Pyridostigmine treatment selectively amplifies the mass of GH secreted per burst without altering GH burst frequency, half-life, basal GH secretion or the orderliness of GH release

Keith Friend1, Ali Iranmanesh1, Ivan S Login2 and Johannes D Veldhuis3

1 Endocrine Section, Medical Service, Veterans' Affairs Medical Center, Salem, Virginia 24153, USA, 2 Department of Neurology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908, USA, and 3 Division of Endocrinology, Department of Internal Medicine, University of Virginia Health Sciences Center, NSF Center for Biological Timing, Charlottesville, Virginia 22908, USA

(Correspondence should be addressed to Johannes D Veldhuis)

(Keith Friend is now at Section of Endocrine Neoplasia and Hormonal Disorders, Department of Medical Specialties, University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA)

Abstract

Growth hormone (GH) release from the anterior pituitary gland is predominantly regulated by the two antagonist hypothalamic peptides, growth hormone-releasing hormone (GHRH) and somatostatin. Appraising endogenous GHRH action is thus made difficult by the confounding effects of (variable) hypothalamic somatostatin inhibitory tone. Accordingly, to evaluate endogenous GHRH actions, we used a clinical model of presumptively acute endogenous somatostatin withdrawal with concomitant GHRH release. To this end, we administered in randomized order placebo or the indirect cholinergic agonist, pyridostigmine, for 48 h to 13 healthy men of varying ages (29–77 years) and body mass indices (21–47 kg/m²). We sampled blood at 10-min intervals for 48 h during both placebo and pyridostigmine (60 mg orally every 6 h) administration, and used an ultrasensitive GH chemiluminescence assay (sensitivity 0.002-0.005 mg/l) to capture GH pulse profiles. Multiparameter deconvolution analysis was applied to quantitate the number, amplitude, mass, and duration of significant underlying GH secretory bursts, and simultaneously estimate the GH half-life and concurrent basal GH secretion. Approximate entropy was utilized as a novel regularity statistic to quantify the relative orderliness of the hormone release process. All measures of GH secretion/half-life and orderliness were statistically invariant across the two consecutive 24-h placebo sessions. In contrast, pyridostigmine treatment significantly increased the mean serum GH concentration from 0.23 ± 0.054 mg/l during placebo to 0.45 ± 0.072 mg/l during the first day of treatment (P < 0.01). There was also a significant rise in the calculated 24-h pulsatile GH production rate from 8.9 ± 1.7 mg/l/day on placebo to 27 ± 5.6 mg/l/day during active drug treatment (P < 0.01). Pyridostigmine significantly and selectively amplified GH secretory burst mass to 1.5 ± 0.35 mg/l compared with 0.74 ± 0.19 mg/l on placebo (P < 0.01). This was attributable to stimulation of GH secretory burst amplitude (maximal rate of GH secretion attained within the release episode) with no prolongation of estimated burst duration. Basal GH secretion and approximate entropy were not altered by pyridostigmine. However, age was strongly related to more disorderly GH release during both days of pyridostigmine treatment (r = 0.79, P = 0.0013). During the second 24-h of continued pyridostigmine treatment, most GH secretory parameters decreased by 15–50%, but in several instances remained significantly elevated above placebo. Body mass index, but not age, was a significantly negative correlate of the pyridostigmine-stimulated increase in GH secretion (r = −0.65, P = 0.017).

In summary, assuming that somatostatin is withdrawn and (rebound) GHRH release is stimulated via pyridostigmine administration, we infer that relatively unopposed GHRH action principally controls GH secretory burst mass and amplitude, rather than apparent GH secretory pulse duration, the basal GH secretion rate, or the serial regularity/orderliness of the GH release process in the human. Moreover, we infer that increasing age is accompanied by greater disorderliness of somatostatin-withdrawn GHRH, and hence rebound GH, release. The strongly negative correlation between pyridostigmine-stimulated GH secretion and body mass index (but not age) further indicates that increased relative adiposity may result in decreased effective (somatostatin-withdrawn) endogenous GHRH stimulus strength.

