Estradiol Potentiates Ghrelin-Stimulated Pulsatile Growth Hormone Secretion in Postmenopausal Women

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Context: Ghrelin and an estrogen-rich milieu individually amplify pulsatile GH secretion by increasing the amount of hormone released per burst. However, how these distinct agonists interact in controlling pulsatile GH output is not known.

Objective: The objective of the study was to test the hypothesis that elevated estradiol (E2) concentrations potentiate hypothalamic-pituitary responses to a near-physiological ghrelin stimulus.

Design: This was a double-blind, placebo-controlled, prospectively randomized, parallel-cohort study.

Setting: The study was conducted at an academic medical center.

Subjects: Twenty-one postmenopausal women participated in the study.

Interventions: Eleven subjects received placebo (Pl) and 10 others E2 transdermally in escalating doses over 3 wk to mimic late follicular-phase E2 concentrations. Saline or a submaximally stimulatory amount of ghrelin (0.3 μg/kg) was infused iv on separate randomly ordered mornings fasting after 17–21 d of Pl or E2 administration.

Outcomes: Outcomes included serum concentrations of E2, ghrelin, GH, IGF-I, IGF binding protein (IGFBP)-1 and IGFBP-3, and the estimated mass and waveform of stimulated GH secretory bursts.

Results: Administration of E2 yielded late follicular-phase E2 concentrations. Compared with Pl, E2 did not alter ghrelin concentrations but reduced IGF-I and IGFBP-3 and elevated IGFBP-1 concentrations. Compared with saline, ghrelin infusion amplified pulsatile GH secretion by 7.1-fold (P < 0.01). The effect of E2 alone was 2.0-fold placebo and that of combined ghrelin/E2 10.4-fold (P < 0.01). Ghrelin and E2 accelerated initial GH release individually but nonadditively by more than 2-fold (P < 0.01).

Conclusions: Estrogen augments ghrelin’s near-physiological stimulation of pulsatile GH secretion and mimics ghrelin’s acceleration of initial GH release. Thus, we hypothesize that estrogen and a GH secretagogue act via independent as well as convergent mechanisms.

GH AND IGF-I production increase by 3- to 10-fold in puberty and decrease progressively thereafter beginning in young adulthood (1). The parallelism between pulsatile GH secretion and sex-steroid concentrations across the human lifetime suggests that waning of gonadal steroidogenesis in older individuals contributes to relative hyposomatotropism in aging (2). This notion is supported by the capability of short-term supplementation with estradiol (E2) or testosterone to double GH concentrations in young and older hypogonadal patients and elderly healthy adults (3–10).

Estrogen stimulates GH secretion by augmenting the amount of hormone released per burst (11–13). The size of GH secretory bursts in turn is determined by at least three peptides, viz., GHRH, ghrelin [a GH-releasing peptide (GHRP)], and somatostatin (an antagonist of both GHRH and GHRP) (14–26). Puberty as well as the administration of estrogen or an aromatizable androgen in prepubertal children enhances responsiveness to synthetic analogs of ghrelin (27, 28). Studies in adults are conflicting, in that E2 supplementation may or may not potentiate stimulation of GH secretion by near-maximally effective doses of ghrelin analogs (reviewed in Ref. 29). Possible explanations for disparities are confounding effects of endogenous ghrelin and/or the use of pharmacological doses of agonists, which would test secretagogue efficacy but not potency (30). Addressing these issues could help clarify why the actions of GHRP are increased more in girls than boys during puberty, reduced in aging individuals with lower sex-steroid concentrations, and gender independent in other settings (18, 21, 27, 28, 31).

The present study tests the hypothesis that a young adulthood-like estrogen-enriched compared with estrogen-deficient milieu in postmenopausal women will amplify hypothalamosomatotrope responses to ghrelin, a naturally occurring GHRP. To mimic a physiological secretagogue signal, a submaximal rather than pharmacological dose of ghrelin was used (25). Analyses were then designed to quantify both the size and shape of induced GH secretory bursts under the hypothesis that E2 and ghrelin may regulate both end points (32–34).

