Impact of two or three daily subcutaneous injections of hexarelin, a synthetic growth hormone (GH) secretagogue, on 24-h GH, prolactin, adrenocorticotropic and cortisol secretion in humans

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Abstract

Objective: To extend the insights on the action of GH secretagogues (GHS) on pituitary function, we studied the impact of intermittent daily s.c. administration of a peptidyl GHS, hexarelin (HEX), on 24-h GH, PRL, ACTH and cortisol secretion in healthy volunteers.

Design: We investigated the impact of two or three times daily s.c. administration of a short-acting peptidyl GHS, the hexapeptide HEX (1.5 mg/kg) on 24-h GH, PRL, ACTH and cortisol secretion (sampling every 20 min) in six normal young men. To monitor possible down-regulation, the effect of 1 mg/kg i.v. HEX at the end of each 24-h sampling period was studied.

Methods: Multi-parameter deconvolution analysis was used to quantitate pulsatile GH, PRL, ACTH and cortisol secretion and estimate the corresponding hormone half-lives. Complementary to deconvolution analysis, approximate entropy was used as a scale- and model-independent statistic to quantify the serial orderliness or pattern regularity of hormone measurements. 

Results: Mean and integrated (24-h) serum GH concentrations were increased from baseline values to the same extent by two and three HEX injections. Both HEX schedules equally increased GH secretory burst mass (but not burst frequency), mean daily GH production rate, GH half-life and irregularity of GH release patterns. No change occurred in the secretion of IGF-I, PRL, ACTH and cortisol. Intra-venous HEX at the end of each spontaneous 24-h profile induced a significant rise in GH, PRL, ACTH and cortisol. Prior HEX administration blunted the GH response, abolished that of ACTH and cortisol and did not modify the PRL increase.

Conclusions: The study showed that two or three daily s.c. injections of HEX augmented 24-h GH secretion equally, amplifying selectively GH secretory pulse mass without altering lactotroph and corticotroph secretion. IGF-I levels were not modified by these 1-day HEX treatment schedules.

Introduction

The neuroendocrine control of pulsatile somatotroph secretion is mediated by hypothalamic signals, intrapituitary mechanisms and multilevel feedback effects (1–3). The interplay between growth hormone-releasing hormone (GHRH) and somatostatin has a major role in the neural control of growth hormone (GH) secretion. In addition, GH secretion is subject to important influences by neurotransmitters, peripheral hormones and metabolic fuels, and possibly the natural ligand of the GH secretagogue (GHS) receptor (GHS-R) (2, 3). The last-mentioned family of agonists was discovered based on studies of synthetic peptidyl and non-peptidyl GHS (4–6). A natural ligand of the GHS-R, ghrelin, was recently purified from the stomach as a 28 amino acid peptide with a unique n-octanoyl esterification of the third serine residue (4). Ghrelin exerts potent stimulatory effects on somatotroph secretion, and is expressed in the brain and is measurable in the blood of rodents and the human (7, 8). The activity of ghrelin as well as that of non-natural GHS is not fully specific for GH; in fact, most GHSs also stimulate lactotroph and corticotroph secretion acutely (1, 5, 9).

Non-natural peptidyl and non-peptidyl GHSs can induce marked GH release after oral administration
Six healthy young male volunteers (age (means ± s.e.m.): 30± 1.2 years, range 27–35 years) were studied after providing written and voluntary informed consent. The study protocol had been approved by an independent local ethical committee.

Subjects and methods

Six healthy young male volunteers (age (means ± s.e.m.): 30± 1.2 years, range 27–35 years) were studied after providing written and voluntary informed consent. The study protocol had been approved by an independent local ethical committee.

Study design

All subjects were studied in three different sessions in a randomly assigned order with a wash-out period of at least 3 days. Volunteers were admitted to the Clinical Unit on the morning of the test session following an overnight fast. The cubital veins were cannulated at 0730 h and kept patent over 24 h by slow infusion of isotonic saline. During the day, subjects received a 200 kcal breakfast (60% carbohydrate) at 0900 h and a 800 kcal (50% carbohydrate, 30% lipid, 20% protein) lunch and dinner at 1300 h and 2000 h respectively.

At 0800, 1600, 2000 and 2400 h subjects received either 1 ml saline or HEX (1.5 μg/kg s.c.; 100 μg per vial; Europeptides, Argenteuil, France) according to the following schema. (A) HEX at 0800 and 2000 h; saline at 1600 and 2400 h. (B) HEX at 0800, 1600 and 2400 h; saline at 2000 h. (C) Saline alone. At the end of each session (i.e. at 0800 h on the following day), all subjects received HEX (1 μg/kg as an i.v. bolus).

