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Preservation of GHRH and GHRP-2 Efficacy in Young Men with Experimentally Induced Hypogonadism

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Abstract

Background—Somatostatin (SS), GH-releasing hormone (GHRH), GH-releasing peptide (GHRP) and the sex-steroid milieu regulate GH secretion.

Objective—To test whether GHRH and GHRP remain effective secretagogues in the face of short-term hypogonadism.

Design—Prospective, randomized double-blind.

Methods—Healthy young men (N = 24) received a GnRH agonist twice 3 weeks apart followed by placebo (N = 13, Pl) or testosterone (N = 11, T) addback. Subjects were then given consecutive i.v. infusions of L-arginine (to restrain SS outflow) and a maximally effective dose of GHRH or GHRP-2 (to test corresponding secretagogue pathways).

Results—GH secretion stimulated by L-arginine/GHRH and by L-arginine/GHRP-2 was unaffected by combined T/E₂ depletion. The low T/E₂ milieu decreased basal (nonpulsatile) GH secretion (P = 0.038), without altering fasting pulsatile GH secretion or IGF-I or IGFBP-3 concentrations. IGFBP-1 (P < 0.0001) and abdominal visceral fat (AVF, P = 0.017) correlated negatively with fasting basal GH secretion. In contrast, IGF-I (P = 0.0012) and IGFBP-3 (P = 0.015) correlated positively with fasting pulsatile GH secretion. AVF (P = 0.0024) was a negative determinant, and IGF-I a positive determinant (P = 0.018), of GHRH-driven GH pulses. Responses to GHRP-2 were unrelated to any of these factors.

Conclusion—L-arginine/GHRP-2 appears to be an especially robust stimulus of GH secretion, since efficacy is unmodified by profound short-term hypogonadism, a range of AVF estimates, and a spectrum of IGF-I, IGFBP-1 and IGFBP-3 concentrations. Whether robustness also applies to chronic hypogonadism is not known.

Keywords

androgen; fat; human; male; GH; IGF-I; pulsatility; secretion

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Introduction

Growth hormone (GH) is secreted predominantly in discrete bursts under the supervision of GH-releasing hormone (GHRH, a 40 and 44 amino-acid peptide synthesized in the arcuate nucleus), GH-releasing peptide (GHRP, of which ghrelin is the endogenous 28 amino-acid prototype produced in the hypothalamus, pituitary gland and stomach), and somatostatin (a 14 amino-acid sequence made in the periventricular nucleus). Pulsatile GH secretion is also regulated strongly by sex-steroid hormones (1), which exert their stimulatory effects to a large degree via SS, GHRH and GHRP (2). Testosterone (T) and its aromatized product, estradiol (E₂), determine the amplitude and mass of GH pulses, whereas pathophysiological factors such as age, IGF-I feedback and relative adiposity repress pulsatile GH secretion (3-5). In particular, administration of T to prepubertal children and hypogonadal men enhances GH secretion after infusion of saline, L-arginine or a maximally effective GHRP stimulus (6-9). On the other hand, the degree to which short-term T or E₂ deficiency affects combined secretagogue actions in men is far less clear. Whereas two studies inferred that short-term pharmacological T/E₂ deprivation does not diminish the efficacy of GHRH infused alone (10;11), how acute hypogonadism affects combined L-arginine/GHRH or L-arginine/GHRP drive is not known. This point is important, inasmuch as Infusion of L-arginine before or with a peptidyl secretagogue has been employed to restrict hypothalamic release of SS (12-15). The motivation for using L-arginine is to permit inferences about the efficacies of GHRH and GHRP in a low-SS milieu (2). Thus, the present study utilizes sequential L-arginine/GHRH vs L-arginine/GHRP-2 infusions to examine their robustness to a low-T/low-E₂ milieu in healthy men. Secondarily, we ask whether abdominal visceral-fat mass and prevailing markers of the IGF-I system influence combined-secretagogue effects in this setting.

Methods

Subjects

Volunteers provided written informed consent approved by the Mayo Institutional Review Board and reviewed by the U.S. Food and Drug Administration under investigator-initiated new drug numbers for GHRP-2 and GHRH. Exclusion criteria were exposure to psychotropic or neuroactive drugs within one yr; BMI less than 18 and more than 32.5 kg/m²; anemia (hemoglobin < 12.8 g/dL); drug or alcohol abuse, psychosis, depression, mania or severe anxiety; acute or chronic organ-system disease; use of T, other anabolic steroids or glucocorticoids; endocrinopathy, other than primary thyroidal failure receiving replacement; nightshift work or recent transmeridian travel (exceeding 3 time zones within 7 days of study); acute weight change (loss or gain of > 2 kg in 6 wk); allergy to administered peptides; and unwillingness to provide written informed consent. Each subject had an unremarkable medical history and physical examination and normal screening laboratory tests of hepatic, renal, endocrine, metabolic and hematologic function. The men reported normal sexual development and function.

