu$g/l) on all occasions (days 1 to 6), except in subject no. 1, who had basal GH levels of $5\,\mu$g/l.

After stimulation with GHRP-2, serum GH concentrations increased significantly in all subjects. They

started to rise within 20 min of the bolus injection, and peak GH concentration was reached within 40–60 min (mean 44 min). The acute GH rise was temporary, and within 180 min, GH had fallen to concentrations approaching pre-GHRP-infusion levels. The GH peaks on days 1, 3 and 5 were 83 ± 31, 59 ± 22 and $51 \pm 13\,\mu$g/l respectively (mean $\pm$ s.d.) (Fig. 1).

Also visible in Fig. 1 is the partial response attenuation that occurred during the test period, which was statistically significant ($P = 0.02$). The decline in peak GH levels was significant between days 1 and 5 ($P < 0.01$), but not between days 1 and 3 ($P = 0.12$).

AUC for GH levels (mean $\pm$ s.d.), calculated for the first 180 min after GHRP-2 administration on day 3, was 3987 ± 1418 mU/l per min and was significantly lower than after GHRP-2 administration on day 1, 6366 ± 2514 mU/l per min ($P < 0.02$).

Between days 3 and 5, AUCs were not significantly different ($P = 0.25$). Test for trend (ANOVA) showed a significant decrease ($P < 0.01$, Fig. 2).

Serum IGF-I concentrations were within the normal range and did not change after GHRP administration (Fig. 3). IGFBP-3 also showed no significant alterations (data not shown). As a consequence, the IGF/IGFBP-3 ratio, reflecting the percentage of free biologically active IGF-I, did not change.

Surprisingly, osteocalcin levels increased significantly during treatment with GHRP-2: 3.2 ± 1.0 and $4.2 \pm 0.4\,\mu$g/l (mean $\pm$ s.d.) on days 1 and 6 respectively ($P = 0.007$).

Basal cortisol and prolactin levels were within the normal range. Prolactin levels showed an acute response following the administration of GHRP-2 (Fig. 4). The slight but significantly increased levels ($P < 0.01$ for days 1 and 5) returned to basal within approximately 60 min. A significant decline in response of prolactin levels was also found on days 1 and 5 ($P = 0.037$), demonstrating the same response attenuation on administration of GHRP-2 as for GH.

Cortisol levels showed a small but significant decline (data not shown), reflecting the normal physiological decrease in serum cortisol during the morning.

To check that the GHRP-2 formulation used retained its potency during the whole study period, two subjects tested in the first week were given a final s.c. injection at the end of the third week. Their maximum GH concentrations were similar to those on the first test day, showing maintained potency of the drug as well as recovery of desensitization.

Correlation between the hGH immunofunctional assay and the Sorin immunometric assay was very high ($r = 0.96$), with no obvious alterations in the ratio of hGH between the two assays between baseline and GH peak, or between peaks at days 1, 3 and 5 within subjects. This finding indicates that the hGH released by GHRP-2 is of normal structure and biological potency. No adverse clinical signs or symptoms were observed during the testing period. No change in blood pressure or pulse rate occurred after the administration of GHRP-2. In the first 10 min after the injection, three subjects experienced a warm pricking feeling at the injection site.