European Journal of Endocrinology 137 377–386

Introduction

The episodic mode of growth hormone (GH) release from the anterior pituitary gland is predominantly driven by intermittent hypothalamic growth hormone-releasing hormone (GHRH) stimulation of somatotrope cells adequately disengaged from hypothalamic somatostatin inhibition. The secretion of these two hypothalamic
pyridostigmine administration over two days on pulsatile GH secretion in humans across a wide span of ages and body compositions. This wide range was selected in order to allow multifold variations in mean serum GH concentrations and thereby evaluate possible regressions of age and body mass on pyridostigmine effects. Multiparameter deconvolution was applied to quantify objectively the number, mass, duration, and amplitude of underlying GH secretory bursts, as well as simultaneously estimating the apparent GH half-life and concurrent basal GH secretion (14–16). Approximate entropy (ApEn), a novel estimate of the relative orderliness of the hormone release process (17–19), was used to quantify the regularity of serial GH measurements over time. Unlike classical deterministic chaos estimates of entropy, ApEn is valid for biological data series as short as 50–300 observations. An automated ultra-high-sensitivity chemiluminescence GH assay (sensitivity 0.002–0.005 μg/l) was applied in this study (20–22) in order to measure all serum GH concentrations in each 48-h sampling session. Under these circumstances of presumptive somatostatin withdrawal and increased GHRH release, we could evaluate the impact of body composition (relative obesity) and age on endogenous GHRH-driven pulsatile GH release.

Materials and methods

Clinical protocol

Thirteen healthy men, ranging in age from 29–77 years, were recruited to participate in this study after providing written informed consent, which was approved by the Human Investigation Committee of the University of Virginia Health Sciences Center. Prior to admission into the study, each subject underwent a complete history and physical examination and had normal screening blood tests of hepatic, renal, hematological, and metabolic function. No subject had any known acute or chronic illness, recent weight gain or loss, was receiving any medications, or had undergone transmeridian travel within 3 weeks. The body mass indices of subjects ranged from 21–47 kg/square meter of surface area. After overnight adaptation to the General Clinical Research Center study unit, subjects had a catheter placed in a forearm vein the next morning at 0700 h. Baseline blood samples were withdrawn at 0800 h. Pyridostigmine (60 mg) or placebo orally every 6 h for 48 h was administered on any given admission in a randomized, double-blind fashion. On the other hand, in sheep, neostigmine stimulates GH release and rapidly increases hypophysical portal blood GHRH concentrations by 8- to 10-fold without altering somatostatin levels monitored concurrently (8). In the absence of antiserum or specific peptide antagonists of somatostatin for use in clinical studies, a GHRH antagonist was used to show that pyridostigmine’s stimulation of GH secretion in the human is dependent upon endogenous GHRH action (9), which may well be enhanced following concurrent somatostatin withdrawal (10–13). These observations in several species can be harmonized by the interpretation that pyridostigmine probably triggers rebound GH release via somatostatin-withdrawn GHRH action (see Discussion).

The current study was undertaken, therefore, to evaluate the effects of repeated pyridostigmine
GH profiles were evaluated earlier for test-retest reliability (23).

**Assays**

Serum GH concentrations were measured in duplicate using a high-sensitivity chemiluminescence-based assay, as described in detail previously (20, 21). A robotics system (Nichols Laboratories, San Juan Capistrano, CA, USA) with automated bead washing and reagent pipetting was used. Assay sensitivity was 0.002 µg/l when defined as 2 S.D. above the zero dose tube, and 0.005 µg/l at 4 S.D. above zero; the within-assay precision ranged from 3.2 to 11.5%. No subject’s sample values fell below 0.01 µg/l (approximately 6–8 S.D. above the zero tube). Standards and quality control tubes were assayed in triplicate and the results analyzed by a new dose-dependent variance model to estimate low-end sensitivity and within-assay precision (22). This methodology generates an apparent standard deviation associated with each sample measurement on the basis of the following three sources of experimental uncertainty: variability between duplicate measures of individual unknown samples; uncertainty in the values of the fitted 4- or 5-parameter monotonic curve describing behavior of the assay; and imprecision within the triplicates of the calibration curve. The overall uncertainty assigned to each sample was used as an inverse weighting value for deconvolution analysis (below). Plasma total insulin-like growth factor-I (IGF-I) concentrations were assayed after acid-ethanol extraction using the reagents of Nichols Laboratory.

