Estradiol regulates GH-releasing peptide's interactions with GH-releasing hormone and somatostatin in postmenopausal women

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Abstract

Objective: Estrogen stimulates pulsatile secretion of GH, via mechanisms that are largely unknown. An untested hypothesis is that estradiol (E₂) drives GH secretion by amplifying interactions among GH-releasing hormone (GHRH), somatostatin (SS), and GH-releasing peptide (GHRP).

Design: The design comprised double-blind randomized prospective administration of transdermal E₂ vs placebo to healthy postmenopausal women ($n=24$) followed by pulsatile GHRH or SS infusions for 13 h overnight with or without continuous GHRP2 stimulation.

Methods: End points were mean concentrations, deconvolved secretion, and approximate entropy (ApEn; a regularity measure) of GH.

Results: By generalized ANOVA models, it was observed that E₂ vs placebo supplementation: i) augmented mean (13-h) GH concentrations ($P=0.023$), GHRH-induced pulsatile GH secretion over the first 3 h ($P=0.0085$) and pulsatile GH secretion over the next 10 h ($P=0.054$); ii) increased GHRP-modulated ($P=0.022$) and SS-modulated ($P<0.001$) GH ApEn; and iii) did not amplify GHRH/GHRP synergy during pulsatile GH secretion. By linear regression, E₂ concentrations were found to be positively correlated with GH secretion during GHRP2 infusion ($P=0.022$), whereas BMI was found to be negatively correlated with GH secretion during GHRH ($P=0.006$) and combined GHRH/GHRP ($P=0.015$) stimulation. E₂ and BMI jointly determined triple (combined L-arginine, GHRH, and GHRP2) stimulation of GH secretion after saline ($R^2=0.44$ and $P=0.003$) and pulsatile GHRH ($R^2=0.39$ and $P=0.013$) infusions.

Conclusion: In summary, in postmenopausal women, E₂ supplementation augments the amount (mass) and alters the pattern (regularity) of GH secretion via interactions among GHRH, SS, GHRP, and BMI. These outcomes introduce a more complex model of E₂ supplementation in coordinating GH secretion in aging women.

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Introduction

Decreased growth hormone (GH) secretion during aging, hypogonadism, and obesity was recognized almost four decades ago (1, 2, 3, 4). Low GH concentrations, in turn, are associated with insulin resistance, dyslipidemia, cardiovascular mortality, sarcopenia, osteopenia, possible

cognitive impairment, and reduced quality of life. Supplementation of estradiol (E₂) in girls or women and that of an aromatizable androgen in boys or men stimulate GH production and alter the regularity of GH secretion patterns (2, 3, 5). The importance of E₂ is inferable in both sexes,

since an ER antagonist lowers GH concentrations in women and men and an aromatase inhibitor blocks GH responses to testosterone in men (2, 3, 6). Nonetheless, the precise clinical mechanisms mediating estrogen's augmentation of GH secretion remain largely unknown.

The effects of gonadal estrogen on the GH axis have been investigated in animal models by ovariectomy and transgenic silencing of the *ERα* (*ESR1*) and aromatase genes (2, 3, 7). In rats, E_2 controls hypothalamic GH-releasing hormone (GHRH) neurons, hypothalamic receptors for GH-releasing peptide (GHRP), pituitary receptors for GHRH and GHRP, somatostatin (SS) receptor subtypes 2 and 5, and gastric ghrelin (native GHRP) expression (2, 3, 7). The various effects of E_2 on GHRH, SS, and ghrelin secretion and action in animal models strongly suggest the estrogenic control of multiple peptide pathways. However, to date, available clinical studies of E_2 action have evaluated primarily single-peptide effects, rather than their interactions. To address this major limitation, the present study combined: i) experimental imposition of young-adult E_2 concentrations in postmenopausal women; ii) individual and combined infusions of hypothalamic peptides (GHRH, SS, and GHRP) for 13 h overnight; iii) deconvolution (secretion-based) and approximate entropy (ApEn, a pattern-regularity measure) analyses of GH concentration–time series; and iv) triple stimulation of GH secretion with L-arginine and GHRH/GHRP to estimate maximal (pharmacological) pituitary GH secretory capacity. The end points are important, because burst-like (pulsatile) GH secretion and ApEn-quantified secretion regularity are physiologically supervised modes of GH secretion (2, 3, 8). The postulate was that both the amount and pattern of GH secretion are jointly specified by E_2 , GHRH, GHRP/ghrelin, and SS, along with BMI as a surrogate of relative adiposity.