Discussion

The present study demonstrates that repetitive daily s.c. administration of GHRP-2 to healthy young men is
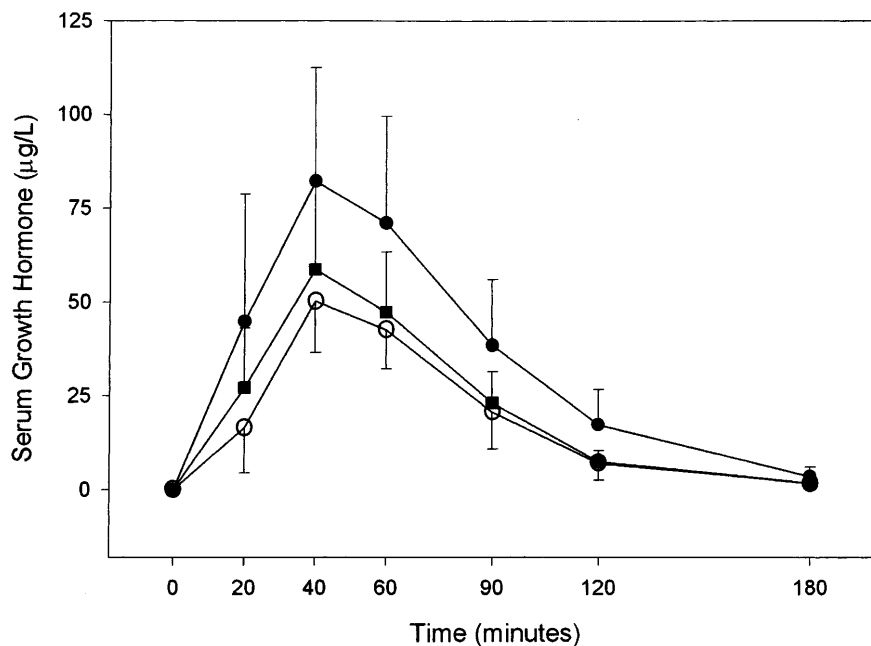
Figure 1 Serum GH concentrations on days 1 (●), 3 (■) and 5 (○) after GHRP-2 administration. Values are mean $\pm$ s.d.
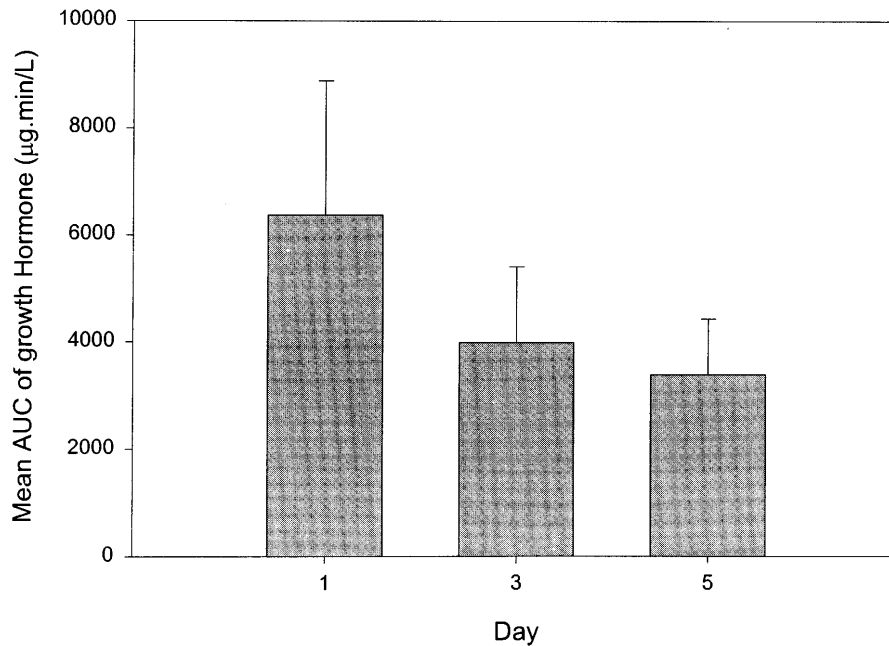
Figure 2 Area under the curve (AUC) for GH levels (mean ± s.d.).

invariably followed by increased GH release. However, a partial response attenuation occurred, which was most pronounced between the first and third day. Between days 3 and 5, the decline in response was only small and not significant. This might indicate that, after having reached a certain level, desensitization will not increase or that responsiveness to GHRP is restored after repetitive administration, as is also documented for responsiveness to GHRH (6, 21). This has also been suggested in other studies (15, 17). It is likely that the mechanisms responsible for GHRP action become partially refractory after chronic exposure to GHRP. The somatotroph remains responsive but is unable to respond to the same extent to a high dose of GHRP. To
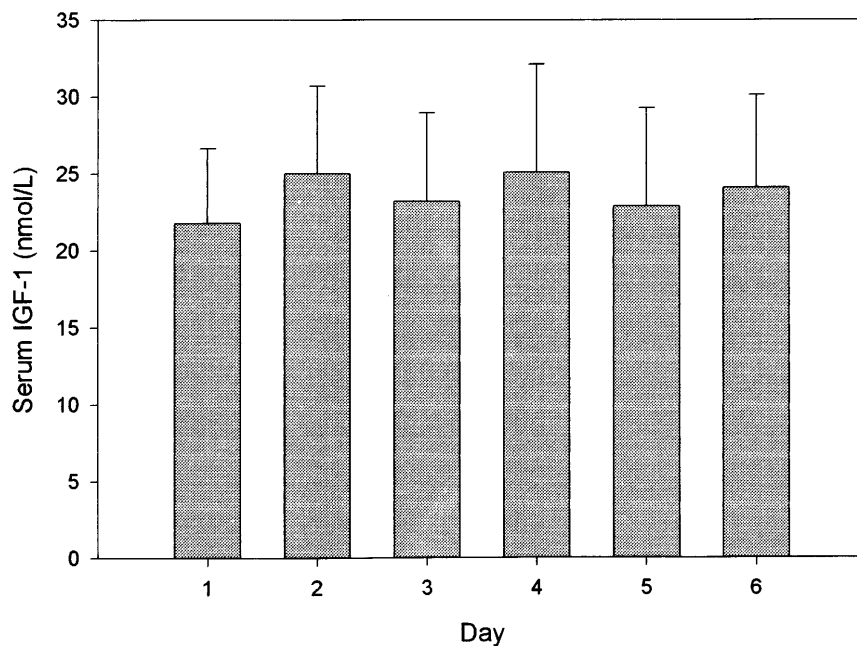