Subjects and Methods

Subjects

Twenty-one healthy postmenopausal women were enrolled in and completed the two study sessions (below). Participants provided written
informed consent approved by the Mayo Institutional Review Board. The protocol was first reviewed by the U.S. Food and Drug Administration under an investigator-initiated new drug number. Exclusion criteria were recent transdermal travel or night-shift work (within 2 wk); significant weight change (>2 kg in 1 month); body mass index less than 20 kg/m² or greater than 30 kg/m²; acute or chronic systemic illness; use of neuroactive agents; psychiatric treatment; substance abuse; known or suspected cardiac, cerebral, or peripheral arterial or venous thromboembolic disease; poorly controlled hypertension or hypertriglyceridemia; any history or suspicion of endometrial or breast cancer; and untreated gallstones. Some enrollees continued to take a thiazide diuretic, an angiotensin-converting enzyme inhibitor, multivitamins, ferrous sulfate, calcium carbonate, aspirin, or ibuprofen on non-study days. Each subject had an unremarkable medical history and physical examination and normal screening laboratory tests of hepatic, renal, endocrine, metabolic, and hematologic function, as described (12, 13). The median (range) age was 64 (50–69) yr in the placebo (Pl) and 62 (52–68) yr in the E₂-treated volunteers. Corresponding body mass indices (kilograms per square meter) were 25 (20–29) and 26 (21–29), respectively. Postmenopausal status was confirmed by concentrations of FSH greater than 50 IU/liter, LH greater than 20 IU/liter, and estradiol less than 20 pg/ml (<74 pmol/liter). Volunteers stopped any post-menopausal hormone replacement at least 6 wk before study.

Statistical design

The study was a parallel-cohort design in which the order of the two infusions was prospectively randomized and double blind. The Food and Drug Administration restricted the infusion of ghrelin to once per subject but approved a parallel design (Pl vs. E₂).

Estrogen clamp

Placebo or E₂ was applied each night in matching transdermal patches. In the case of E₂ administration, the schedule achieves stepwise increases to good standards of clinical practice (13).

Sampling paradigm

Volunteers were admitted to the Mayo General Clinical Research Center on the evening before study to allow overnight adaptation. To obviate food-related confounds, subjects received a constant meal (turkey, brown rice, and green vegetables, or vegetarian alternative) of 500 kcal containing 55% carbohydrate, 15% protein, and 30% fat at 1800 h. Participants then remained fasting overnight until 1200 h the next day. On the day of combined sampling and infusion, catheters were inserted in contralateral forearm veins at 0700 h. Blood was withdrawn at 0800 h for later assay of serum estradiol, LH, FSH, IGF-I, IGF-binding protein (IGFBP)-1, and IGFBP-3 concentrations. Further samples (1.5 ml) were collected in chilled plastic tubes containing EDTA every 10 min for 4 h between 0800 and 1200 h. At 0900 h, a single bolus was injected of saline or ghrelin (0.3 μg/kg, delivered in 3.7% mannitol). Plasma was separated on ice and frozen at −70°C within 30 min. Lunch was provided before discharge.

Dosimetry

The ghrelin stimulus is approximately half maximal according to dose-response estimates in young adults (25, 35, 36).

Hormone assays

Plasma GH concentrations were measured in duplicate by automated ultrasensitive double-monoclonal immunoenzymatic chemiluminescence assay using 22-kDa recombinant human GH as assay standard (Sanofi Diagnostics Pasteur Access, Chaska, MN) (12). All samples (n = 50) from any given subject were analyzed together. Sensitivity is 0.010 μg/liter (defined as 3 σ above the zero-dose tube). Interassay coefficients of variation were 7.9 and 6.3% at GH concentrations of 3.4 and 12 μg/liter, respectively. Intraasssay coefficients of variation were 4.9 and 4.5% at 1.1 and 20 μg/liter GH. No values fell less than 0.020 μg/liter. Cross-reactivity with GHB or 20kDa GH is less than 5%. Serum LH and FSH concentrations were quantitated by automated chemiluminescence assay (ACS 180; Bayer, Norwood, MA), using as standards the first and second international reference preparations, respectively (13). E₂ was quantitated using the same platform, as described (37). Prolactin, total IGF-I, IGFBP-1, and IGFBP-3 concentrations were measured by immunoradiometric assay (Diagnostic Systems Laboratories, Webster, TX), as reported (12, 38).

Total ghrelin concentrations were assayed on a serum pool collected 10, 20, and 30 min after bolus ghrelin injection using RIA kits purchased from Linco Research, Inc. (St. Charles, MO) (catalog no. GHRA-H841K). Inter- and intraassay coefficients of variation were, respectively, 5.2 and 4.7%, and the detection threshold was 280 pg/ml.