Subjects and methods

Study design and volunteers

The study protocol was approved by the Mayo Institutional Review Board (IRB) and reviewed by the U.S. Food and Drug Administration. This was a double-blind, placebo-controlled, prospectively randomized study of 24 healthy, community-based, ambulatory women conducted in the Center for Translational Science Activities (CTSA). The subjects were healthy postmenopausal women (follicle-stimulating hormone (FSH) levels >30 IU/l and E_2 levels <50 pmol/l) in the allowable age range of 50–80 years. The subjects provided witnessed voluntary informed consent

approved by the Mayo IRB. Screening consisted of medical history, physical examination, and biochemical testing. Eligibility required normal hepatic, renal, hematological, metabolic, and endocrine function and a negative mammogram within the last 12 months. Exclusion criteria were hypopituitarism, hyperthyroidism, hyperprolactinemia, concurrent use of neuroactive medications or sex hormones, acute or chronic systemic illness, diabetes mellitus, weight loss (>2 kg) in the last 3 months, systemic inflammatory disease, BMI <20 or >32 kg/m², greater than three time-zone transmeridian travel in the last week, shift-work schedule, concurrent involvement in any other study, drug or alcohol abuse, hemoglobin levels <2 g/dl, thrombotic arterial disease (stroke, transient ischemic attack (TIA), myocardial infarction (MI), and angina), pulmonary embolism or thrombophlebitis, and history or suspicion of cancer or neoplasm (except for basal cell carcinoma if localized and treated surgically).

E_2 treatment

E_2 supplementation was via daily transdermal E_2 patches (Novartis) at escalating increasing doses, viz. 0.05 mg/day (184 nmol) for the first 4 days, 0.10 mg/day (368 nmol) for the next 4 days, and 0.15 mg/day (552 nmol) for 20 additional days (9). Overnight infusion studies were initiated after at least 4 days on the highest (0.15 mg) E_2 dose. Medroxyprogesterone acetate (5 mg; Pharmacia and Upjohn, New York, NY, USA) was given orally daily for 12 days after E_2 supplementation, when all infusion/sampling sessions were completed.

Sampling schedule

The participants were admitted to the CTSA by 1530 h on the evening of the study. To limit nutritional confounds, a standardized meal (33 kJ/kg of 50% carbohydrate, 20% protein, and 30% fat) was served at 1600 h. The subjects then remained fasting, alcohol abstinent, and caffeine free until noon the next day. Vigorous exercise and daytime sleep were not allowed. Bilateral cannulas were placed in forearm veins at or before 1900 h to permit concurrent overnight blood sampling and peptide infusions.

Infusion and sampling paradigm

Each subject participated in a total of six (6) separate randomly ordered double-blind overnight consecutive 13- and 3-h (total 16-h) infusion sessions (2000 h until noon the next day) scheduled at least 48 h apart in the

CTSA (Fig. 1). Three infusion sessions comprised continuous i.v. saline infusions (30 ml/h) and the other three sessions comprised continuous i.v. GHRP2 infusions (1 µg/kg per h) for 13 h (2000–0900 h). Based upon prior dose–response curves obtained in older women (10, 11, 12), bolus i.v. saline (Sal; 1 ml), GHRH (1.0 µg/kg), or SS (0.67 µg/kg) pulses were superimposed upon the continuous saline or GHRP2 infusions every 90 min for a total of nine pulses during the inclusive time window 2000–0850 h. GHRP2 was obtained from Kaken Pharmaceuticals (Tokyo, Japan), and GHRH and SS were obtained from Bachem Laboratories (Torrance, CA, USA).

Immediately after the 13-h overnight pulsatile peptide infusions, a triple secretagogue was administered, followed by three more hours of sampling. This was done to test maximal (pharmacological) GH release. The triple stimulus comprised sequential i.v. infusion of L-arginine (30 g continuously from 0900 to 0930 h). This was immediately followed by combined bolus injections of

GHRH and GHRP2 (both i.v. at 1.0 µg/kg) at 0930 h. This combination has been used by others (8).