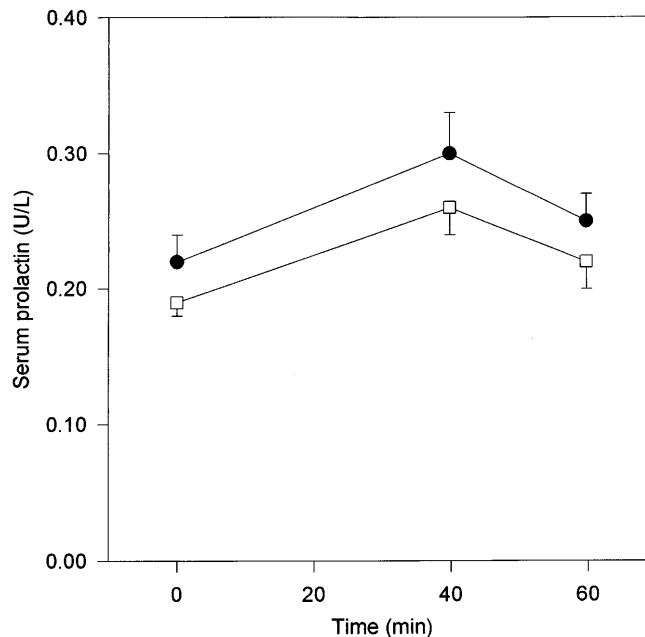**Figure 3** Serum IGF-I concentrations after GHRP-2 administration. Values are mean ± s.d.

Figure 4 Serum prolactin concentrations on days 1 (●) and 5 (□) after GHRP-2 administration. Values are mean ± s.d.

our knowledge we are the first to demonstrate the partial decrease in prolactin response. This may be due to the fact that both prolactin structure and its receptor are very similar to those of GH (22).

After oral administration of GHRP, no desensitization of the GH response was reported (4, 9). This could have been a result of either only moderate bioavailability after oral administration, leading to submaximal stimulation of somatotroph cells, or the fact that the GH receptor was not maximally stimulated, as indicated by a peak GH release of only 20–30 μg/l, leading to reduced negative feedback of GHRP-receptor-mediated GH release after oral administration.

We were surprised to find no change in IGF-I levels during the test period, because IGF-I levels are known to reflect overall GH activity in various target tissues. In GH replacement therapy for GH deficiency, there is good agreement between serum IGF-I and GH dosage (23).

On the basis of results from several other studies in which rhGH was administered, we concluded that a GH AUC of the magnitude found in our study should give rise to an IGF-I response (24, 25). Moreover, the study of Brixen *et al.* (26) also demonstrated that IGF-I levels showed an earlier and more significant response to GH administered s.c. than did osteocalcin, and therefore seemed to be a more sensitive marker.

In a study in which twice daily s.c. GHRH injections were given to elderly men, serum IGF-I levels had increased significantly after 2 weeks (27). As GHRPs have been shown to have the same or even better activity than GHRH, we expected serum IGF-I levels to rise after repetitive stimulation with GHRP-2 (1, 8, 13, 28).

The lack of increase in IGF-I production can be explained in various ways. First, the GH released may be biologically inactive. To exclude this possibility we examined extensively its biological integrity, and the various assays demonstrated that GHRP-stimulated GH is perfectly normal endogenous GH.

Secondly, perhaps an increase in IGF-I should not be expected in the nine people tested, all being young healthy volunteers with IGF-I levels in the optimal (maximal) range. Jørgensen *et al.* (29) demonstrated in a dose–response study on GH-deficient adults that, after IGF-I levels had reached the normal range with a certain dose of GH, only a modest further increase was obtained with a higher dose.

Another possible explanation is that we missed a transient IGF-I increase by measuring it only once daily, approximately 24 h after the GHRP injection. One daily injection of GHRP, resulting in one peak GH release, may result in fluctuating IGF-I levels. However, when serum GH exceeds a certain level, also shown in the study mentioned above, IGF-I concentrations in the serum reach a plateau after 12 h and stay virtually constant. The GH concentrations in our study after GHRP-2 stimulation were similar to high-dose serum GH concentrations in the study mentioned above, but no overall increase in IGF-I levels was seen, therefore this explanation seems very unlikely.

The most plausible explanation for the lack of change in serum IGF-I levels is that the administered dose was not high enough and/or injections should have been given more frequently and/or a longer treatment period was needed. Prolonged exposure to continuously elevated GH levels has been shown to be more effective

in raising serum IGF-I levels than exposure to the same amount of GH in pulses separated by periods of low or undetectable GH levels (30, 31). However, Copinschi *et al.* (19), who found an increase in IGF-I levels in the absense of any increase in GH peak levels after oral administration of MK-677, suggest that increased IGF-I levels are caused by elevated 'basal' GH secretion resulting from an increase in the number and/or amplitude of small GH pulses.

Osteocalcin, not seen as the first choice of marker of GH action, showed a substantial increase between days 1 and 6. It is not clear whether this is a paracrine effect of IGF-I generated locally in bone tissue or a direct effect of either GH or GHRP on the osteoblasts.

In conclusion, GHRP-2 is a powerful stimulator of GH generation, showing partial attenuation of the response over time. However, the dose of GHRP-2, the frequency of administration and the length of treatment needed to sufficiently stimulate IGF-I production still have to be defined. It would be worth investigating the possibility of direct effects of GHRP-2 on various tissues, e.g. bone.

Acknowledgements

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