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Interactive Regulation of Postmenopausal Growth Hormone Insulin-Like Growth Factor Axis by Estrogen and Growth Hormone–Releasing Peptide-2

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Estrogen is the proximate sex steroid sustaining GH secretion throughout the human life span in both sexes. However, very little is known about the specific neuroendocrine mechanisms by which estrogen activates and maintains GH secretion in the young or aging human. The identification of somatostatin in 1973 as a key negative peptidyl regulator of the GH axis and the discovery of GH-releasing hormone (GHRH) in 1982 as a dominant feedforward agonist of GH secretion provided an initial basic science foundation for exploring sex-steroid control of the GH-IGF-1 axis. Although GH-releasing peptides (GHRPs) were first recognized in 1977–1981, subsequent cloning of hypothalamopituitary receptors transducing potent secretagogue actions of GHRPs in 1996 and of an endogenous ligand for this effector pathway in 1999 now extend the framework for examining the mechanisms of estrogen-driven GH secretion in aging. Herein, we review several novel and multifaceted interactions in postmenopausal women between estrogen and GHRP-2. We combine these observations into a simplified construct of GH-axis neuroregulation comprising the somatostatin, GHRH, and GHRP effector pathways, as well as GH and IGF-1 autofeedback. We suggest the thesis that estrogen controls the interfaces among these pivotal regulatory peptides in hyposomatotropic postmenopausal individuals.

Key Words: Somatotropin; growth hormone–releasing peptide; growth hormone secretagogue; aging; women.

Introduction

In addition to age, body composition, aerobic fitness, and nutritional status, the sex-hormone estradiol is a dominant positive regulator of growth hormone (GH) secretion in the healthy human *(1–40)*. Indeed, estrogen likely mediates the gender contrast in GH secretion in both young and older adults *(1,2,7,11–13,31,41–72)*. We have shown that this sex difference includes a twofold protection against the age-related decline in daily GH production in premenopausal women compared with similarly aged men *(33)*. Combined deprivation of GH and estrogen in postmenopausal individuals heralds altered body composition, variable dyslipidemia, increased cardiovascular risk, cognitive changes, diminished bone mass, relative sarcopenia, and a potentially reduced quality of life *(2,29,73–81)*. Data in experimental animals and some clinical studies demonstrate trophic complementation of the effects of estrogen and GH (or insulin-like growth factor-1 [IGF-1]) at the target tissue level, as well as prominent hypothalamopituitary regulatory actions of estrogen on the GH-IGF-1 axis *(1)*. However, the precise neuroregulatory mechanisms that link estrogen depletion and hyposomatotropism are not known. One clinical model for exploring the pathophysiology of ovariprival hyposomatotropism is estrogen's drive of GH secretion in postmenopausal women. New clinical insights into the nature of estrogen's control of this major trophic axis may be relevant to the unfolding of more rational preventive and interventional strategies to avert and treat hyposomatotropism associated with aging and hypogonadism.

The present overview highlights selected interactions between potent growth hormone–releasing peptides (GHRPs) (oligopeptidyl GH-releasing peptides, or GH secretagogues [GHSs]) and estrogen in regulating GH secretion. Perhaps unexpectedly the combined stimulatory actions of estradiol and GHRP-2 in postmenopausal individuals closely emulate the unique pulsatile, 24-h rhythmic, and entropic dynamics of GH release in healthy pubertal girls (and boys) (16,82–85). These data and the available literature point to a novel effect of estradiol on the human GHRP-receptor/effector pathway. Accordingly, herein we articulate an emergent thesis of estrogen's plausible role in modulating the threefold peptidyl control of GH secretion by somatostatin, GH-releasing hormone (GHRH), and GHRP.

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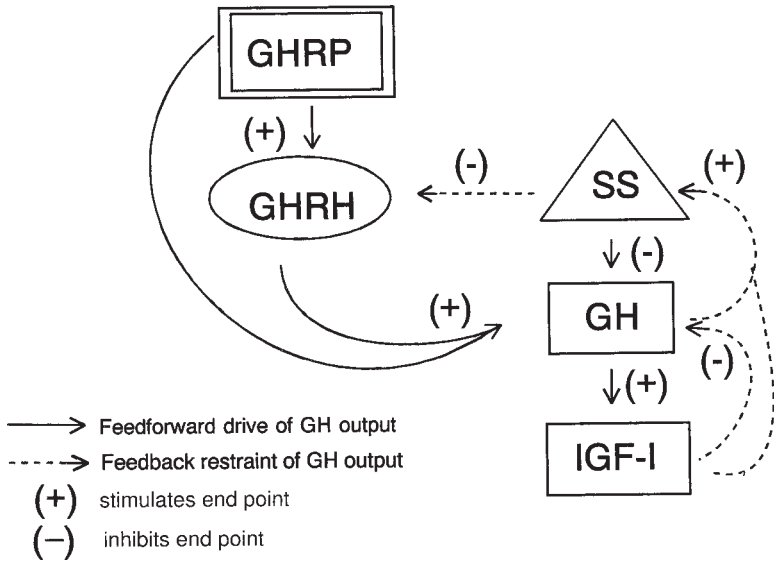