Blood (0.75 ml/sample) was withdrawn every 10 min for 16 h (2000 h–noon) from all 24 subjects during all the six sessions. Sleep was not assessed in this study. Serum that was separated was frozen at –70 °C until assay.

Assays

GH concentrations were determined as a batch of 582 samples/subject via a robotics-assisted two-site monoclonal immunoenzymatic chemiluminescence assay (sensitivity 0.010 µg/l). The assay standard was 22 kDa recombinant human GH. Inter-assay coefficients of variation (CV) for GH concentrations of 3.4 and 12 µg/l were 7.9 and 6.3% respectively. Intra-assay CV values for GH concentrations of 1.1 and 20 µg/l were 4.9 and 4.5% respectively. No values were <0.020 µg/l. Screening thyroid-stimulating hormone (TSH), prolactin, luteinizing hormone (LH), and FSH concentrations were quantified by an automated chemiluminescence assay (ACS 180; Bayer), using as standards recombinant TSH and prolactin and the First and Second International Gonadotropin Reference Preparations. Liquid chromatography–tandem mass spectrometry was used to quantify E₂ and testosterone concentrations in serum samples collected at 0800 h at screening and at each of the six (6) CRU visits (13). SHBG, insulin-like growth factor 1 (IGF1), IGF-binding protein 1 (IGFBP1), and IGFBP3 concentrations were assayed by an immunoradiometric assay (Diagnostic Systems Laboratories, Webster, TX, USA).

Analytical methods

Deconvolution analysis was applied to the initial 3-h (2000–2250 h inclusive) and the next 10-h (2300–0900 h) windows of the 13 h of 10-min sampling sessions during peptide/saline infusions, as well as to the final 3 h (0900 h–noon) of 10-min sampling sessions during the triple stimulus. The goal was to estimate basal (nonpulsatile) and pulsatile (burst-like) GH secretion and thereby total GH secretion. The first 3-h GH responses to peptide infusions (2000–2250 h) were used to estimate readily releasable GH (14). GH secretion was quantified using published biexponential GH-elimination kinetics (*viz.* 3.5- and 20.8-min half-lives (15)). The Matlab model is conditioned mathematically on *a priori* identification of candidate sets of pulse-onset times using an image boundary-detection technique. Pulse number is optimized by the Akaike information criterion (16). Both sensitivity and specificity are 93%.

Figure 1

Schematic representation of 16-h overnight peptide infusion/blood sampling protocol conducted in 24 postmenopausal women randomized to 20 days of transdermal E₂ or placebo (PI) exposure. During E₂ (n = 10) or PI (n = 14) exposure, each subject participated in six randomly ordered 13-h i.v. infusion sessions with overnight fasting: three with continuous saline stimulation and three with continuous GHRP2 stimulation. Pulses of saline (Sal), GHRH, or SS were superimposed every 90 min i.v. during the 13-h infusion sessions. Sampling was continued for an additional 3 h to test pituitary GH secretory capacity using a triple-secretagogue stimulus (consecutive L-arginine and bolus GHRH/GHRP2 injections).

Approximate entropy

ApEn (1, 20%) was used as a scale- and model-independent statistic to quantify the relative orderliness (regularity) vs disorderliness (irregularity). Higher ApEn denotes greater relative randomness (irregularity) of the secretion process. Mathematical models and clinical experiments establish that greater irregularity signifies decreased feedback control with high sensitivity and specificity (both >90%) (17).

Biostatistical analysis

The experimental design was consistent with the general principles of a split-plot design. The whole plots were the primary treatments of E₂ or placebo. Within each condition, the participants received six different infusions (GHRP2 vs Sal infusion crossed with Sal, GHRH, or SS infusion). The nesting of the treatments within patients was addressed statistically by means of a mixed fixed- and random-effects model using SAS PROC MIXED. Main effects for each level of the split-plot design along with their two- and three-variable interactions were modeled as fixed effects. The random effect consisted of a random participant (blocking) factor. To limit heterogeneity of variance, parameters were first log-transformed. Model-based means were compared with the Tukey–Kramer *post hoc* correction factor. The degrees of freedom for the fixed effects were estimated using the Kenward–Roger method. Experiment-wise (adjusted) *P* values <0.05 were considered statistically significant, except for multiple comparisons when *P*<0.01 (protected) was used (18). *Post hoc* analysis used Tukey's honestly significantly different (HSD) test. Analyses were conducted using the SAS System, version 9.3 (Cary, NC, USA).