Protocol

The study design was parallel-cohort, double-blind and prospectively randomized. Twenty-four healthy young men [age 24 ± 0.72 (SEM) yr, BMI 25 ± 0.91 kg/m²] received 2 consecutive im injections of depot leuprolide acetate (3.75 mg im 3 wk apart) to deplete systemic T and E₂ concentrations. The synergy between GHRH and GHRP-2 in these subjects was presented earlier (16). Beginning on the day of the second leuprolide injection, volunteers were given either saline (N = 13) or a pharmacological dose of 200 mg T enanthate (N = 11) im weekly for 3 doses (designated as days 0, 7, and 14) double-blind. Secretagogue infusions were scheduled during the time window 10-18 days. Each participant was studied twice in the

Clinical-Translational Research Unit (CRU) at least 48 hr apart on separate mornings after a standardized evening meal and subsequent overnight fast.

Volunteers continued their usual level of daily physical activities, except the evening before and on the day of study. Subjects were admitted to the CRU before 1700 hr and stayed overnight. Sleep was not monitored. Room lights were put out at 2230 hr. To limit nutritional confounds, single a constant meal (vegetarian or nonvegetarian) was given in the CRU at 1800 hr the night before study comprising 12 kcal/kg distributed as 50% carbohydrate, 20% protein, and 30% fat. Volunteers then remained fasting, alcohol-abstinent, and caffeine-free overnight until the end of the infusion the next day.

In the CRU, catheters were placed in contralateral forearm veins at 0700 hr to allow simultaneous infusion of secretagogues and blood sampling every 10 min for a total of 6 hr beginning at 0800 hr. Sampling encompassed a 3-hr baseline and 3-hr stimulation interval. Infusions comprised L-arginine 30 gm delivered iv over 30 min, followed immediately by iv bolus of either 1 µg/kg GHRH (GEREF; Serono, Norwalk, MA) or 3 µg/kg GHRP-2 (Takeda Pharmaceuticals, Deerfield, Illinois). The doses of L-arginine and both peptides are maximally stimulatory in adults (17;18). L-arginine was employed to antagonize central somatostatin outflow (12;13).

Blood was also withdrawn at 0800 hr for later assay of serum estradiol (E₂), T, LH, FSH, IGF-I, IGFBP-1, IGFBP-3, albumin and SHBG concentrations. Lunch was provided after sampling before discharge from the CRU.

Hormone assays

Serum GH concentrations were determined in duplicate by automated ultrasensitive two-site immunoenzymatic chemiluminescence assay performed on the DxI automated system (Beckman Instruments, Chaska, MN 55318). Interassay CV's were 6.1% at 0.46 µg/L, 4.3% at 3.0 µg/L, 5.0% at 7.2 µg/L and 4.8% at 13.6 µg/L. Intraassay CV's were 4.7% at 0.37 µg/L, 3.5% at 2.5 µg/L and 3.2% at 14.8 µg/L. The lowest detectable GH concentration at 95% confidence is 0.008 µg/L determined by processing a 6-point calibration curve, 5 quality controls and 10 replicates of zero calibrator in multiple assays. The GH standard was recombinant human 22 kDa GH.

E₂ and T were measured by tandem liquid-chromatography ion-spray mass spectrometry. For E₂, intraassay CV's were 18%, 3.8% and 7.2% at concentrations of 3.6, 40 and 297 pg/mL (multiply by 3.68 for pmol/L). Interassay CV's were 8.1, 4.7, and 4.9% at 16, 31 and 119 pg/mL, respectively. For T, the analytic range is 7-2000 ng/dL (multiply by 0.0347 for nmol/L) for a 0.1 mL volume. Intraassay CV's were 3.3, 2.8, 2.2 and 2.0% at T concentrations of 16, 64, 184 and 927 ng/dL, respectively. Corresponding interassay CV's were 5.1, 3.8, 3.7 and 2.8%. Free and bioavailable T concentrations were calculated as described earlier (19).

IGFBP-1, IGFBP-3 and total IGF-I concentrations were quantified by immunoradiometric assays (Diagnostic Systems Laboratories, Webster, TX) as described (20). Intra- and interassay CV's were 6.1 and 8.4% for IGFBP-1, and 5.8 and 8.5% for IGFBP-3, respectively. Intraassay CV's for IGF-I were 3.4% at 9.4, 3% at 55 and 1.5% at 264 µg/L, and interassay CV's 9% at 64 µg/L and 6.2% at 157 µg/L.

Statistical analysis

An unpaired Student's *t* test was used to compare age, BMI and AVF as well as baseline fasting hormone concentrations in the 2 groups. Two-way ANOVA (2×2 factorial design) was used to examine the individual and interactive effects of normal vs low T/E₂ (2 factors) and secretagogue type (2 factors) on the summed mass of GH secreted in pulses over the 3 hr after

secretagogue infusion. *Post hoc* contrasts were made via Fischer's least-significant difference test (21). Log transformation was used to limit the dispersion of residual variance. Linear regression analysis and Pearson's correlation-coefficient P value were applied to examine the relationship between GH secretion and age, AVF, IGF-I, IGFBP-1 and IGFBP-3 concentrations (Systat, Point Richmond, CA). Bonferroni correction was applied to the 3 IGF-related measures ($P \leq 0.0167$). Significant differences were corroborated by the nonparametric rank-sum and Kruskal-Wallis tests (21).

Data are presented as the mean \pm SEM. Experiment-wise $P < 0.05$ was construed as statistically significant.