Fig. 1. Simplified schema of inferred primary peptidyl control of the core human GH-IGF-1 axis. SS, somatostatin.

Feedback Ensemble Perspective of GH-IGF-1 Axis

The GH-IGF-1 axis can be viewed as a core feedback ensemble that includes the following:

1. Pulsatile drive of somatotrophs by endogenous GHRH, as established directly in vivo by GHRH immunoneutralization and antagonist studies, molecular mutations of the GHRH receptor, and hypothalamopituitary portal-venous blood sampling.
2. Variably episodic somatostatin secretion, whose precise pulsatile features vary among different mammalian species.
3. A recently cloned GH-releasing oligopeptide (GHRP) receptor and endogenous ligand capable of potently and specifically stimulating GH secretion.
4. IGF-1 negative feedback of the hypothalamopituitary unit.
5. GH autonegative feedback, presumptively mediated via GH's induction of hypothalamic somatostatin secretion (and, inferentially, by concomitant suppression of GHRH release) (1,86–112). Figure 1 illustrates this simplified schema.

Current knowledge of the structure-activity relationships, receptorology, and endogenous ligand properties of GHRPs began with in vitro analyses of potent pentapeptide (metenkephalin-derived) secretagogues approximately two decades ago (113–118). In 1977–1981, GHRPs were viewed as novel entities of uncertain biologic relevance *(108,113–115,117–119)*. The array of known GHRPs now includes multiple oligo- (e.g., tri-, penta-, hexa-, and hepta-) peptides *(120)*, as well as several distinct classes of novel nonpeptidyl agonists of this secretagogue pathway *(111, 117,118,120–125)*. In 1996, Howard et al. *(126)* expression-cloned a cDNA encoding a (type Ia) GHRP-receptor transcript in the pig and human. The receptor is a novel G-protein-coupled hepathelical transmembrane protein, which is functionally expressed in the hypothalamus and pituitary gland (and nearly ubiquitously elsewhere) in species as diverse as the human, rat, rabbit, cow, pig, chicken, and Pufferfish *(111,117,120,126–137)*.

The GHRP receptor has only modest (~30–33%) sequence identity with the neurotensin and thyrotropin-releasing hormone receptors, and moderate (~50%) homology with the motilin and galanin receptors *(138)*. This transducing protein has a distinct structure, and signal activating and regulatory function. Unlike somatostatin and GHRH receptors, the GHRP receptor transduces G_{q11}-dependent inositol phosphate release, biphasic $[Ca^{2+}]_i$ signaling, and protein kinase C activation *(139–144)*. The cloned somatostatin receptor family mediates K^+ channel-driven membrane hyperpolarization, repression of $[Ca^{2+}]_i$ spiking, and G_i-dependent inhibition of cyclic adenosine monophosphate (cAMP) production in somatotrophs *(145,146)*, thereby antagonizing both GHRP and GHRH-stimulated GH secretion. However, somatostatin does not inhibit pituitary GH synthesis *per se (147–150)*. Further distinguishable, the GHRH receptor stimulates cAMP formation, GH gene transcription, *de novo* synthesis of GH protein, and rapid exocytotic release of GH *(151–156)*. Finally, the topography of GHRP, somatostatin, and GHRH receptor expression within the hypothalamopituitary unit is overlapping but nonidentical *(1,86)*. In short, the foregoing three dominant (albeit nonexclusive) neuropeptidyl regulators of the GH-IGF-1 axis operate via distinct but complementary receptor- and signaling-specific pathways, thus allowing significant biologic interactions and potentially multilocus control by sex steroid hormones.

GHRP receptors are expressed in hypothalamic GHRH and neuropeptide Y (NPY) neurons in the arcuate nucleus, and in approx 50% of rodent somatotrophs *(111,127,157)*. GHRPs exert prominent central nervous system (CNS) effects including the stimulation of electrical firing of

GHRH neurons, c-fos gene activation in GHRH and NPY neurons, acute secretion of GHRH, and sleep and appetite changes *(1,123,158–165)*. GHRP-like secretagogues also drive in vivo pituitary GH gene transcription in the infant rat and elicit in vitro GH secretion from adult and neonatal pituitary cells *(115,135,166)*.