Epidemiological data are presented as means ± S.E.M. (medians and ranges) to provide a full view of variability. Backward stepwise-elimination linear regression was carried out to identify the independent or joint contributions of IGF1, IGFBP1, IGFBP3, and E₂ concentrations and/or BMI to the modulation of GH production.

Statistical power

By way of statistical power estimates, earlier studies had quantified pulsatile GH secretion stimulated by: i) 90-min pulses of GHRH in 19 men; ii) continuous i.v. infusion of GHRH and/or GHRP2 in 12 women; and iii) i.v. injections of SS in 11 other women (10). On combining these studies, the respective amplifying actions of E₂ and GHRH were

2.1 ± 0.18 (s.d.) and 4.9 ± 0.53-fold placebo/saline; the effect of GHRP2 was 8.5 ± 2.2-fold saline; and the effect of SS suppression was 0.5 ± 0.11-fold saline (5, 10, 11, 12). For two-way ANOVA, the estimated statistical power to detect 50% augmentation of GH responses to either GHRH or SS pulses by E₂ during saline or GHRP2 stimulation equaled or exceeded 80% if 24 women completed the study.

Results

Subject characteristics at baseline screening

Epidemiological and baseline endocrine data are given in Table 1. Absolute age range for the 24 participants was 50–76 years. There was no age or BMI difference in the randomized groups (protected *P*<0.01 for multiple tests). Total testosterone, E₂, LH, FSH, prolactin, SHBG, and TSH concentrations were all normal for age (Mayo Medical Reference Laboratories, Rochester, MN, USA). After E₂ vs Pl supplementation, E₂ (*P*<0.001) and mean GH concentrations during Sal/Sal infusions (*P*=0.023) and SHBG concentrations (*P*<0.01) increased, whereas IGF1, IGFBP1, and IGFBP3 concentrations did not change significantly (Table 2).

GH responses to 13-h peptide or saline infusions

Rapid (3-h) GH responses ► The response observed in the first 3 h of each 13-h peptide/saline infusion session was arbitrarily considered an immediate or rapid response. Analysis of 3-h pulsatile GH secretion revealed strong effects

Table 1 Baseline epidemiological and endocrine data: pre-randomization (fasting 0800-h values). Data are means ± S.E.M. (median, range).

Variables	Pre-randomization (fasting 0800-h values)	
	Subsequent placebo exposure (<i>n</i> = 14)	Subsequent E ₂ exposure (<i>n</i> = 10)
Age (years)	66 ± 1.5 (66) (55–76)	60 ± 2.0 (60) (50–69)
BMI (kg/m ²)	26 ± 0.85 (26) (22–30)	25 ± 1.1 (25) (21–32)
Hb (g/dl)	13 ± 0.22 (13) (12–15)	13 ± 0.40 (14) (12–16)
Albumin (g/dl)	4.3 ± 0.05 (4.3) (3.9–4.6)	4.5 ± 0.05 (4.4) (4.2–4.8)
Total testosterone (nmol/l)	0.83 ± 0.13 (0.58) (0.26–1.8)	0.66 ± 0.15 (0.52) (0.28–1.8)
E ₂ (pmol/l)	69 ± 12 (69) (39–128)	68 ± 11 (81) (35–128)
LH (IU/l)	25 ± 2.6 (26) (12–44)	31 ± 2.0 (29) (23–43)
FSH (IU/l)	88 ± 13 (75) (41–171)	74 ± 6.7 (69) (37–125)
Prolactin (μg/l)	6.1 ± 0.74 (5.5) (3–11)	7.5 ± 0.56 (8.0) (4–9)
SHBG (nM)	53 ± 5.5 (48) (31–98)	59 ± 10 (53) (24–136)
TSH (mU/l)	1.8 ± 0.22 (1.6) (0.5–3.2)	1.8 ± 0.27 (1.5) (0.8–3.1)

Table 2 Baseline epidemiological and endocrine data: post-randomization (mean of six visits per subject). Data are means $\pm$ S.E.M. (median, range).