Blood samples were withdrawn every 20 min for 26 h from 0800 on day 1 to 1000 h on the following day. GH, PRL, ACTH and cortisol were assayed in each sample (see below).

Hormone assays

Serum GH concentrations (μg/l) were measured by immunoradiometric assay (hGH-CTK; Sorin Biomedica, Saluggia, Italy). All samples from an individual subject were analyzed together in duplicate. The sensitivity of the assay was 0.15 μg/l. The ranges of inter- and intra-assay coefficients of variation were 4.9–6.5% and 1.5–2.9% respectively.

Serum PRL levels (μg/l) were measured by immunoradiometric assay (PROLCTK; Sorin Biomedica), as above. The sensitivity of the assay was 0.45 μg/l. The ranges of inter- and intra-assay coefficients of variation were 7.7–10.8% and 2.4–3.4% respectively.

Plasma ACTH levels (ng/l) were measured by immunoradiometric assay (ACTH; Nichols Institute Diagnostic, San Juan Capistrano, CA, USA). The sensitivity of the assay was 1 ng/l. The ranges of inter- and intra-assay coefficients of variation were 2.4–8.5% and 3.9–9.9% respectively.

Cortisol (nmol/l) was measured by radioimmunoassay (CORT-CTK125; Diasorin Diagnostic, Saluggia, Italy). The sensitivity of the assay was 0.66 nmol/l. The inter- and intra-assay coefficients of variation ranged from 4.3 to 14.6% and from 4.2 to 8.96% respectively.

Data analysis

Multiparameter deconvolution analysis was used to quantitate pulsatile GH, PRL, ACTH and cortisol secretion and estimate the corresponding hormone
half-lives (36). Basal hormone secretion represents the calculated time-invariant interpulse component of the release profile. The daily pulsatile production rate is the product of the number of secretory bursts and the mean mass of GH released per pulse. Secretory pulse identification required that secretory burst amplitudes and the basal secretion rate exceed zero by 95% statistical confidence. The analyst was blinded to the randomization scheme.

**Approximate entropy**

ApEn was used as a scale- and model-independent statistic, which is complementary to deconvolution analysis (34). ApEn quantifies the serial orderliness or pattern regularity of hormone measurements. Normalized ApEn parameters of m = 1 (series length) and r = 20% (threshold) of the intraseries s.d. were used, as previously validated for 24-h time series (37). This statistic is thus designated ApEn (1, 20%). Increased ApEn (at equal series lengths and similar parameter values, as used here) indicates greater secretory process irregularity, consistent with altered within-axis feedforward feedback control (34).

The results are reported as the means±S.E.M. of absolute values as well as areas under curve (AUC) calculated by trapezoidal integration.

**Statistical analysis**

ANOVA with repeated measures was used to compare results among the different sessions. If a significant difference had occurred a post hoc analysis was carried out using Newman–Keuls test. P < 0.05 was considered statistically significant.

**Results**

Mean and integrated (24-h) serum GH concentrations were increased (P < 0.03) from baseline values of 0.6±0.2 μg/l and 890±220 μg/l per min to the same extent by two and three HEX injections daily:

**Table 1** Deconvolution analysis of repetitively sampled serum GH, PRL, ACTH and cortisol concentration profiles during saline or two (HEX2) or three (HEX3) HEX injections daily.