GHRPs show strong physiologic interactions with established effectors of the GH axis *(1,86,162,167,168)*. First, these agonists interact critically with GHRH *(169–176)*. For example, GHRP receptors are expressed on GHRH neurons *(127,177)*, GHRP secretagogue activity requires functional GHRH receptors in mice (lit/lit mutation) and humans (dwarfs of Sindh) *(90,178)*, a higher dose of a GHRH antagonist opposes GHRP's actions in young adults *(179,180)*, and available GHRPs stimulate GHRH secretion from the rat hypothalamus in vitro *(181)* and into sheep hypothalamopituitary portal blood in vivo *(179,180,182)*. One or more of these combinatorial interactions would likely account for the in vivo synergy between GHRP and GHRH, which together evoke massive outpouring of GH in the human, pig, and rodent *(162,183–194)*. How sex steroids, in particular, modulate these in vivo interactions remains sparingly known.

GHRPs also interact significantly with somatostatin, as so-called functional somatostatin antagonists. This term reflects the ability of GHRPs to reduce the ID_{50} of somatostatin's inhibition of GHRH actions by ~2- to 3-fold in vitro and by as much as 8- to 10-fold in vivo in the adult male rat *(183,193)*. In vitro, GHRPs and somatostatin exert antagonistic effects on $[Ca^{2+}]_i$ spiking and on selected protein phosphorylation/dephosphorylation reactions, possibly including secretagogue receptors and K^+ channels *(111, 162)*. Analogously, somatostatin also partially opposes several CNS actions of GHRPs *(111,141,144,160,195)*. However, somatostatin does not block GHRP binding *per se*, and, conversely, GHRPs do not inhibit somatostatin binding or suppress somatostatin secretion into hypothalamopituitary portal blood *(182,183,193,196)*.

Virtually all clinical studies and in vivo animal experiments, as well as one in vitro hypothalamopituitary coincubation analysis, indicate that maximal actions of GHRPs, and the in vivo synergy between a GHRP and GHRH, require a functionally connected hypothalamopituitary unit *(1,197,198)*. Additivity of the effects of GHRP and GHRH, but not true synergy, is typically observed in dispersed pituitary cultures in vitro *(199–202)*. The magnitude of the supraadditive interaction between GHRP/GHRH is enhanced further in most (but not all) experiments in the rat and human by a reduction in somatostatinergic activity, e.g., via passive somatostatin immunoneutralization or L-arginine infusion *(111,134,172,175,183,184,190,193, 203,204)*. For example, in the adult rodent, threefold administration of GHRP-6, GHRH, and somatostatin antisera increases serum GH concentrations synergistically to 2500 ng/mL *(183)*. Likewise, in older humans, triple injection of hexarelin (a GHRP), GHRH, and L-arginine (to relieve somatostatin inhibition) evokes supraadditive secretion of GH, which in most studies exceeds that induced by any pair (or any one) of these three secretagogues *(172–175,205)*.

These species-independent observations indicate that GHRP/GHRH synergy is expressed in part via a somatostatin-independent mechanism and, conversely, subject to partial somatostatin restraint. The precise mechanisms that mediate the supraadditive interaction between GHRP and GHRH remain unknown *(1,162,186,190,204,206,207)*. However, based on somatostatin-independent synergy, Bowers *(193)* postulated possible mediation by an unidentified hypothalamic ("U" or unknown) factor. Independently of the neural mechanisms involved, the joint GHRP/GHRH synergy, and the single actions of GHRP and GHRH, decline markedly (by approx twofold) in the majority of studies in older humans *(1,30,172,193,205)*. The basis for this decline in aging is not established. Accordingly, we believe that all three—GHRH, somatostatin, and GRHP—must be considered in attempting to understand the regulated secretion of GH in aging and gonadoprival states.

Although an endogenous ligand for the GHRP effector system was long anticipated to exist, Kojima et al. *(88)* only recently reported cloning of a natural ligand for the human and rat GHRP receptorsL ghrelin. This oligopeptidyl agonist specifically stimulates GH secretion by pituitary somatotrophs in vitro and in the adult rat in vivo, and activates $[Ca^{2+}]_i$ signaling by GHRP (MK0677)-receptor transfected CHPO cells. Ghrelin is a unique 28-residue ^{3}Ser-octanoylated peptide cloned from the stomach but expressed by immunocytochemistry in the hypothalamic arcuate nucleus and by reverse transcriptase polymerase chain reaction in the brain. Its ED_{50} is ~2.1 n*M* for releasing GH in vitro, which is comparable to that of GHRH (~0.6 n*M*). Radioimmunoassay further revealed that Ghrelin circulates in low (0.2 n*M*) concentrations in human blood *(88)*.