Variables	Post-randomization (mean of six visits per subject)	
	During placebo exposure	During E ₂ exposure
E ₂ (pmol/l)	66 $\pm$ 13 (58)	450 $\pm$ 110 (355)*
IGFBP1 (μ g/l)	35 $\pm$ 4.6 (28)	43 $\pm$ 6.1 (42)
IGFBP3 (mg/l)	3.6 $\pm$ 0.71 (3.5)	3.2 $\pm$ 0.23 (3.1)
IGF1 (nmol/l)	21 $\pm$ 2.6 (18)	17 $\pm$ 2.0 (16)
SHBG (nM)	42 $\pm$ 4.3 (39)	93 $\pm$ 17 (91)*
GH (μ g/l)	0.48 $\pm$ 0.073 (0.42)	0.88 $\pm$ 0.16 (0.72) ^a

Protected $P < 0.01$ vs placebo.

^aMean of 13-h sampling during Sal/Sal infusions ($P = 0.023$ for PI vs E₂ exposure).

of: i) E₂ vs PI supplementation ($P = 0.0085$); ii) GHRP2 vs Sal infusion ($P < 0.0001$); iii) GHRH vs Sal infusion ($P < 0.0001$); and iv) GHRH vs SS infusion ($P < 0.0001$) (Table 3, column 1). SS infusion had no significant effect on this end point.

Sustained (10-h) responses to peptide infusions ► The response observed in the last 10 h of each 13-h infusion session was considered a sustained response. Median 10-min GH concentrations (PI, $n = 14$; E₂, $n = 10$) over this interval (2300–0900 h) under saline infusion are shown in green in Fig. 2. GHRH pulses elicited marked pulsatile GH responses (blue lines) in all four major conditions: PI (no E₂)/Sal, E₂/Sal, PI/GHRP2, and E₂/GHRP2. The unique combination of pulses of GHRH and continuous GHRP2 infusions with or without E₂ supplementation was maximally stimulatory. SS pulses were inhibitory (red lines) only in the presence of PI/GHRP2 and E₂/GHRP2. Deconvolution analysis revealed significant treatment effects on 10-h pulsatile GH secretion. Figure 3 shows box-and-whisker plots (medians, interquartile ranges, 95% CIs, and extreme ranges (solid dots)) for the 12 arms: six with PI supplementation (left panel) and six with E₂ supplementation (right panel). Statistical main effects existed for E₂ ($P = 0.054$), GHRP2 ($P < 0.0001$), and GHRH (vs saline and SS; $P < 0.0001$) (Table 3, column 2). SS vs Sal infusion had no effect. There was a prominent amplifying interaction between GHRP2 and GHRH ($P < 0.0001$) and a nonsignificantly trending potentiating interaction between E₂ and GHRP2 ($P = 0.059$) for 10-h pulsatile GH secretion.

ApEn (degree of irregularity)

The ApEn of GH secretion over the sustained 10-h period of saline or peptide infusions (2300–0900 h) revealed main effects of E₂ ($P = 0.028$), continuous GHRP2 ($P < 0.0001$),

pulsed GHRH ($P < 0.0001$), and pulsed SS ($P = 0.013$) to increase ApEn (Table 3, column 3). In particular, E₂ vs PI supplementation elevated GH ApEn under Sal/SS ($P < 0.001$) and GHRP2/Sal ($P < 0.01$) infusions, strongly suggesting decreased feedback. Moreover, there were significant interactions between E₂ and GHRP2 ($P = 0.022$), between E₂ and GHRH ($P = 0.019$), and among E₂, GHRP2, and GHRH/SS ($P = 0.027$) in increasing ApEn.