Statistical power analysis

Data from 18 studies in hypogonadal or normal males ($N = 149$ subjects total) indicate that parenteral T supplementation increases mean GH concentrations by a weighted-mean effect size (standard-deviate score) of 1.8 (6;8;9;11;22-26). Power analysis assumed that T/E₂ depletion exerts an opposite effect of similar relative magnitude. If comparison is made via a one-tailed (based upon the prior hypothesis) unpaired Student's *t* test at protected $P \leq 0.01$, then analysis of data from a total of 24 subjects (approximately 12 in each group) would achieve $> 99\%$ power to detect this effect size.

Deconvolution analysis

GH concentration time series were analyzed using a recently developed automated deconvolution method, which was verified mathematically by direct statistical proof and validated empirically by hypothalamo-pituitary sampling and simulated pulsatile time series (27;28). The Matlab-based algorithm first detrends the data and normalizes concentrations to the unit interval [0, 1]. Second, the program creates multiple sets of potential pulse times via an incremental smoothing process (a nonlinear adaptation of the heat-diffusion equation). Third, a maximum-likelihood estimation (MLE) method calculates all secretion and elimination parameters for each of the multiple candidate pulse-time sets. Deconvolution parameters comprise basal secretion (β_0), two half-lives (α_1, α_2), secretory-burst mass (η_0, η_1), random effects on burst mass (σ_A), procedural/measurement error (σ_ϵ), and a 3-parameter flexible Gamma secretory-burst waveform ($\beta_1, \beta_2, \beta_3$). The fast half-life of GH was represented as 3.5 min constituting 37% of the decay amplitude and the slow half-life as 20.8 min (29). The Akaike information criterion (30) is used to select the optimal pulse-time set from the multiple candidate sets. Other parameters are basal and pulsatile secretion rates (concentration units/3 hr), mass secreted per burst (concentration units), and waveform mode (time delay to maximal secretion after burst onset).

Visceral fat mass

Intraabdominal visceral fat mass was estimated by single-slice abdominal CT scan at L3-L4, exactly as reported (4).

Results

As described earlier and in a Supplemental (Appendix) Table, baseline subject characteristics did not differ in volunteers assigned to the leuprolide/placebo (Pl) and leuprolide/T treatment groups (16). In contrast, post-leuprolide vs post-placebo hormone concentrations differed significantly with respect to: (a) IGFBP-1 (higher in the T addback group, $P = 0.027$); (b) prolactin (higher in the T group, $P = 0.001$); (c) FSH (lower in the T group, $P < 0.001$); and (d) E₂, total T, bioavailable T and free T (each higher by $P < 0.001$ in the T group): **Figure 1**. The data suggest that eugonadal T concentrations contribute to maintaining IGFBP-1 and prolactin concentrations, and in suppressing FSH concentrations. IGF-I, IGFBP-3, SHBG and

LH concentrations were similar in the PI and T cohorts after leuprolide injection, whereas FSH was lower after leuprolide plus T (0.35 ± 0.11 IU/L) than leuprolide plus PI (2.3 ± 0.43 IU/L, $P = 0.001$). Therefore, T potentiated suppression of FSH by leuprolide, without further affecting LH or SHBG. Moreover, IGF-I and IGFBP-3 were not affected.

Figure 2 presents (mean \pm SEM) GH concentrations sampled every 10 min for 6 h, comprising 3-hr saline-infused and 3-hr secretagogue-stimulated profiles. Data were obtained after leuprolide injection plus PI vs T addback. The peak and time-course of GH responses were comparable visually in the two cohorts. Unstimulated (pre-secretagogue) mean GH concentrations were 0.61 ± 0.19 (PI) and 1.3 ± 0.49 μ g/L (T) [$P = 0.046$], and estimated basal (nonpulsatile) GH secretion rates were 2.3 ± 0.52 (PI) vs 4.0 ± 0.94 μ g/L/3 hr (T) [$P = 0.038$]. Unstimulated fasting pulsatile GH secretion was not affected by the sex-steroid milieu ($P = 0.37$): **Table 1**. Thus, short-term T depletion mainly reduced basal GH secretion. In relation to sequential secretagogue-stimulated GH release, two-way ANOVA identified a strong effect of L-arginine/GHRP-2 over L-arginine/GHRP ($P < 0.001$), but no effect of T vs PI addback ($P = 0.79$) and no interaction between secretagogue and sex-steroid milieu ($P = 0.49$). The effect of L-arginine combined with GHRP-2 on pulsatile GH secretion was 2.0 and 2.7-fold that of L-arginine/GHRH under low T and high T, respectively. Unpaired statistical comparisons of pulsatile GH secretion (μ g/L/3 hr) in the PI vs T-addback cohorts during the separate infusion of L-arginine/GHRH and L-arginine/GHRP-2 confirmed no differences due to T/E₂ availability for either secretagogue: **Table 1**.

Lack of statistical difference (at good statistical power, here $> 95\%$) between PI and T-addback responses permitted combining the data ($N = 24$ subjects) statistically (21). Linear-regression analyses in the combined cohorts revealed that basal (nonpulsatile) GH secretion was inversely related to AVF ($R^2 = 0.23$, $P = 0.017$) and directly to IGFBP-1 concentrations ($R^2 = 0.53$, $P < 0.0001$): **Figure 3**. Fasting unstimulated (saline-infused) pulsatile GH secretion correlated positively with IGF-I ($R^2 = 0.39$, $P = 0.0012$) and IGFBP-3 ($R^2 = 0.25$, $P = 0.015$) concentrations: **Figure 4**. These outcomes point to potentially different modulation of basal vs pulsatile GH secretion in the unstimulated fasting state. These results remained significant at Bonferroni-restricted $P \leq 0.0167$ for assessment of the 3 IGF-related measures.