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>HEX2</th>
<th>HEX3</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal secretion (concentration/day)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>86±72</td>
<td>29±14</td>
<td>14±14</td>
<td>ns</td>
</tr>
<tr>
<td>PRL</td>
<td>1.4±14</td>
<td>29±29</td>
<td>14±1.4</td>
<td>ns</td>
</tr>
<tr>
<td>ACTH</td>
<td>245±29</td>
<td>245±58</td>
<td>216±86</td>
<td>ns</td>
</tr>
<tr>
<td>Cortisol*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td><strong>Mass (concentration units)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>4.7±1.2</td>
<td>23±3.3</td>
<td>18±1.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PRL</td>
<td>5.6±1.0</td>
<td>4.4±0.6</td>
<td>5.8±0.5</td>
<td>ns</td>
</tr>
<tr>
<td>ACTH</td>
<td>64±11.4</td>
<td>44±16.7</td>
<td>57±22.8</td>
<td>ns</td>
</tr>
<tr>
<td>Cortisol</td>
<td>154±13</td>
<td>149±21</td>
<td>182±25</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Frequency (pulses/24h)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>7.5±1.0</td>
<td>7.0±0.6</td>
<td>6.7±1.7</td>
<td>1.7 ns</td>
</tr>
<tr>
<td>PRL</td>
<td>13.1±0.7</td>
<td>15.8±0.8</td>
<td>12.5±1.0</td>
<td>ns</td>
</tr>
<tr>
<td>ACTH</td>
<td>17.2±1.0</td>
<td>19.8±0.7</td>
<td>18.5±1.1</td>
<td>ns</td>
</tr>
<tr>
<td>Cortisol</td>
<td>12.3±0.8</td>
<td>11.7±0.7</td>
<td>12.5±0.6</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Pulsatile production rate</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>36±8.3</td>
<td>154±19.3</td>
<td>121±28</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PRL</td>
<td>68±9.5</td>
<td>69±7.5</td>
<td>73±9.3</td>
<td>ns</td>
</tr>
<tr>
<td>ACTH</td>
<td>1.1±0.2</td>
<td>0.9±0.3</td>
<td>1.1±0.5</td>
<td>ns</td>
</tr>
<tr>
<td>Cortisol</td>
<td>1.9±0.1</td>
<td>1.7±0.3</td>
<td>2.2±0.3</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Mean concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>0.6±0.2</td>
<td>3.7±0.7</td>
<td>3.3±1.1</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>PRL</td>
<td>5.6±0.7</td>
<td>6.0±0.5</td>
<td>6.1±0.9</td>
<td>ns</td>
</tr>
<tr>
<td>ACTH</td>
<td>19±2.3</td>
<td>18±1.7</td>
<td>18±1.4</td>
<td>ns</td>
</tr>
<tr>
<td>Cortisol</td>
<td>188±12</td>
<td>173±11</td>
<td>207±29</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Integrated concentration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>0.9±0.2</td>
<td>5.3±1.0</td>
<td>4.3±1.2</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>PRLs</td>
<td>6.1±1.0</td>
<td>8.5±0.7</td>
<td>8.6±1.3</td>
<td>ns</td>
</tr>
<tr>
<td>ACTH</td>
<td>26±3.1</td>
<td>25±2.4</td>
<td>26±1.9</td>
<td>ns</td>
</tr>
<tr>
<td>Cortisol</td>
<td>265±18</td>
<td>243±16</td>
<td>248±58</td>
<td>ns</td>
</tr>
</tbody>
</table>

* Cortisol profiles were fit according to a model of zero basal secretion (see Methods).

** Integrated concentration of GH and PRL are reported in mg/l per min, ACTH in ng/l per min and cortisol in nmol/l per min.

*** GH production rate is reported in μg/l per day, ACTH in ng/l per day and cortisol in nmol/l per day.

ns, not significant.
i.e. to 3.7±0.7 vs 3.3±1.1 μg/l and 5300±1030 vs 4300±1200 μg/l per min (Fig. 1).

Based on deconvolution analysis (Table 1), HEX augmented, to the same extent in response to two and three HEX treatments (P < 0.01), the daily pulsatile GH production rate by three- to fourfold and mean GH secretory burst mass from 4.7±1.2 μg/l at baseline to 23±3.3 and 18±1.7 μg/l per min respectively. Pulse frequency did not change. GH half-life increased slightly from 15±0.8 at baseline to 19±1.5 and 22±1.9 min after two or three HEX administrations respectively (P < 0.05) (Figs 1 and 2).

Both two and three HEX injections elevated ApEn values of the 24-h GH profiles (P < 0.01) (Table 2 and Fig. 1).

GH peaks following the first two s.c. HEX stimuli were comparable, whereas that following the third injection was decreased.

Deconvolution analysis showed no changes in the parameters of PRL, ACTH and cortisol secretion after two or three HEX infusions. ApEn values of these hormones were also independent of intervention (Tables 1 and 2).

Cross (X)-ApEn analysis showed that there was a disjunction (i.e. synchronicity loss) of ACTH and cortisol secretion during two vs three daily HEX injections.

Acute challenge with i.v. HEX at the end of the spontaneous 24-h profile induced a marked GH rise (peak: 60±4.4 μg/l) and a significant increase in serum PRL (13±1.9 μg/l), ACTH (77±7.7 ng/l) and cortisol (552±26 nmol/l) concentrations (Table 3 and Fig. 3).