Triple-stimulus effects

A triple stimulus of consecutive L-arginine and combined GHRP2/GHRH infusion was used to evaluate pituitary GH secretory capacity at the end of the 13-h pulsatile peptide infusion session (Fig. 4). Prior 13-h pulsatile GHRH infusion potentiated pulsatile GH secretion after the triple stimulus ($P = 0.0015$), whether or not E₂ was administered (blue lines in the top two panels). Concomitant GHRP2 infusion (blue lines, bottom two panels) unexpectedly suppressed ($P < 0.0001$) the GHRH effect (bottom two panels). Statistical outcomes are summarized in column 4 of Table 3. Specifically, E₂ supplementation increased the GH-inhibitory effect of GHRP2 on GHRH's priming of pulsatile GH secretion ($P = 0.049$). E₂ supplementation did not affect median GH concentrations after GHRH infusion, but it did increase the simple peak GH concentration (arithmetic maximum) response to the triple stimulus following 13-h Sal/SS ($P < 0.01$), GHRP2/Sal ($P < 0.05$), and GHRP2/GHRH ($P < 0.05$) infusions. Comparable median levels but higher peak values after GHRH pretreatment probably reflect the fact that peak values occur at non-uniform time points after the triple stimulus.

Table 3 Pulsatile GH secretion and ApEn in 24 women. Split-plot analysis with main effects and interactions.

	Pulsatile (acute/3 h)	Pulsatile (sustained/ 10 h)	ApEn (irregularity)	Pulsatile after triple stimulus
Main effects				
i) E ₂	0.0085	0.054	0.028	0.92
ii) GHRP2	<0.0001	<0.0001	<0.0001	<0.0001
iii) Pulsed peptides (GHRH/SS/Sal)	<0.0001	<0.0001	<0.0001	0.0028
GHRH vs Sal	<0.0001	<0.0001	<0.001	0.0015
SS vs Sal	0.63	0.95	0.013	0.32
GHRH vs SS	<0.0001	<0.0001	0.012	0.091
Interactions				
E ₂ $\times$ GHRP2	0.61	0.059	0.022	0.049
E ₂ $\times$ GHRH/SS/Sal	0.45	0.17	0.019	0.10
GHRP2 $\times$ GHRH/ SS/Sal	0.091	<0.0001	0.31	0.55
All three factors	0.77	0.67	0.027	0.74

Data are P values for main effects and interactions.

Figure 2

Median GH concentration profiles obtained by sampling blood every 10 min overnight during the last 10 h of a 13-h interval (2300–0900 h) of constant saline (top row) or GHRP2 (bottom row) infusions in 14 women treated with placebo (no E_2) (left column) and ten other women treated with E_2 (right column). The colors green, blue, and red denote superimposed pulsatile infusions of saline, GHRH, and S respectively. X-axis zero time is 2250 h. The corresponding box-and-whisker plots of the matching statistical outcomes are shown in Fig. 3.

Linear regression analysis

A stepwise backward-elimination multivariate regression analysis was used to explore the effects of BMI, age, E_2 , IGF1, IGFBP1, and IGFBP3 on GH secretory responses to the overnight peptide infusions and the subsequent triple stimulus. In relation to 10-h data, BMI was a negative correlate of total (basal plus pulsatile) GH secretion during Sal/GHRH infusions ($P=0.006$ and $R^2=0.29$) as well as GHRP2/GHRH infusions ($P=0.015$ and $R^2=0.24$), and E_2 was a positive correlate of 10-h total GH secretion under GHRP2/Sal infusions ($R^2=-0.22$ and $P=0.022$). In relation to the triple stimulus, BMI significantly reduced and E_2 increased the total GH secretory response after 13 h of Sal/Sal infusions (joint $P=0.003$ and $R^2=0.44$) and Sal/GHRH infusions ($P=0.013$ and $R^2=0.39$). On the other hand, BMI and age together determined the triple-stimulus effect on total GH secretion after 13 h of combined GHRP2/GHRH infusion ($P=0.001$ and $R^2=0.46$). BMI negatively predicted GH responses to the triple stimulus after GHRP2/Sal infusions ($P=0.030$ and $R^2=0.20$).