In the setting of secretagogue infusions, regression analysis revealed a markedly negative effect of AVF ($R^2 = 0.35$, $P = 0.0024$) on L-arginine/GHRH's stimulation of pulsatile GH secretion: **Figure 5 (top)**. In contrast, stimulation by L-arginine/GHRP-2 was not influenced by either age or AVF (both $P > 0.10$). There was a strong trend toward IGF-I's being a positive statistical determinant of pulsatile GH secretion driven by sequential L-arginine/GHRH infusion ($R^2 = 0.23$, $P = 0.018$): **Figure 5 (bottom)**. On the other hand, none of IGF-I, IGFBP-1 or IGFBP-3 concentrations, age or AVF correlated with GH responses to L-arginine/GHRP-2. Accordingly, AVF is a major negative determinant of GHRH but not GHRP-2 action in this setting.

The waveform (time-dependent shape) of GH secretory bursts is estimated as the analytical mode of the secretory burst. Instead of being a measure of mass (amount of GH released), the mode denotes the time in min to attain maximal secretion after objectively estimated burst onset. By linear regression analysis and in the case of L-arginine/GHRH infusion only, AVF ($R^2 = 0.26$, $P = 0.011$) correlated negatively, and IGFBP-1 positively ($R^2 = 0.27$, $P = 0.0094$), with GH secretory-burst mode: **Figure 6**. Thus, the shape (waveform) of GH secretory events is influenced in some manner by AVF and IGF-I, but not only when the secretagogue is GHRH, thereby illustrating a further distinction in the mode of action of GHRH and GHRP-2.

Discussion

The present study affirms with high statistical power the *a priori* hypothesis that GH responses to sequential L-arginine/GHRH and L-arginine/GHRP-2 infusions are resistant to marked short-term reduction of T and E₂ concentrations. The outcomes indicate that L-arginine infusion followed by either GHRH or GHRP-2 constitutes an effective secretagogue combination in acutely (within 1 mo) hypogonadal men. On the other hand, only sequential L-arginine/GHRP-2 also stimulated GH secretion independently of AVF and IGF markers. Thus, L-arginine/GHRP-2 may be a useful secretagogue pair under clinical conditions in which hypogonadism is of recent onset and/or possibly overweightness exists.

The gold-standard insulin-tolerance test has some practical limitations, thus prompting evaluation of other powerful GH-releasing agents, such as the combination of L-arginine and GHRP-2 assessed here. For clinical utility, efficacy (maximal response) has been the conventional endpoint. However, complete dose-response estimates would be required to quantify potency and sensitivity as well. Maximal hormone concentrations (height of peak) in general correlate directly with the mass of hormone secreted per burst (31). Thus responses to the secretagogues utilized here yielded consistent inferences for secretory-burst mass (tables and figures) and maximal GH peak height (not shown).

The leuprolide-clamp paradigm also demonstrated that—independently of T/E₂ status—unstimulated basal (nonpulsatile) GH secretion was determined positively by IGFBP-1 concentrations and negatively by AVF, whereas unstimulated pulsatile GH secretion was related positively to both IGF-I and IGFBP-3 concentrations. IGF-I concentrations also represented a strongly positive predictor, and AVF a strongly negative predictor, of L-arginine/GHRH efficacy. In contradistinction, none of these factors influenced L-arginine/GHRP-2 stimulation in this cohort of 24 individuals. More studies will be required to assess the generality of this inference in other cohorts.

A parsimonious explanation for the observed nonsteroidal determinants of GHRH efficacy could be that high GHRH receptor-effector function enhances pulsatile GH output, which increases IGF-I and IGFBP-3 concentrations and decreases AVF. This scenario could explain both the positive association between pulsatile GH secretion and IGF-I and IGFBP-3 concentrations, and the negative association between pulsatile GH secretion and AVF. The precise reasons why GHRP does not exhibit the same multiple interdependencies with AVF and IGF-related factors as GHRH are not so clear. A main difference from GHRH is that ghrelin/GHRP exerts hypothalamic effects required for synergism with GHRH (2).

Sex-steroid hormones in women regulate not only the amount of GH secreted in bursts, but also the waveform or time-pattern of GH released within individual bursts (32). We could not detect T/E₂ effects on secretory-burst shape in men. The mode represents a shape or time-sensitive estimate of the delay to maximal secretion. This waveform term is independent of the amount (mass) of hormone secreted (33). However, AVF (negatively) and IGFBP-1 (positively) correlated with the time delay to maximal GH secretion under L-arginine/GHRH drive. Analyses using atomic-force microscopy indicate that GH-containing vesicles in pituitary cells must fuse with membrane pores to allow exocytosis (34). How AVF and IGFBP-1 modulate these processes is unknown.