Deconvolution analyses of pulsatile and basal (nonpulsatile) GH secretion

Earlier deconvolution methods in some cases yield nonunique estimates of hormone secretion and elimination rates (39). To address this technical impasse, pulsatile and basal GH secretions were estimated simultaneously using a new variable-waveform secretory-burst model statistically conditioned on biexponential kinetics and a priori estimated pulse times, as recently validated (40–43). The mass of GH secreted per burst was defined as the product of secretory-burst amplitude, a unit-area normalized waveform (burst shape) and a weak linear function of the preceding interpulse interval. The secretory-burst waveform (three-parameter generalized gamma density) was considered common to any given intervention and either cohort, as stated algebraically in the supplemental data published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org. GH pulse-onset times were created for each 4-h GH time series by a selective smoothing (anisotropic diffusion) algorithm, as described (42). All secretion parameters were then estimated simultaneously, conditioned on the selected pulse-time sets. The principal analytical outcomes are pulsatile GH secretion (micromegrams per liter per 3 h), defined as the summed mass of GH secreted in bursts after saline or secretagogue infusion, and the modal time latency (minutes) to achieve maximal secretion within any given burst (12, 13, 44).

Other statistical comparisons

The rank-sum test was used to contrast the intradividual incremental effect of ghrelin stimulation over that of saline in the two study cohorts. In view of the unbalanced statistical design, exact P values were estimated by random permutations on ranks. Fifty thousand permutations were performed for each comparison by random realignment of interventional ranks in the combined cohorts (n = 21 subjects). Purely chance assortment would predict a median normalized rank of 10.5. The null hypothesis of no E₂ effect over Pl further forecasts a difference (E₂ − Pl) in ranks of zero. The critical P value of the observed difference was one sided, given the a priori postulate that E₂ potentiates the ghrelin-induced response. The se of the mode of the GH secretory-burst waveform was calculated analytically, as described and verified earlier by bootstrap estimates (12, 13).

Hormone concentrations were compared in the E₂ and Pl cohorts via an unpaired Student’s t test. Data are presented as the mean ± SEM (45).

Results

E₂ administration caused a sense of abdominal bloating, breast tenderness, headache, or mild pedal edema in three volunteers. None discontinued participation. Saline and ghrelin infusions were occasionally (four subjects each) associated with facial warmth.

Figure 1A gives mean fasting hormone concentrations in the two cohorts. E₂ concentrations (picograms per milliliter) averaged 7.5 ± 0.51 (Pl) and 352 ± 9.1 (E₂) (P < 0.001, multiply by 3.67 for units of picomoles per liter). Total IGF-I concentrations of variation were 7.9 and 6.3% at GH concentrations of 3.4 and 12 μg/liter, respectively. Intraassay coefficients of variation were 4.9 and 4.5% at 1.1 and 20 μg/liter GH. No values fell less than 0.020 μg/liter. Cross-reactivity with GHB or 20kDa GH is less than 5%. Serum LH and FSH concentrations were quantitated by automated chemiluminescence assay (ACS 180; Bayer, Norwood, MA), using as standards the first and second international reference preparations, respectively (13). E₂ was quantitated using the same platform, as described (37). Prolactin, total IGF-I, IGFBP-1, and IGFBP-3 concentrations were measured by immunoradiometric assay (Diagnostic Systems Laboratories, Webster, TX), as reported (12, 38).

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TABLE 1. Impact of E₂ vs. Pl administration on GH and ghrelin concentrations

<table>
<thead>
<tr>
<th>Interventions</th>
<th>Placebo (n = 11)</th>
<th>E₂ (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH (μg/liter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline infusion</td>
<td>1.2 ± 0.33</td>
<td>2.0 ± 0.94</td>
</tr>
<tr>
<td>Ghrelin infusion</td>
<td>8.1 ± 1.9³</td>
<td>15 ± 1.5³</td>
</tr>
<tr>
<td>Peak GH to ghrelin ratio</td>
<td>0.80 ± 0.21</td>
<td>1.7 ± 0.57</td>
</tr>
<tr>
<td>Saline infusion</td>
<td>3.4 ± 0.26²</td>
<td>5.8 ± 0.51²</td>
</tr>
</tbody>
</table>

Data are the mean ± SEM for indicated n.
³ P < 0.05 vs. saline/placebo.
² P < 0.05 vs. placebo.

rose with ghrelin infusion, consistent with the properties of a nonlinear dose-responsive curve (30). E₂ vs. Pl significantly increased peak GH to ghrelin ratios during ghrelin infusion (P < 0.05), pointing to increased potency of ghrelin in the presence of E₂.