In view of the foregoing expanding basic science knowledge base, we envision the three primary peptidyl effectors of GH output (GHRP, GHRH, and somatostatin) as a functionally interlinked ensemble (Fig. 1). Gerontologic studies also make an ensemble perspective essential, since only L-arginine combined with GHRP, GHRH, or both GHRP and GHRH can normalize acutely stimulated GH secretion in elderly humans, when judged against similarly infused young adults *(172,173,203,207–213)*. Acutely, neither GHRPs nor GHRH can individually restore GH output consistently in older volunteers *(1,214)*. However, continuous sc GHRP-2 administration for up to 30 d in elderly men and women tends to reconstitute the GH-IGF-1 axis substantially *(30,125,170,215–220)*. Likewise, 72 h of recurrent iv GHRH pulses and 14–90 d of daily, twice daily or continuous sc GHRH injections can elevate serum GH, IGF-1, and IGF binding protein-3 concentrations signifi-

cantly in older humans but fail to normalize the GH-IGF-1 axis compared with similarly stimulated young adults *(27,211,212,221,222)*. Isolated excess somatostatin also does not readily explicate the so-called somatopause, because no somatostatin antagonist administered alone in either the human or experimental animal relieves hyposomatotropism in the elderly *(1,29,30,203,208,223–225)*. Thus, we hypothesize that the age-associated decline in GH secretion in humans embodies threefold neuroregulatory failure; that is, presumptive excess somatostatin combined with putative GHRH and/or GHRP deficiency, probably exacerbated by relative or absolute sex hormone deficiency, increased visceral adiposity and reduced aerobic capacity in aging individuals *(1)*.

This trilogy of neuroregulatory peptides also participates in modulating the negative feedback control of this axis by GH or IGF-1 *(1,91,97,226–230)*. In most studies, acute infusion of GH or IGF-1 in humans and experimental animals imposes time-limited (e.g., 45 min to 3 h) suppression of spontaneous (endogenously driven) and exogenously stimulated GH secretion *(226,231–238)*. We can reproduce time-delayed (~2 h) autofeedback inhibition of spontaneous and GHRP- and GHRH-stimulated GH secretion via a single 6-min iv pulse of rhGH in young and older humans *(239)*. Basic science studies have suggested that such GH-IGF-1 autoregulatory biology is pivotal in generating normal GH pulsatility *(104,162,178,240–243)*. Intrahypothalamic reciprocal connectivity between somatostatin and GHRH neurons likely further maintains physiologic GH pulsatility *(111,178,240,244)*. Whereas infused IGF-1 appears to suppress spontaneous GH secretion normally in older humans *(235)*, whether GH autofeedback or endogenous IGF-1 inhibition of the GH-IGF-1 axis is altered in aging or gonadoprival individuals is not known.

GH autofeedback control is prominently sexually dimorphic in the rat *(105,178,245–252)*. Analogous gender-specific feedback data are not available in the human at present. However, spontaneous pulsatile GH secretion patterns show readily quantifiable gender contrasts in women and men, as well as postpubertal girls and boys *(14–16,82,253–256)*. In addition, (peripheral) IGF-1 negative feedback efficacy differs in young women and men *(257)*. Accordingly, it will be important to access how sex hormones regulate GHRP and GHRH's supravention of GH and IGF-1 negative feedback in the aging human.

Clinical analyses using intensive blood-sampling schedules and IRMA or chemiluminescence-based assays of GH have documented marked amplitude-specific damping of pulsatile GH secretion in elderly individuals *(22,27,29,32, 254,258)*. From a mechanistic perspective, preferential suppression of GH pulse amplitude is a predicted outcome of excess somatostatin and GHRP or GHRH deficiency *(1)*. Regarding possible excess somatostatin, we have shown that iv somatostatin and sc octreotide infusions in men and women selectively attenuate GH pulse amplitude *(93,259, 260)*. In the rat, hypothalamic somatostatin secretion also secondarily inhibits GHRH release via direct somatostatinergic synapses on GHRH neurons *(241,261,262)*. Based on these neuroregulatory concepts, the recognized damping of GH pulse amplitude in aging humans could arise from a relative excess somatostatin with or without secondary GHRH deficiency or GHRP depletion. For example, GHRH responsiveness declines with age in virtually all studies *(1,86)*. In corollary, diminished hypothalamic GHRP receptor density in older humans, and substantial reconstitution of the GH-IGF-1 axis in elderly men and women administered sc GHRP-2 for 30–90 d or an orally active (nonpeptidyl) GHRP secretagogue for 2–4 wk, make a partial GHRP deficiency in aging clinically plausible *(216,217)*. Thus, further clinical experiments will be important to affirm or refute a notion of excess somatostatin and GHRH and GHRP deficiency in aging and gonadal sex steroid–deficient individuals.