Little is known about the regulation of basal (nonpulsatile) GH secretion (1). In the present study, this measure correlated positively with IGFBP-1 concentrations ($R^2 = 0.53$, $P < 0.0001$). The precise basis for this new association is not known. One plausible mechanism linking IGFBP-1 and basal GH secretion would be free IGF concentrations, which appear to inhibit GH secretion (35).

Caveats include the relatively small cohort studied (N = 24), possible unknown effects of leuprolide per se, and the need to eventually extend paradigm duration.

In summary, L-arginine/GHRH and L-arginine/GHRP-2 are robust stimulators of GH secretion in a short-term hypogonadal setting. L-arginine/GHRH efficacy is negatively determined by AVF and positively by IGF-I concentrations. No such effects were evident for L-arginine/GHRP-2 stimulation. AVF and IGFBP-1 are significant covariates of the waveform duration of L-arginine/GHRH (but not L-arginine/GHRP-2) induced GH secretory bursts. The present outcomes delineate new distinctions between GHRH and GHRP actions, which may influence the choice of optimal secretagogue in a particular clinical setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Definitions

basal, nonpulsatile secretion; baseline, prior to intervention.

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Post-Intervention Hormone Concentrations in Young Men

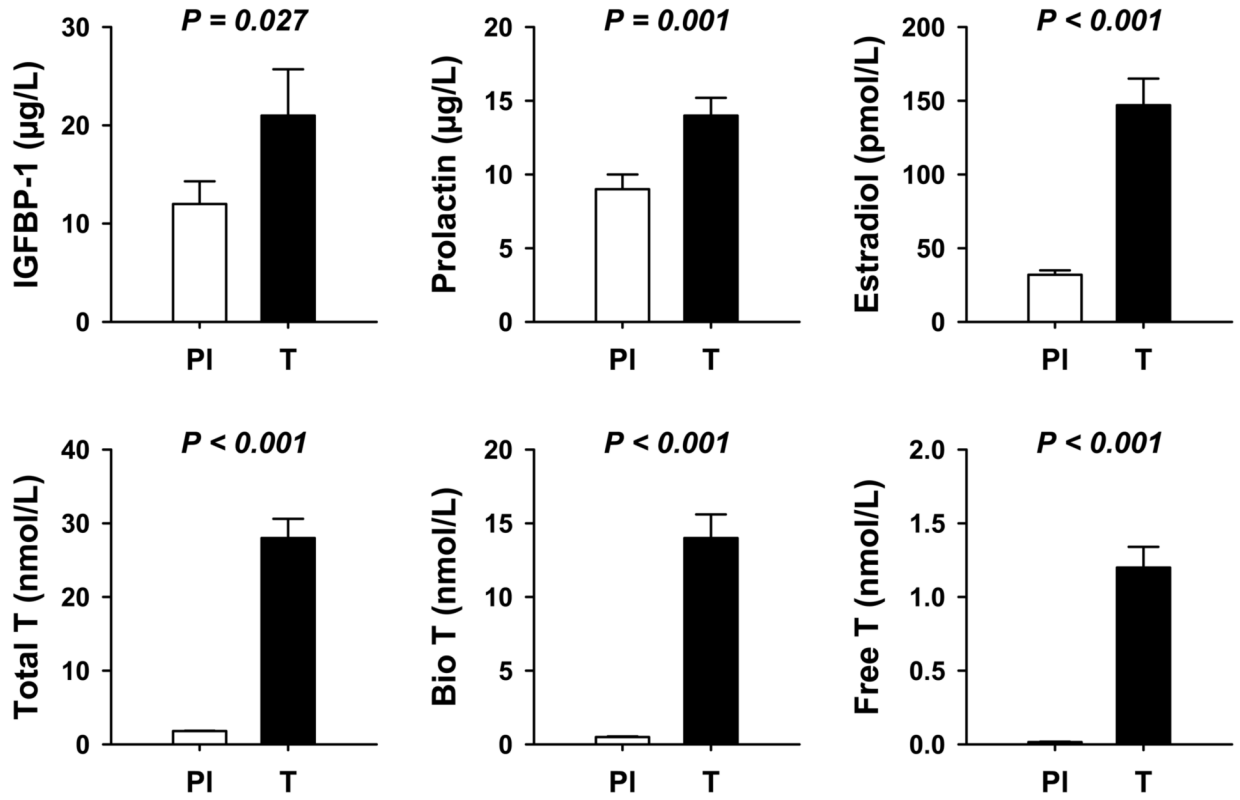


Figure 1. Significant hormonal differences in young men given leuprolide plus placebo vs leuprolide plus T addback.