Deconvolution analysis was applied to assess GH secretion. Figure 2A illustrates GH concentration time series in three subjects from each cohort, whose responses are typical (the median and flanking two ranks of responses in the Pl and E₂ groups). Deconvolution-based estimates revealed that injection of ghrelin in the Pl setting stimulated pulsatile burst-like GH secretion 7.1-fold more than saline/Pl (P < 0.01); E₂ alone amplified GH secretion 2-fold more than saline/Pl (P < 0.10); and combined ghrelin/E₂ did so by 10.4-fold (P < 0.01) (Fig. 2B). Formal statistical comparison of responses to E₂ to ghrelin and Pl to ghrelin was made using rank permutations. The null hypothesis assumes chance assortment of the strength (rank) of incremental GH responses to ghrelin over saline among the 21 women studied independently of estrogen status. Thus, for individual Pl and E₂ interventions, the median random rank would be 10.5, and the (E₂ minus Pl) difference in random ranks would be zero. However, the actual difference in ranks was 4.6 (P < 0.05), which rejects the null hypothesis of no treatment effect (Fig. 3). Thus, E₂ increases (nearly doubles) the amount of GH secreted in the presence of ghrelin (P < 0.05).

GH secretory-burst waveform (shape), defined as the time evolution of GH release within bursts, was quantified by the modal time delay (minutes) to attain maximal GH secretion (Fig. 4). The mode denotes maximal secretion in the burst. The time (minutes) when the mode occurs reflects how rapidly GH is released. Statistical comparisons of the modes disclosed that, in the Pl group, infusion of ghrelin, compared with saline, reduced the time latency to achieve peak GH secretion by 2.3-fold (P < 0.01). Exposure to E₂ vs. Pl shortened the time delay to maximal GH secretion by 2.3-fold after saline (P < 0.01) but did not further abbreviate the delay after ghrelin stimulation (P > 0.80).

Discussion

The present investigations reveal that E₂ and ghrelin stimulate pulsatile GH secretion via both distinctive (independent) and shared (convergent) mechanisms in postmenopausal women. First, a distinct interaction was identified by the capability of E₂ supplementation to amplify the amount of ghrelin-induced GH secretion without altering ghrelin concentrations. In particular, E₂ supplementation increased...
the ratio of peak GH concentrations to total ghrelin concentrations (Table 1), consistent with an enhancement of ghrelin’s potency. Second, shared effects were inferred by the finding that E2 and ghrelin individually but nonadditively accelerate initial GH release within secretory bursts (Fig. 5). The collective outcomes indicate that E2 and ghrelin act via independent and convergent mechanisms to augment the size of GH secretory bursts and trigger more rapid exocytosis of GH stores, respectively.

A novel analytical tool was applied to estimate both the shape and the size of GH secretory bursts. The shape of bursts has been viewed traditionally as symmetric to simplify calculations (46). Direct estimates show that this is not the case (41, 47, 48). In fact, estrogen and ghrelin nonadditively change the shape of secretory bursts to favor rapid initial release of available GH-containing granules in pituitary cells. In addition, the two hormones additively increase the amount of GH released in each burst, quite independently of changing its shape. By this means, the accompanying analyses suggest that E2 and ghrelin share a common mechanism to facilitate prompt GH release but activate different mechanisms to augment the amount of GH secreted.

The paradigm of graded transdermal E2 administration reduced IGF-I and IGFBP-3 and elevated IGFBP-1 concentrations. This tripartite response pattern has been recognized after oral estrogen administration but not typically after conventional transdermal E2 replacement (1, 49). As discussed by Friend et al. (9), the difference may be explicable in part by the fact that higher E2 concentrations are usually attained after oral than transdermal regimens. The current protocol was designed to mimic late-follicular phase E2 concentrations (12, 13), which are sufficient to lower free IGF-I concentrations measured by ultrafiltration dialysis (50). Whether transdermally delivered E2 also lowers free IGF-I levels is not known. Lower IGF-I availability could withdraw negative feedback and thereby contribute to the greater stimulatory action of ghrelin observed here. Confirmation of this postulate would require quantifying ghrelin-stimulated GH secretion during experimental infusion of IGF-I in a low and high estrogenic milieu. One study using GHRH as the secretagogue reported that E2 administration paradoxically augments feedback inhibition by exogenous IGF-I (44). No comparable data exist to clarify how ghrelin, E2, and IGF-I act in concert to regulate pulsatile GH secretion.