Discussion

The present clinical investigation in postmenopausal women unveils the major effects of E_2 supplementation on GH secretory dynamics driven by an ensemble of GH-regulating peptides, *viz.* repetitive i.v. pulses of GHRH, SS, and saline with or without continuous GHRP stimulation. Peptide doses were chosen based on prior peptide dose–response curves obtained in older women (10, 11, 12). The salient effects of E_2 were as follows: i) amplification of rapid (initial 3-h) pulsatile GH secretion during overnight pulsatile Sal/GHRH stimulation ($P=0.0085$), with a strong analogous trend for E_2 to augment prolonged (10-h) pulsatile GH secretion during GHRP2 drive ($P=0.059$), signifying a greater mass of GH released per burst; ii) elevation of GH ApEn during GHRP2 ($P=0.022$) and SS ($P<0.001$) infusions, thus suggesting decreased feedback effects under E_2 supplementation; and iii) opposition in an E_2 concentration-dependent manner the negative effect of BMI on the GH secretory response to a massive triple stimulus. The last point becomes relevant for interpreting pharmacological testing of GH reserve capacity (8), suggesting that

Figure 3

Box-and-whisker plots of 10-h pulsatile GH secretion. Data are medians, interquartile ranges, 95% CIs, and extreme ranges (separate dots). Fourteen women were randomized to receive placebo (six bars on the left) and ten women E_2 (six bars on the right) for 20 days. The general linear model P value was <0.0001 . Boxes with unique (unshared) alphabetic superscripts differ significantly by *post hoc* Tukey's HSD multiple-comparison test (e.g. A differs from B and C, but not from AB).

Figure 4

Median GH concentration profiles obtained by sampling blood every 10 min over 3 h (0900–1200 h) during (0900–0930 h) and following (0930–1200 h) the triple stimulus (Fig. 1).

both BMI and E_2 administration may alter the cutoffs for a maximal GH response at least in older women.

GH regulation is important in aging, in as much as small increments in GH availability in older adults decrease visceral adiposity, diastolic blood pressure, and LDL concentrations and increase lean body mass (19). Pulsatile secretion is a key locus of physiological GH regulation by hypothalamo-pituitary GHRH, SS, and GHRP (2, 20). In turn, GH pulses specifically control liver and muscle gene expression, mediate GH feedback on brain, and enhance lipolysis in fat (3). Thus, tissue responses to GH peaks may be more significant than those to mean/median GH concentrations *per se*, especially in relation to growth and anabolism (3). The generation of GH pulses is believed to require SS, GHRH, and GHRP/ghrelin. First, SS withdrawal seems necessary before GHRH pulses can initiate prominent GH secretory bursts (3, 20, 21). Second, GHRH is essential for stimulating both GH release and GH synthesis (2). SS and GHRH act in concert, since SS restrains exocytosis (but not synthesis) of GH, thus allowing the accumulation of releasable GH stores under ongoing GHRH-stimulated GH synthesis (14). Third, whereas GHRP does not augment GH synthesis, it markedly potentiates GHRH-evoked GH release, thus serving as an *in vivo* amplifier of GH pulsatility (3, 22, 23, 24). We tested the amplifying interaction between GHRH and GHRP by infusing GHRH pulses every 90 min for 13 h along with saline or GHRP2. GHRP2 and a physiologically pulsatile GHRH stimulus were strongly synergistic over 10 h

independently of E_2 availability. The importance of GHRH for maximal GHRP drive has been established in patients with inactivating mutations of the GHRH receptor (21) or hypothalamo-pituitary disconnection (23). Thus, a working model of GH pulsatility would include SS as a determinant of the timing and size of GH secretory bursts and ghrelin/GHRP and GHRH as individual and synergistic amplifiers of the size of GH pulses (20, 25, 26, 27, 28).

SS is a powerful inhibitor of GH release. In the presence of E_2 , repeated SS pulses increased GH ApEn (Table 3), a statistical measure of the relative randomness, irregularity, or disorderliness of secretory patterns (29). The ability of E_2 and SS to increase GH ApEn would be consistent with the ability of E_2 to attenuate GH and IGF1 feedback and SS potency, as well as to enhance submaximal GHRH and GHRP stimulation in women (3, 10, 23, 30). Nonetheless, SS pulses at the dose used in the present study in women had no detectable effect on GH ApEn in an earlier study in older men in the presence and absence of supplemental testosterone (31). This raises the question whether pituitary sensitivity to SS differs by gender in humans.