**Deconvolution analysis/approximate entropy**

Multiparameter deconvolution analysis was performed in a blinded manner to measure objectively the number, duration, amplitude, and mass of statistically significant GH secretory bursts over each 48-h observation interval (14–16). Basal hormone secretion was simultaneously estimated as was the apparent GH half-life. The fitting pathway used has been described previously (23), and was demonstrated to achieve approximately 90% or greater sensitivity, specificity, and positive and negative
Figure 2  Illustrative consecutive 24-h serum GH concentrations and deconvolution-calculated GH secretory profiles in three placebo and three drug-treated representative healthy men sampled every 10 min for 48 h during the two days of placebo and days 1 and 2 of pyridostigmine (Mestinon) administration. The curves drawn through the observed serum GH concentrations are those predicted by multiparameter deconvolution analysis (12–14). The vertical bars associated with each sample value are within-assay s.d. estimated by a dose-dependent variance model that combines experimental uncertainty within the triplicate standards, the fitted parameters of the standard curve, and the within-sample duplicates (22). Calculated GH secretory rates are shown on matching panels.
Mean serum GH concentrations and daily GH production rates

Pyridostigmine significantly increased the mean serum GH concentration from 0.23 ± 0.054 μg/l during placebo day 1 to 0.45 ± 0.072 μg/l during the first day of drug treatment (P<0.01). During the second day of continued drug treatment, the mean serum GH concentration decreased to 0.39 ± 0.081 μg/l, which was not significantly different from that seen during the second day of placebo (P = 0.068). There was also a significant increase in the calculated daily GH production rate from a mean of 8.9 ± 1.7 on placebo day one to 27 ± 5.6 μg/l/day on the first day of drug treatment (P<0.01) (Fig. 1A). Eleven of thirteen subjects showed this increase. Again, there was a decrease during the second day to 17.2 ± 3.4, but this remained significantly above placebo day 2 (P < 0.01). The difference in the GH production rates between pyridostigmine day 1 and 2 was also significant (P < 0.01) (Fig. 1A).

The 24-h serum GH concentration profiles from 3 individual men during placebo and pyridostigmine treatment representative of the patterns observed in the majority of subjects who participated in this study are shown in Fig. 2. Volunteers exhibited a marked increase in GH release during the first day of pyridostigmine treatment and a modest decline from this value on the subsequent treatment day.

Plasma IGF-I concentrations

Plasma total IGF-I concentrations were 180 ± 26 and 160 ± 16 μg/l on the two consecutive placebo days (P = not significant), and 190 ± 25 vs 180 ± 28 μg/l on pyridostigmine days 1 and 2 (P = not significant). Plasma IGF-I levels during drug treatment were highly correlated on the 2 days (R = +0.822, P = 0.007).

Attributes of pulsatile GH release

GH secretory burst mass rose from 0.74 ± 0.19 μg/l on placebo day 1 to 1.5 ± 0.35 μg/l on pyridostigmine day 1 (P < 0.01) (Fig. 1B). Eleven of the thirteen volunteers manifested this increase. The mean decreased to 1.2 ± 0.28 μg/l during pyridostigmine day 2, which was not significantly different from that of placebo day 2 (P = 0.068). The difference between pyridostigmine days 1 and 2 approached statistical significance (P = 0.052). The increase in burst mass was primarily attributable to stimulation of GH secretory burst amplitude (maximal rate of GH secretion attained within the release episode) with no prolongation of calculated burst duration. The burst amplitude value increased from 0.033 ± 0.08 μg/l/min during placebo day 1 to 0.08 ± 0.023 μg/l/min during pyridostigmine day 1 (P < 0.05). Again, there was no significant difference between placebo day 2 and pyridostigmine day 2 where the burst amplitude was 0.041 ± 0.007 μg/l/min (P = 0.305), but there was a

**Statistical analysis**

Possible differences in median values for placebo vs drug treatment groups within subjects were evaluated by paired non-parametric (Wilcoxon) testing of specific measures of GH secretion or half-life between two corresponding 24-h periods, e.g. placebo day 1 vs pyridostigmine day 1. Unless otherwise noted, results are expressed as the mean ± S.E.M. (n = 13). Linear or mono-exponential regression analysis was used to evaluate possible relationships between GH responses to drug and body mass index (BMI) or age.