Robustness of Combined Secretagogue Actions to Hypogonadal State

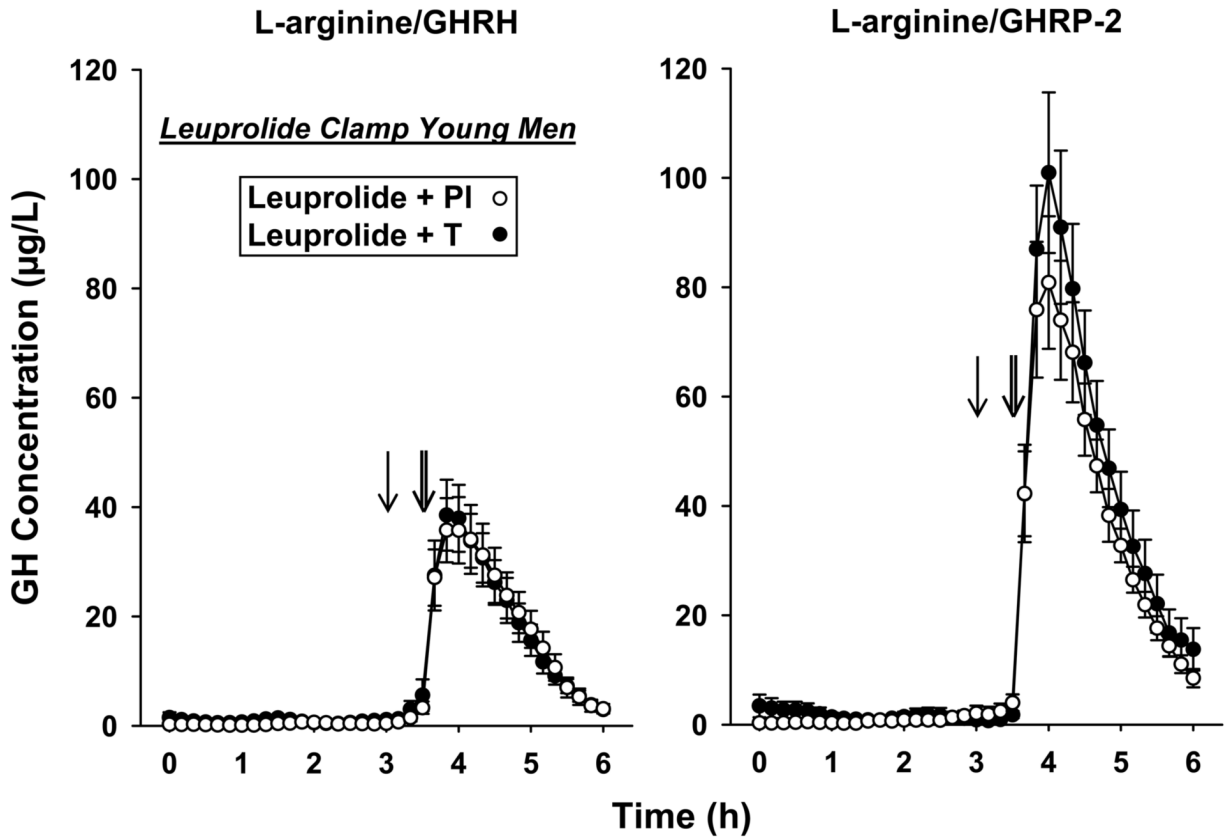


Figure 2.

Time profiles of GH concentrations sampled every 10 min for 6 hr fasting in 13 young men given leuprolide and placebo (PI) and 11 others given leuprolide and T injections. Saline was infused iv for 3 h, then L-arginine for 0.5 hr (*single arrows*) followed by bolus GHRH or GHRP-2 (*double arrows*). Data are the mean \pm SEM.