Estrogen does not appear to influence blood total ghrelin concentrations. In one study, 2 d of orally administered con-
jugated estrogens in peripubertal girls did not alter total ghrelin concentrations (51). The present data in postmenopausal women given transdermal E2 are congruent with such findings. In contrast, in women with anorexia nervosa, combined administration of a synthetic estrogen and progestin elevated total ghrelin concentrations (52). In another analysis, 6 months of oral E2 supplementation in postmenopausal women increased active ghrelin concentrations by 14%, without affecting total ghrelin concentrations (53). Thus, no study using estrogen alone has demonstrated a significant increase in plasma total ghrelin concentrations. Whether acute transdermal E2 supplementation as studied here alters acylated ghrelin levels is not known but could be important by way of influencing ghrelin bioactivity. At present the mechanisms that control ghrelin's acylation status are not well known (1).

Infusion of a submaximally stimulatory dose of ghrelin (0.3 μg/kg) (25, 35, 36) induced peak plasma GH concentrations of 8.1 μg/liter in the Pl setting and 15 μg/liter in the E2 setting. These pulse sizes are attained normally during sleep or short-term fasting in young adults (1, 2). The purpose of mimicking physiological GH pulses was to obviate the difficulty in interpreting pharmacological responses, which reflect secretagogue efficacy only (30). Therefore, the finding that E2 potentiates the GH response to a submaximal ghrelin stimulus could denote that estrogens increase secretagogue potency or enhance pituitary sensitivity to ghrelin. Other studies indicate that E2 supplementation increases the stimulatory potency of and somatotrope sensitivity to GHRH and conversely reduces the inhibitory potency of and pituitary sensitivity to somatostatin (54, 55).

Ghrelin and E2 individually but nonadditively accelerated the initial release of GH within secretory bursts by 2-fold. Laboratory observations raise the possibilities that E2 may trigger rapid GH release by up-regulating pituitary GHRP receptors, down-regulating somatostatin subtype-5 receptors, or augmenting pituitary GH stores (1, 29, 56–59). In addition, atomic force microscopy indicates that GH release by porcine somatotropes requires transient fusion of exocytotic vesicles with membrane porosomes (60). If this cellular model also applies to the human, then ghrelin and E2 may individually recruit secretory vesicles or facilitate their fusion with the membrane, thus driving rapid initial GH release. The fact that the enhancing effects of ghrelin and E2 were not additive suggests that the
two effectors may regulate similar steps in the release process. In contradiction, $E_2$ administration accelerated GH release within individual secretory bursts driven by combined infusion of GHRH/GHRP-2 or GHRH/-arginine (13). Pulses of ghrelin also evoke GH secretory bursts of more rapid onset and offset than those stimulated by GHRH under in vitro conditions (34). These observations suggest that the mechanisms by ghrelin and $E_2$ stimulate GH, but not ghrelin and $E_2$ trigger initial GH release differ.

Certain caveats should be considered. First, the present investigation used a submaximally stimulatory dose of ghrelin to mimick physiological secretagogue action. Serum concentrations of ghrelin confirmed this expectation. When investigationaly practicable, detailed dose-response analyses should allow one to quantify the full nonlinear interaction between $E_2$ and ghrelin. In the only dose-response study of this nature, administration of $E_2$ potentiated GHRP-2-driven GH secretion at the highest dose of secretagogue tested (3 $\mu$g/kg), which was still not maximal (33). Second, the mean concentration of $E_2$ attained experimentally was intended to and did approach that in the late-follicular and preovulatory phase of the normal menstrual cycle when pulsatile GH secretion typically doubles (61–63). This model differs from clinical replacement doses of estrogen in postmenopausal women. The present data should have relevance to understanding the basis of large GH pulses in healthy pubertal girls and young women studied in the late follicular phase of the menstrual cycle (1, 11). Third, given the growth-related and anabolic significance of constitutive signaling by the human ghrelin receptor (64), an important future question is how constitutive receptor function, $E_2$ concentrations, and ghrelin availability jointly determine GH secretion.

In summary, experimental elevation of $E_2$ concentrations in postmenopausal women amplifies ghrelin’s near-physiological stimulation of pulsatile GH secretion, consistent with independent mechanisms. Conversely, elevated $E_2$ concentrations mimic the action of ghrelin by evoking rapid initial GH release, suggesting shared stimulus-response mechanisms.

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