**Results**

All measures of GH secretion including the mass of GH secreted per burst, the daily endogenous GH secretion rate, the mean serum GH concentration over 24 h, and approximate entropy as a measure of the serial regularity of GH release were statistically indistinguishable in the two consecutive 24-h placebo sessions, as reported earlier (23).

Pyridostigmine treatment elicited abdominal cramps in five subjects, which subsided during the second day and did not necessitate interruption of the study.
significant decrease between pyridostigmine days 1 and 2 ($P < 0.01$). There were no statistical differences in the estimated endogenous GH half-life, GH burst frequency, or basal secretion rates among admissions (Fig. 1C and D). These data are summarized in Table 1.

### Approximate entropy

As noted previously, ApEn provides a quantitative estimate of the relative disorderliness of hormone concentration profiles. Previous studies have demonstrated that the serial orderliness of the GH release process over 24 h is not significantly different in individual subjects studied on successive days (21). ApEn was $0.47 \pm 0.054$ during placebo day 1 and $0.58 \pm 0.050$ during pyridostigmine day 1 ($P = 0.068$). The mean ApEn value on placebo day 2 of $0.53 \pm 0.059$ was not statistically different from that seen on pyridostigmine day 2, namely $0.56 \pm 0.071$ ($P = 0.233$). There was also no statistical difference between ApEn measures on pyridostigmine days 1 and 2 ($P = 0.376$).

### Regression analysis

Linear regression analysis showed strongly positive correlations between pyridostigmine day 1 and day 2 treatment effects, whether assessed as GH mass secreted per burst ($r = +0.917, P = 0.00001$) or the daily GH production rate ($r = +0.839, P = 0.00034$).

As demonstrated in Fig. 3A, there was an inverse linear correlation between BMI and the increase in GH secretory burst mass on pyridostigmine day 1 vs placebo day 1. A negative exponential relationship, which provided a better fit by F ratio testing than a linear plot, existed between BMI and GH production rate (Fig. 3B). No significant correlations existed for GH response to pyridostigmine vs age. In addition, during pyridostigmine treatment, ApEn values were strongly positively correlated with age on both days of drug treatment ($r = +0.79, P = 0.0013$) (Fig. 4).

### Discussion

We have used an indirect cholinergic agonist, pyridostigmine, as a clinical probe of somatostatin withdrawal with rebound GH release presumptively dependent on endogenous GHRH action, in view of pyridostigmine’s ability to achieve effective GHRH release in the sheep and putatively in the human (8, 9), and, in the rat and possibly human, to heighten somatostatin withdrawal (1–7, 10–13, 31–35). We hypothesized that unmasking of endogenous GHRH actions, e.g. by withdrawing somatostatin and enhancing GHRH secretion action via this muscarinic agonist, would disclose the impact of age and/or relative adiposity on (endogenous) hypothalamic GHRH-driven pulsatile GH secretion in a diverse cohort of healthy men. During the first 24 h of treatment, pyridostigmine clearly increased GH secretory burst mass, and thereby the daily GH secretion rate and the mean (24-h) serum GH concentration without altering GH half-life, basal GH secretion, or GH secretory burst duration or frequency. During the second 24 h of continued pyridostigmine treatment, GH secretion measures decreased by 15–50%, but in some instances still remained significantly elevated above placebo values. This delayed decrease might be secondary to tachyphylaxis and/or increased GH and (free) IGF-I negative feedback, although plasma total IGF-I concentrations did not change significantly. Approximate entropy, a quantitative estimate of the relative orderliness of hormone concentration profiles, was not significantly altered by pyridostigmine treatment, indicating that the specific effect of pyridostigmine is to increase normally organized pulsatile GH release. This

### Table 1

Attributes of 24-h pulsatile GH release in 13 healthy men evaluated by deconvolution analysis during 2 successive days of either placebo or pyridostigmine treatment.