AVF and IGFBP-1 Determine Basal GH Secretion

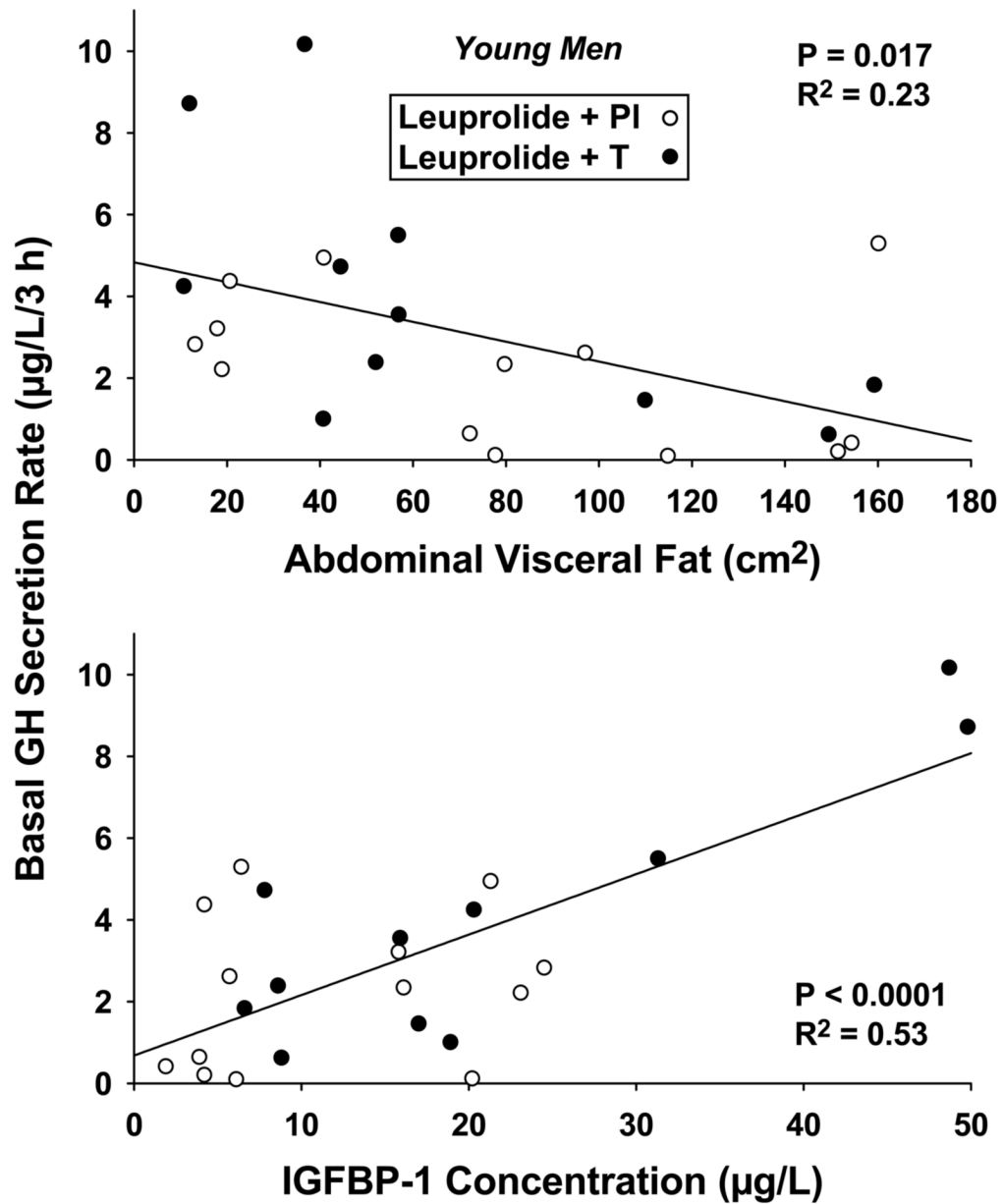


Figure 3.

Regression of unstimulated basal (nonpulsatile) GH secretion rates on CT-estimated abdominal visceral fat (AVF) and serum IGFBP-1 concentrations in 24 young men treated with leuprolide and PI (open circles) or T (closed circles). Two-tailed P values are given with the correlation coefficients.

IGF-I and IGFBP-3 Predict Pulsatile GH Secretion

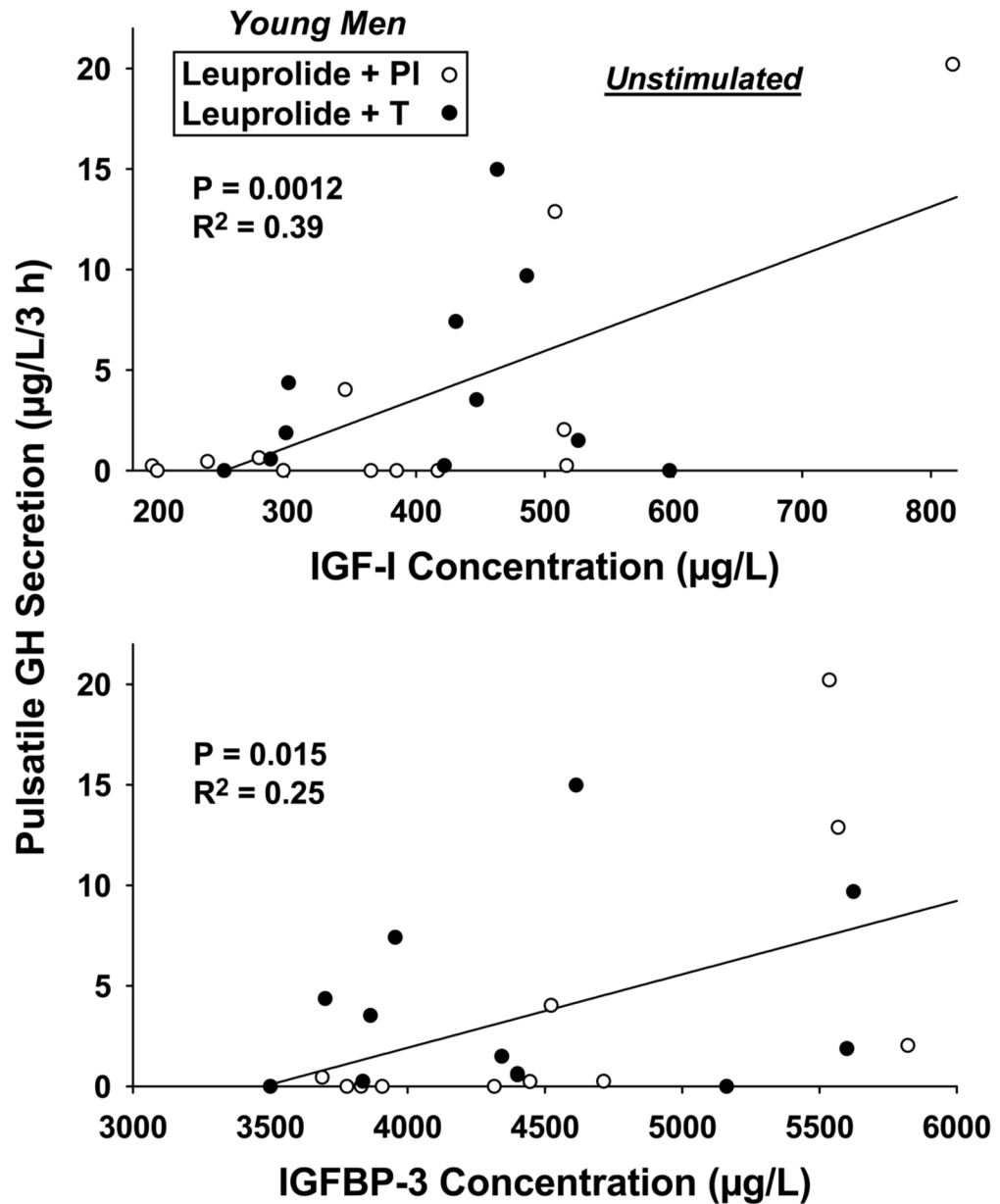
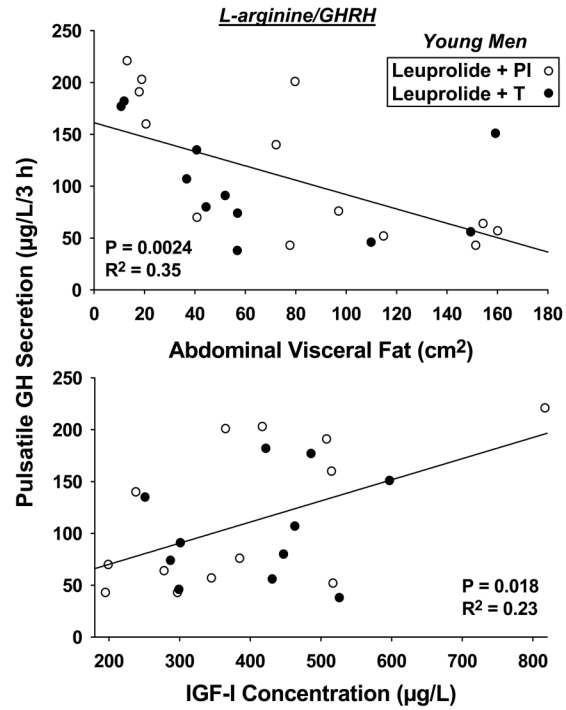