The triple-secretagogue paradigm has been suggested as a probe of age-independent GH release (8). In the present study, we used it to rule out possible ceiling effects of the double-peptide clamps. The average peak GH concentration after the triple stimulus in the present study was 339 ± 53 (146–648 range) $\mu\text{g/l}$ after overnight pulsatile Sal/GHRH infusions. This excluded the downregulation of GH secretion under the overnight GHRH clamp, rather showing the GHRH priming effect. In the combined group of 24 women, E_2 concentrations positively determined the peak GH response to the triple stimulus after exposure to Sal/Sal and Sal/GHRH, indicating a selective influence of E_2 on GHRH's priming of acute GH secretion.

When continuous GHRP2 stimulation was combined with a train of GHRH pulses, GHRH's potentiating effect on the acute triple stimulus was suppressed by more than 50%. This was unexpected. The attenuation of the triple-secretagogue effect by GHRP2 could be due, in principle, to homologous GHRP receptor desensitization or heterologous GHRP/GHRH receptor downregulation, as reported in animal models (32). Since heterologous GHRH/GHRP receptor upregulation also occurs, our results introduce the clinical question: what determines GHRH/GHRP up- vs downregulation? The present data further indicate that E_2 increases the negative effect of GHRP2 on GHRH's amplification of the triple stimulus. Since E_2 elevates GH levels, high GH concentrations may have exerted negative feedback by hypothalamic SS (20). Nonetheless, L-arginine, as

part of the triple stimulus used in the present study, would be predicted to mute GH negative feedback (2, 3, 33).

The significance of this study is that target tissues responded to circulating GH in a pulse-defined and concentration-dependent fashion (2). Complexity arises, since E_2 can both inhibit and augment GH's actions on target tissues (e.g. on liver, brain, breast, fat, or bone) (34). The tendency of IGF1 levels to decrease during E_2 administration, especially orally, may constitute another mechanism for amplified pulsatile GH secretion beyond that of muting dose-dependent IGF1 feedback actions (30). The joint capabilities of E_2 to drive pulsatile patterns of GH production and to modulate GH's actions on target tissues should confer precise metabolic and anabolic effects. This may be particularly important clinically in otherwise estrogen-deprived women, who are secondarily relatively GH deficient as well.

BMI was a major and consistently negative determinant of total (pulsatile plus basal) GH secretion during pulsatile Sal/GHRH infusions and after triple-secretagogue stimulation. During overnight pulsatile GHRH infusion, BMI (negatively) accounted for 29% of the variance in total GH secretion. The negative and positive effects of BMI and E_2 together explained 44% of inter-subject variability in triple stimulus-driven total GH secretion after Sal/Sal infusions. BMI also interacted with age reciprocally in controlling 46% of the variance in the triple-stimulus effect after combined GHRH/GHRP priming. Thus, even pharmacological multi-stimuli tests of GH secretory reserve are damped by relative obesity. Further studies are needed to test the reproducibility of, and elucidate the exact bases for, these unique interactions.

Limitations include the ultimate need to: elucidate the dose dependence and long-term sustainability of E_2 effects on multiple peptide-regulated GH secretion; examine a wide range of pulsed SS and GHRH doses; assess possible effects on sleep as well as GH; and extend the short-term (weeks) paradigm to long-term (months) hormone-replacement regimens.

In summary, controlled E_2 supplementation in postmenopausal women exerts positive multipathway effects on GHRH/GHRP/SS-regulated GH secretion. E_2 also counteracts the suppressive effect of BMI on stimulated GH secretion. These outcomes suggest multiple loci of estrogenic regulation of the adult female GH axis, viewed as an ensemble of GHRH, GHRP/ghrelin, and SS signals.

Declaration of interest

Dr Dana Erickson is on the Advisory board for Ipsen. Otherwise, the authors have nothing to disclose.

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Author contribution statement

C Norman carried out the experiments, drafted the manuscript, and approved the final version of the manuscript; N L Rollene, D Erickson, and J M Miles carried out the experiments and approved the final version of the manuscript; C Y Bowers was responsible for the conception and design of the research, interpreted the results of the experiments, and approved the final version of the manuscript; J D Veldhuis was responsible for the conception and design of the research, carried out the experiments, analyzed the data, interpreted the results of the experiments, prepared the figures, revised the manuscript, edited and revised the manuscript, and approved the final version of the manuscript.

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