Impact of Estrogen Supplementation on GHRP-2 Driven GH Secretion in Healthy Postmenopausal Women

In June 1998, Leong et al. *(263)* reported that sheep portal blood contains pulses of an unknown substance capable of stimulating $[Ca^{2+}]_i$ in HEK 293 cells stably transfected with the full-length human GHRP (MK0677) receptor. In December 1999, Kojima et al. *(88)* extended this pilot observation by using a Xenopus oocyte expression-cloning strategy to identify the human and rat cDNAs encoding an endogenous ligand for the GHRP receptor, Ghrelin. Neither the native peptide nor a derivative antagonist is yet available for clinical studies, and extensive safety data will be required before such molecules can be employed investigationally. Moreover, the physiologic role of Ghrelin is unknown at present. On the other hand, selective and specific GHRPs are unique probes of the human GHRP-receptor effector pathway. To this end, we have utilized GHRP-2 as the most potent currently available GHRP-receptor agonistic peptide for iv injection in humans *(84,85,92,109,190,191,193,264–271)*.

Our preliminary bolus GHRP-2 dose-responsive and continuous GHRP-2 infusion studies in postmenopausal women document significant and reciprocal estrogen-GHRP-2 interactions; for example, estradiol-17 β supplementation at a dose of 1 mg orally twice daily for 1 wk increases both the sensitivity and maximal responsiveness of the hypothalamopituitary axis to GHRP-2 acutely; estrogen replacement modulates GHRP-2-driven basal, entropic, and nyctohemeral GH secretion; and iv GHRP-2 infusion over 24 h opposes oral estrogen's suppression of plasma IGF-1 concentrations *(85,239,268–271)* Table 1. Other investigations of GHRP's effects across the human lifetime show maximal secretory actions in mid-to-late puberty when sex steroid hormone concentrations rise

Table 1
Precis of Significant Estrogen-GHRP-2 Interactions Observed in Postmenopausal Women

1. Estradiol enhances GHRP-2's potency.
2. Estradiol enhances GHRP-2's efficacy.
3. Estradiol doubles GHRP-2's stimulation of the basal (interpulse) GH secretion rate.
4. Estradiol amplifies the GHRP-2-stimulated mesor (mean) and amplitude of the 24-h serum GH rhythm.
5. Estradiol restores the marked GH phase advance driven by iv GHRP-2 infusion.
6. Estradiol normalizes the 24-h rhythm of GHRP-2-damped GH interburst intervals.
7. Estradiol and GHRP-2 jointly increase the approximate entropy of GH secretory profiles.
8. GHRP-2 infusion overcomes oral estrogen's suppression of plasma IGF-1 concentrations.
9. GHRP-2 infusion synchronizes within-subject daily GH pulse profiles.

(272,273). Interventional experiments in prepubertal children further indicate that only 3 d of oral ethinyl estradiol administration in girls or a single im injection of testosterone enanthate in boys will double responsivity to hexarelin, a GHRP *(274,275)*. Estrogen- and gender-GHRP contrasts are also evident in the adult rat *(199)*, during postnatal development *(276)*, and in the GH transgenic mouse *(277)*. Interestingly, the human GHRP-receptor gene promoter contains a putative half-estrogen-responsive *cis*-DNA element *(278)*. These data collectively prompt the postulate that estrogen might be able to "rescue" the apparent attenuation of responsiveness to GHRP agonism in the older female.