Figure 4. Positive relationships between unstimulated (saline-infused) pulsatile GH secretion and serum IGF-I (*top*) and IGFBP-3 (*bottom*) concentrations in 24 young men. Data are presented as described in **Figure 3**.

AVF and IGF-I Modify GHRH-Stimulated GH Secretion

**Figure 5.**

Efficacy of L-arginine/GHRH in stimulating pulsatile GH secretion correlates negatively with AVF (*top*) and positively with IGF-I concentrations (*bottom*) ($N = 24$ men, see **Figure 3**). This was not true for L-arginine/GHRP-2.

AVF and IGFBP-1 Determine Secretory-Burst Duration

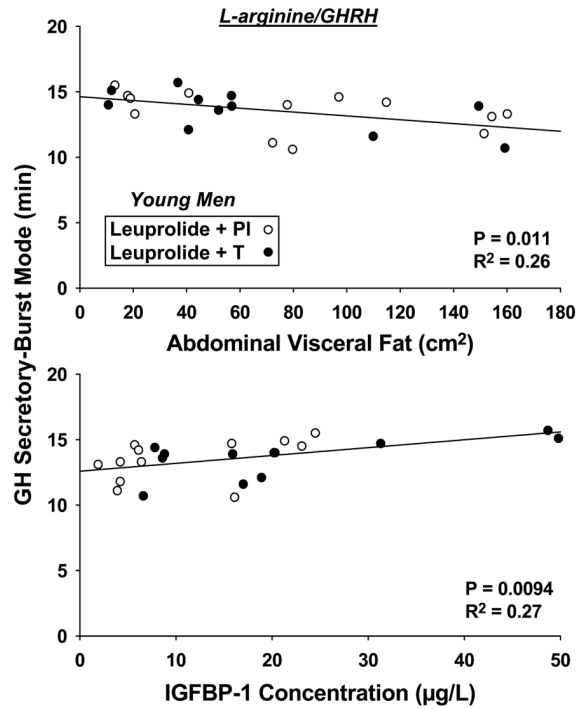


Figure 6. Impact of AVF (*top*) and IGFBP-1 concentrations (*bottom*) on L-arginine/GHRH-stimulated GH secretory-burst waveform. The mode of the waveform is the time delay to maximal GH secretion. No such effects were evident for L-arginine/GHRP-2 stimulation..

Table 1Impact of T/E₂ Depletion vs Repletion on GH Secretion *

Endpoint	Leuprolide + Pl (N = 13)	Leuprolide + T (N = 11)	P value
Basal (nonpulsatile) GH secretion	2.3 ± 0.52	4.0 ± 0.94	0.019
Unstimulated pulsatile GH secretion	5.1 ± 2.6	4.9 ± 1.6	P > 0.10
L-arginine/GHRH stimulation	117 ± 19	103 ± 15	P > 0.10
L-arginine/GHRP-2 stimulation	235 ± 27	273 ± 41	P > 0.10

Data are the mean ± SEM.

P values were estimated by an unpaired one-tailed Student's *t*-test under the prior hypothesis that T/E₂ depletion lowers GH output.

* Units are µg/L/3 h.