Two and three s.c. HEX administrations the day before blunted (P < 0.05) the GH response to acute i.v. HEX challenge to the same extent (peak: 39 and 35 μg/l; AUC: 2.5±0.6 and 2.1±0.5 mg/l per min) when compared with the response recorded after 24-h saline administration (peak: 66 μg/l; AUC: 4.4±0.4 mg/l per min). In addition, two and three s.c. HEX administrations abolished the ACTH and cortisol but did not modify the PRL response to the acute i.v. HEX challenge (Table 3 and Fig. 3).

Serum IGF-I concentrations at the end of the various testing sessions were similar.

Side-effects
No side-effects were evident after two or three s.c. injections of HEX daily. On the other hand, i.v. administration of HEX induced transient facial flushing in three subjects.

Discussion
The present study establishes the ability of two or three s.c. injections of a peptidyl GHS, HEX, to augment 24-h pulsatile GH secretion equivalently and significantly in young men. The increase in daily GH secretion driven by HEX reflected a preferential (three- to fourfold) increase in burst mass without any significant change in frequency. HEX also elevated the ApEn (irregularity) of GH pulsatility and blunted the GH response to a subsequent acute i.v. challenge with the hexapeptide at the end of 24 h. Circulating total IGF-I levels did not change over this interval. Likewise, lactotroph and corticotroph secretion was not modified by either two or three s.c. injections of HEX. However, HEX pretreatment abolished the acute ACTH and cortisol (but not PRL) response to a delayed challenge with this hexapeptide.

In an earlier study in healthy older humans, continuous i.v. infusion of GH-releasing peptide-2 (GHRP-2) also augmented 24-h serum GH concentrations due to an enhancement of the physiological pattern

Table 2 ApEn of GH, PRL, ACTH and cortisol profiles during saline or two (HEX2) or three (HEX3) HEX injections daily. Data are the means ± S.E.M. (n = 6).

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>HEX2</th>
<th>HEX3</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>0.16±0.04</td>
<td>0.32±0.03</td>
<td>0.34±0.03</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>PRL</td>
<td>1.01±0.07</td>
<td>1.07±0.05</td>
<td>1.09±0.04</td>
<td>ns</td>
</tr>
<tr>
<td>ACTH</td>
<td>0.91±0.06</td>
<td>1.05±0.07</td>
<td>1.05±0.09</td>
<td>ns</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.91±0.06</td>
<td>0.96±0.03</td>
<td>0.95±0.05</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns, not significant.
of somatotroph secretion with an increase in pulse mass without any change in mean interpulse interval or pulse frequency (12, 15, 38). Subcutaneous GHRP-2 delivery and oral administration of non-peptidyl GHS likewise augmented 24-h GH secretion by increasing GH pulse amplitude without any change in pulse duration, interpulse interval, frequency and half-life (14, 15, 20). However, an increase in pulse frequency has been reported in one other analysis (23).

The present investigation highlights the stimulatory impact of two or three daily acute s.c. injections of a peptidyl GHS on 24-h GH pulsatility. First, our results show that intermittent HEX treatment increases 24-h GH secretion by three- to fourfold in normal young subjects, which is comparable to responses achieved by 24-h continuous i.v. infusion of GHRP-2 (15) or single daily oral administration of MK-0677 (20). The increase in daily GH secretion driven by HEX treatment primarily reflected augmented burst mass (see above) and a small (4–5 min) increase in GH half-life. The latter change probably reflects the concentration dependence of GH kinetics (17). Based on kinetics principles, the rise in 24-h integrated serum GH concentrations was due predominantly (>85%) to increased GH secretion (39).

The elevation of GH ApEn values is consistent with more disorderly patterns of 24-h GH release, as observed during fixed i.v. infusions of GHRH and GHRP-2 (15, 38, 40) and in healthy pubertal girls (41) and boys (41, 42). Aromatizable androgens and oestrogens also elevate GH ApEn (42, 43).

The magnitude of the effects of HEX on 24-h GH secretion was essentially equivalent whether two or three injections were administered, although the peak GH response to three consecutive s.c. injections was reduced. These results indicate that, at least under acute conditions, twice daily stimulation with a peptidyl GHS is sufficient to drive marked somatotroph secretion in young men, and that no further augmentation can be obtained by increasing the frequency of administration. Whether stimulation is maintained with more extended treatment, as observed for continuous s.c. GHRP-2 infusion (12), remains to be established.