Not all clinical studies describe a sex dependency of GHRP's effects; in one report the amount of GH released after a single iv bolus of hexarelin did not vary across the menstrual cycle *(279)*, in women and men *(279)*, or following a 3-mo exposure to low-dose (0.05 mg daily) transdermal estradiol *(203)*. However, to date, all clinical studies of sex steroid control of the actions of GHRPs have been conducted in the presence of unknown (and hence potentially estrogen-modulated) endogenous somatostatinergic and GHRH activity. Indeed, estrogen's reported heightening of GHRP-2's actions in postmenopausal women and of hexarelin's effects in adolescent girls could reflect nonexclusively reduced somatostatinergic activity and/or facilitation of endogenous GHRH or GHRP-like activities. The first of these hypotheses is plausible, since somatostatin withdrawal in the rat and human significantly augments the stimulatory effect of GHRP, and, conversely, somatostatin can partially antagonize GHRP's action *(160,171,172,183,186,190,193,195, 280,281)*. The second postulate is significant, inasmuch as infusion of GHRP evokes hypothalamic GHRH secretion *(182,196)* and acts additively with GHRH in vitro and supraadditively (synergistically) in vivo (above). The third postulate is pertinent also, because hypothalamic GHRP receptor expression is sexually dimorphic in the rodent and estrogen-inducible (above). Further studies are thus required to distinguish among these postulated pathophysiologic mechanisms of estrogen action.

Oral estradiol replacement in postmenopausal women stimulates pulsatile, 24-h rhythmic, and entropic GH secretion by 1.8- to 6.2-fold consistently and also amplifies GHRP-2's acute dose-dependent actions *(83–85,239,268–271,282)*. By contrast, estrogen supplementation does not directly alter pituitary responsiveness to either continuous 24-h GHRH or graded 3-h somatostatin infusions *(186,215,239,260,271)*. Based on these considerations, we are using a combined strategy of simultaneous GHRH infusion and somatostatin rebound to test the ability of estrogen replacement to enhance GHRP-stimulated GH secretion without confounding by variable endogenous GHRH and somatostatin. This experimental reasoning assumes (1) the now well-established premise that estrogen itself exerts little if any known direct effects on pituitary GH gene expression, and (2) the absence of major actions of estrogen on GHRH-stimulated or somatostatin-inhibited pituitary GH secretion, as we have verified recently by, respectively, 24-h GHRH and 3-h somatostatin infusions *(186,215,260)*.

Possible Role of Estradiol Replacement in Postmenopausal Women to Enhance GH Secretion by Restricting the Hypothalamic Release of Somatostatin

Peripheral blood concentrations of somatostatin do not reflect secretion of this regulatory peptide into hypothalamopituitary portal blood *(283)*. However, somatostatin is a critical negative modulator of somatotroph exocytotic release of GH and of hypothalamic arcuate nucleus GHRH secretion *(178,249,284–289)*. Conversely, the recent finding in the rat that short-term administration of a synthetic linear hexapeptide somatostatin antagonist actually inhibits GH secretion and impairs linear growth *(99)* crystallizes an emergent and complementary concept that recurrent somatostatin input plays a critical role in maintaining physiologic pituitary responsiveness to GHSs *(100,149,150,240, 290–293)*. A recent postulate explicating this initial paradox is that recurrent somatostatin exposure reverses agonist-induced and phosphorylation-dependent downregulation of GHRH and the GHRP signaling *(111,294)* by activating

cellular phosphatases that restore somatotroph cell responsiveness *(295,296)*. Thus, further study of estrogen's possible control of endogenous somatostatinergic activity should be relevant to better understand the neuroendocrine mechanisms of estrogen-stimulated GH secretion.

Based on a simplified three-factor model of GH neuroregulation (i.e., comprising principally somatostatin, GHRH, and GHRP) (Fig. 1), we are evaluating estrogen's putative regulation of endogenous somatostatinergic activity further by clamping the stimulatory inputs of both GHRH and GRHP. In the adult rat, GH secretion in response to a combined (near-maximal) GHRH/GHRP stimulus is a highly sensitive experimental index of in vivo somatostatinergic activity (*see* Feedback Ensemble Perspective of GH-IGF-1 Axis). For example, attenuating somatostatinergic input to somatotrophs via passive somatostatin immunoneutralization typically augments the in vivo synergy of GHRH/GHRP-6 by two- to threefold. Hence, we are testing whether estrogen can attenuate endogenous somatostatinergic activity by examining its ability to further amplify the evident synergy between GHRP-2 and GHRH.

Appraising the role of somatostatinergic restraint in estrogen's stimulation of GH secretion in the human is particularly pertinent in view of some evident differences in somatostatin physiology in the rodent and human *(243,297,298)*. For example, in the rat, testosterone and nonaromatizable androgens, but not estrogen, upregulate hypothalamic somatostatin gene and peptide expression *(98)*. However, in the human, the nonaromatizable androgen 5α-dihydrotestosterone (DHT) heptanoate is ineffectual whereas estrogen is strongly stimulatory of sexually dimorphic GH pulsatility *(16,299)*. Further clinical studies are therefore needed to clarify whether and how estrogen governs somatostatinergic activity in older humans, in whom accentuated somatostatin inhibition has been inferred *(249,284–286)*.