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Pyridostigmine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>24-h mean GH</td>
<td>0.23 ± 0.054</td>
<td>0.27 ± 0.067</td>
</tr>
<tr>
<td>concentration (µg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily GH production</td>
<td>8.9 ± 0.89</td>
<td>13.0 ± 3.5</td>
</tr>
<tr>
<td>rate (µg/24 h)</td>
<td>18.5 ± 0.86</td>
<td>17.2 ± 0.79</td>
</tr>
<tr>
<td>GH half-life (min)</td>
<td>14.5 ± 1.6</td>
<td>14.4 ± 1.3</td>
</tr>
<tr>
<td>Burst frequency</td>
<td>0.74 ± 0.19</td>
<td>0.92 ± 0.24</td>
</tr>
<tr>
<td>(per 24 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass of GH secreted</td>
<td>0.033 ± 0.008</td>
<td>0.035 ± 0.0077</td>
</tr>
<tr>
<td>per burst (µg/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GH secretory burst</td>
<td>0.0012 ± 0.0003</td>
<td>0.0011 ± 0.0024</td>
</tr>
<tr>
<td>amplitude (µg/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal secretion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rate (µg/min)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^aP < 0.05$ vs placebo day 1, $^bP < 0.01$ vs placebo day 1, $^cP < 0.05$ vs placebo day 2, $^dP < 0.01$ vs placebo day 2, $^eP < 0.05$ vs pyridostigmine day 1, $^fP < 0.01$ vs pyridostigmine day 2.
occurs mechanistically by enhancing GH secretory burst mass, which is quite reasonably interpreted as due to endogenous GHRH release possibly with concomitant partial somatostatin withdrawal. The latter is more difficult to evaluate in the human given the current lack of selective somatostatin antagonists available for clinical use.

By ultrasensitive GH chemiluminescence assay and deconvolution analysis of combined pulsatile and basal GH secretion over 24 and 48 h, we observed that pyridostigmine treatment did not alter GH secretory burst duration or frequency or GH half-life or the basal (interpulse) GH secretion rate. The last finding suggests that, while not yet well understood, the low rates of basal GH secretion disclosed by ultrasensitive GH assays (20, 21, 25) may not be as sensitive as pulsatile GH release to inhibitory somatostatin influences or to stimulatory GHRH actions. As the mode of pyridostigmine action, particularly in the human, is not determined with absolute certainty, this interesting possibility should be explored further when selective somatostatin and GHRH antagonists become available for use in clinical investigations.

The 24-h mean serum GH concentration in this healthy ambulatory outpatient adult population during placebo administration varied from 0.053 to 0.78 μg/l, which spans a 15-fold range. This probably reflects the deliberately diverse composition of the cohort of men studied here, spanning a wide range of ages (29–77 years) and body mass indices (21–47 kg/square meters), since both factors are known to influence pulsatile GH release (1, 3, 20, 23, 25, 36–42). Because our study included only healthy otherwise unmedicated men, previously described differences in GH release due to gender or underlying illness were not variables in this investigation. Although pyridostigmine administration has been demonstrated to potentiate spontaneous diurnal GH secretion in short children (43) and to counteract the blunted GH response to GHRH in both obese children and adults (6), regression analysis of our data suggested that this cholinergic agonist is unable to reverse completely the suppression of GH release that is associated with increasing adiposity. Indeed, BMI was a strongly negative determinant of both pyridostigmine-stimulated GH secretory burst mass and daily GH production (Fig. 3). This may indicate that obesity results in relative suppression of endogenous GHRH release and/or action. In addition, somatostatinergic inhibitory tone may be a significant factor contributing to diminished GH release in obesity, and pyridostigmine
may not be (completely) effective in decreasing hypothalamic somatostatin release in the human, akin to inferences in sheep (8). Available data cannot distinguish between these hypotheses. Whether these relationships apply equally in the non-obese population is also not known.