At the end of the 24-h profile, the GH response to acute i.v. administration of the peptidyl GHS was blunted independently of the number of previous s.c. injections. Previous studies with continuous infusion (23, 28) or prolonged s.c. administration of GHSs (44) showed that the GH response to a following acute challenge with GHS is reduced, reflecting homologous desensitization. As we did not test subjects with stimuli other than acute i.v. HEX at the end of 24-h sessions, we cannot definitely rule out the possibility that reduction of the GH response to an acute bolus of the hexapeptide reflects some depletion of the GH-releasable pool after two or three s.c. HEX administrations in the previous 24 h. Interestingly, the response attenuation was comparable for two and three injections, thus not establishing a frequency dependence for this effect. However, in another study in elderly subjects, the attenuation of the GH response to HEX, during chronic treatment with the hexapeptide was found to be correlated with the duration of the treatment (44).

Unlike single oral administration of MK-0677 (20) and continuous 24-h i.v. infusion of peptidyl GHS (23), the present study showed no change in circulating total IGF-I levels after HEX injection, despite effective stimulation of daily somatotroph secretion. This finding agrees with the results of Rahim et al. (44) who reported that even after 16 weeks of s.c. injection of HEX, serum IGF-I (and IGF-binding protein-3) levels did not change significantly. The basis of this disparity is not known, but could reflect differences in the secretagogue type or delivery mode and/or the populations studied. According to studies performed either with a single and protracted oral administration of MK-0677 (20, 31) or with a prolonged treatment with HEX administered subcutaneously once a day (45), HEX did not alter daily lactotroph or corticotroph secretion. In contrast, the acute PRL, ACTH and cortisol responses to i.v. HEX at the end of the 24-h control profiles confirmed an immediate stimulatory activity of GHS on lactotroph and corticotroph secretion (5, 6, 9). Moreover, in the present study, 24-h HEX treatment eliminated subsequent acute ACTH and cortisol responses to the same hexapeptide. Down-regulation of ACTH responsiveness was selective, since the acute PRL response was fully preserved. In fact, lactotroph secretion can be slightly enhanced by longer-term GHS administration (46, 47).

### Table 3: Two-hour hormone release recorded at baseline (Basal, 2-h integrated (0800–1000 h) data from day 1 saline) and after acute challenge with i.v. HEX after each 24-h session (Saline, HEX2, HEX3). Data are the mean±S.E.M. (n = 6) integrated values.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Saline</th>
<th>HEX2</th>
<th>HEX3</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH (mg/l per min)</td>
<td>0.01±0.0</td>
<td>4.4±0.4#</td>
<td>2.5±0.6#</td>
<td>2.1±0.5#</td>
</tr>
<tr>
<td>PRL (mg/l per min)</td>
<td>0.4±0.1</td>
<td>1.0±0.2#</td>
<td>0.8±0.01#</td>
<td>1.0±0.2#</td>
</tr>
<tr>
<td>ACTH (ng/l per min)</td>
<td>2.5±0.4</td>
<td>4.8±0.4#</td>
<td>3.6±0.5*</td>
<td>3.1±0.3*</td>
</tr>
<tr>
<td>Cortisol (nmol/l per min)</td>
<td>36±4.4</td>
<td>55±2.2#</td>
<td>41±3.6*</td>
<td>36±3.1*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs Saline; #P < 0.05 vs Basal.
Figure 3 Hormone responses to an acute i.v. challenge with 1 μg/kg HEX after each 24-h session in normal volunteers. GH, PRL and cortisol levels are reported in μg/l, ACTH in ng/l. Values are means ± S.E.M.
In conclusion, the present clinical investigation revealed that two or three daily s.c. injections of HEX increase 24-h GH secretion to an equivalent degree in healthy young men without altering concomitant lactotroph and corticotroph secretion. This dosing schedule blunts subsequent acute GH and abolishes delayed ACTH (but not PRL) responsiveness to an i.v. dose of the same hexapeptide. HEX selectively augments GH pulse mass and increases the irregularity of the GH secretory process but does not increase circulating IGF-I levels.

Acknowledgements

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References


27. Clark RG, Carlsson MS, Trojanjar J & Robinson IC. The effects of a growth hormone-releasing peptide and growth hormone-
36 Veldhuis JD, Carlson ML & Johnson ML. The pituitary gland secretes in bursts: appraising the nature of glandular secretory impulses by simultaneous multiple-parameter deconvolution of plasma hormone concentrations. PNAS 1987 84 7686–7690.
37 Friend K, Iranmanesh A & Veldhuis JD. The orderliness of the growth hormone (GH) release process and the mean mass of GH secreted per burst are highly conserved in individual men on successive days. Journal of Clinical Endocrinology and Metabolism 1996 81 3746–3753.

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