We recently showed that estrogen repletion in postmenopausal women does not alter the inhibitory efficacy of infused somatostatin but does consistently stimulate spontaneous pulsatile, entropic, and nyctohemeral GH secretion *(83)*. Somatostatin infusion data thus exclude a major role of estrogen to relieve the direct pituitary inhibitory actions of somatostatin. Therefore, estrogen's stimulation of GH secretion more likely involves other important actions of estrogen, e.g., restraining hypothalamic somatostatin secretion and modulating its central interactions with GHRH or GHRP *(160,186,195,271,300)*.

Other clinical insights further motivate a better understanding of estrogen's putative control of somatostatinergic activity, since estrogen replacement significantly augments the 24-h rhythmicity of GH secretion *(83)*. Nyctohemeral GH rhythmicity is thought to be under endogenous somatostatin (rather than solely GHRH) control, because it clearly persists in the face of unvarying GHRH stimulation *(27,215,220,284,301)*. Diurnal GH rhythmicity also endures during continuous 24-h iv and 30-d sc GHRP-2 infusions in elderly men and women *(84,215,217,268)*. These clinical observations thus allow for 24-h rhythms in either somatostatin or an endogenous cosecretagogue of GH. Thus, we reason that simultaneous GHRH and GHRP-2 drive is required to more persuasively isolate a hypothesized impact of estrogen on endogenous somatostatin rhythms.

The notion that estrogen antagonizes somatostatin release in the human, albeit unproven, is not new. This hypothesis was first suggested 30 yr ago, inasmuch as the administration of diethylstilbestrol in young men and the estrogen-enriched preovulatory milieu in young women amplified the stimulatory effects of L-arginine, exercise, and insulin-induced hypoglycemia on GH secretion *(2,5,6,302–304)*. These secretagogue effects are notable, because each is believed to require significant suppression of hypothalamic somatostatin release *(1,86)*. Another study indicated that estrogen addback in leuprolide-suppressed young women limits apparent refractoriness to an acute GHRH stimulus, which also is believed to mirror somatostatinergic activity *(36)*. Our analyses further delineate that, whereas aging preferentially represses, estrogen selectively augments GH secretory burst mass. These findings are consistent with somatostatinergic predominance in aging and its possible normalization by estrogen *(13,83, 305)*. Conversely, iv somatostatin and sc octreotide infusions suppress GH pulse mass (and, at very high concentrations, also GH pulse frequency) in men and postmenopausal women *(93,259,260)*. Albeit indirect, these clinical data in aggregate are concordant with a postulate of estrogen-reversible somatostatinergic hyperactivity in postmenopausal women.

Based on theoretical analyses, neuroregulatory input by somatostatin should strongly control the pattern regularity (or approximate entropy) of GH secretory output *(14,253, 306,307)*. Indeed, estrogen treatment in girls and women promptly and markedly reduces the orderliness of GH release *(14,16,83,268)*. These insights would also allow for somatostatinergic regulation by estrogenic steroids.

Possible Impact of Estrogen on GH Autonegative Feedback

A plausible role for estrogen in restraining GH autoinhibition is supported indirectly by the striking sexual dimorphism of GH autofeedback in the rodent *(105,228, 308)*. In particular, the adult female (ovary-intact) animal shows little GH feedback restraint. The predicted consequence would be more persistent (less episodic) secretion of somatostatin along with lower amplitude and less regular patterns of GH secretion in the female, as observed *(249,309,310)*. We recently quantified this gender distinction vividly in the rat via the approximate entropy statistic *(14,253,297)*. We could also document statistically more

irregular GH secretory patterns in pubertal girls than boys, as well as in women compared with men, as assessed by IRMA, IFA, and chemiluminescent GH assay *(14,16,82)*. This sex difference was corroborated by others in estrogen-unreplaced postmenopausal women *(311)*.

We have confirmed the specific role of estrogen in mediating these (putatively GH feedback-dependent) sex differences by showing that short-term administration of (ethinyl) estradiol evokes disorderly GH secretion patterns in prepubertal girls and postmenopausal women *(14,16,83)*. Intramuscular injection of an aromatizable androgen did likewise in prepubertal boys and older men *(16,312,313)*. By contrast, 5α-DHT-heptanoate did not alter GH release patterns *(16)*.