Of further interest, across an age span of 29–77 years, the present cohort of men showed no effect of age on GH responsiveness to a cholinergic agonist, even though pyridostigmine actions in older individuals might actually be prolonged by its longer elimination half-life in the elderly (44). This indicates specificity of the negative impact of BMI (and hence presumptively relative adiposity spanning a broad range) on endogenous somatostatin release and/or GHRH action to stimulate GH secretory burst mass. In contrast, the maximal secretory capacity of somatotrope cells as assessed by combined GHRH and growth hormone-releasing peptide-6 stimulation is preserved in human obesity (45), indicating the absence of a fixed pituitary defect.

Approximate entropy, unlike deterministic chaos measures such as Kolmogorov-Sinai entropy that require a vast number of observations, provides an objective quantification of visually apparent irregularity or disorderliness of hormone release in 24-h profiles (17–19, 26–30, 46). For example, higher ApEn values for 5- and 10-min GH sampling data, thus indicating greater irregularity or disorderliness of (GH) release over 24 h, are observed consistently in active acromegaly (19), with lesser elevations in fasting, obesity, and healthy aging (47). Greater disorderliness of pituitary hormone secretion is also evident for adrenocorticotropic and prolactin release in Cushing’s disease and prolactinoma patients respectively (48, and F Roelfsema & J D Veldhuis, unpublished observations), suggesting that tumor autonomy – or diminished releasing factor/feedback regulation – produces more disorderly hormone release over time. In contrast, our finding of unchanged ApEn values for GH during presumptively increased GHRH release with or without partial somatostatin withdrawal indicates that amplified GH secretion can occur without a change in orderliness of the GH release process. This observation is important, since increased GH secretion is accompanied by increased ApEn (greater irregularity) in women compared with men (46), in testosterone-treated prepubertal boys (49), in fasting men, and in acromegalic men and women (19). Of additional interest, pyridostigmine unexpectedly elicited a more strongly positive
relationship in our cohort of subjects between age and ApEn (increased disorderliness) (Fig. 4). Although we observed this relationship earlier without drug treatment in a larger independent sample of healthy aging men (25), the present comparison of ApEn’s relationship to age during placebo versus cholinergic agonist treatment showed that this agent can enhance the robustness of the correlation. Assuming that pyridostigmine promotes somatostatin withdrawal and rebound GHRH, and hence GH release, our ApEn data would suggest that increasing age is accompanied by decreased pattern reproducibility of the (endogenous) hypothalamic somatostatin–GHRH interaction. In this regard, recent analyses of overnight luteinizing hormone (LH) and testosterone release profiles as a function of age in men have also revealed marked age-related increases in the disorderliness of release of these individual reproductive hormones as well as greater asynchrony (conditional irregularity) of joint LH-testosterone secretion (50). Thus, a potentially more general property of neuroendocrine aging is suggested by ApEn changes in the gonadotropic and somatotropic axes, namely, diminished orderliness of hormone release, probably reflecting reduced network synchrony and/or erosion of feedback and feed-forward coordination within the neuroendocrine axis.

Acknowledgements

We thank Patsy Craig for her skilful preparation of the manuscript and Paula P Azimi for the artwork. This work was supported in part by NIH Grant No. RR-00847 to the Clinical Research Center of the University of Virginia, RCDA 1 KO4 HD00634 (J D V), the Diabetes and Endocrinology Research Center Grant NIH DK-38942, NIH NCI CA38228 (I S L), the NIH-supported release, probably reflecting reduced network synchrony and/or erosion of feedback and feed-forward coordination within the neuroendocrine axis.

Indirect cholinergic agonist stimulates GH pulse mass

References

21 Chapman IM, Hartman ML, Straume M, Johnson ML, Veldhuis JD & Thorner MO. Enhanced sensitivity growth hormone


Received 20 January 1997
Accepted 10 April 1997