The significance of understanding estrogen's regulation of GH autofeedback is additionally noteworthy in older adults in view of the following recent findings:

1. The number of hypothalamic (but not pituitary) binding sites for GHRP declines in elderly individuals *(137)*.
2. The human GHRP-receptor gene promoter exhibits a presumptive half-estrogen-responsive DNA sequence *(276)*.
3. Puberty, estrogen, and aromatizable androgens can induce GHRP responsiveness and GHRP receptors in the rodent and human *(124,275,278,294,314)*.
4. Brain GH receptor density also decreases in older men and women *(315)*, thereby forecasting that GH autofeedback may wane in aging.
5. Estrogen inhibits brain GH receptor expression in the rat *(316)*, which could imply further that estrogen treatment could attenuate GH autonegative feedback, and thereby heighten secretagogue (both GHRP and GHRH) efficacy.
6. The ovariprival state at any age in adults is associated with upregulated hypothalamic estrogen-receptor gene expression *(317)*.

Possible Estrogenic Stimulation of GH Secretion in Postmenopausal Women Independently of IGF-1 Feedback Withdrawal

Earlier studies in the rat using partially purified somatomedin C suggested that IGF-1-like peptides suppress GH secretion via dual (hypothalamic and pituitary) sites of action *(91,97,318)*. In this regard, receptors for IGF-1 are expressed in both the brain and pituitary gland *(319)*. IGF-1 can directly inhibit GH biosynthesis and secretion by pituitary cells in vitro *(103,320)*. However, more recent studies in the rat and sheep using in vivo intracerebroventricular infusions and in vitro incubations of hypothalamic explants with rhIGF-1 disclosed less striking inhibition of GH secretion (or less induction of hypothalamic somatostatin secretion) by this peptide, unless it was coadministered with rhIGF-2 *(227,321,322)*. Recombinant IGF-2 itself is inactive *(323)*. In the human, partial IGF-1 deficiency owing to fasting and profound IGF-1 deficiency owing to IGF-1 gene deletion stimulate GH hypersecretion, which can be suppressed by systemic injections of IGF-1 *(324,325)*. In healthy young adults, preinfusion of 30 g of L-arginine (to putatively limit somatostatin release) opposes the negative feedback effect of peripherally infused GH (above) and IGF-1 *(226)*, thus also allowing possible IGF-1 autoinhibition via somatostatinergic mechanisms.

Whether the foregoing inferences apply physiologically to circulating IGF-1 levels (in contrast with CNS IGF-1 levels) is not clear, because systemic infusions of IGF-1 (10 μg/[kg·h]) generate pharmacologic free IGF-1 concentrations, which correlate with the degree of GH suppression induced *(235)*. During systemic infusion, inhibitory amounts of (free) IGF-1 might enter the CNS by way of transcapillary endocytosis *(1)*. On the other hand, at low or normal blood IGF-1 levels, brain GH receptors might be more relevant in mediating negative feedback. Indeed, an inducible liver-specific CRE/lox knockout of IGF-1 gene activity postnatally in the mouse reduced plasma IGF-1 concentrations by 60–70%, while only minimally elevating blood GH concentrations *(326)*. This initially unexpected result could be explained by preservation of brain GH receptor–dependent feedback in this model. In a thematically analogous clinical study, administration of an rhGH-receptor antagonist (Pegvisomant, Trovert, B2036-PEG) depleted plasma IGF-1 concentrations in young men by 45–55% within 5 d but only slightly increased serum GH concentrations *(327)*. These paradoxes are explicable, if the polyethylene glycol–modified (pegylated) peptide does not penetrate the blood-brain barrier, thereby leaving brain GH receptors accessible to autofeedback. Thus, we believe that the role of physiologically circulating IGF-1 in controlling the GH axis via autonegative feedback is uncertain.

The foregoing query is critical to the understanding of estrogen's mechanism of stimulating GH secretion in postmenopausal individuals. In virtually all clinical studies, oral estrogen administration reduces plasma IGF-1 concentrations by 15–35% *(83,282,328,329)*, likely reflecting decreased hepatic IGF-1 and/or GH-receptor gene expression *(330–332)*. Prevailing (but unproven) clinical dogma is that oral estrogen replacement drives GH secretion solely or predominantly in postmenopausal women by withdrawing peripheral IGF-1 negative feedback *(5,29,257,328,333)*. We are testing this concept by utilizing a peripherally acting rhGH-receptor antagonist to deplete circulating IGF-1 concentrations in the absence vs presence of estrogen replacement.

Recent Clinical Studies

Dynamic Control of the Postmenopausal GH Axis by Estrogen

To quantitate the low serum GH concentrations observed in aging, we first validated an ultrahigh sensitivity robotics-controlled and chemiluminescence-based GH assay (sensitivity of 0.005 μg/L of 22-kDa rhGH) *(22,258)*. Concurrently, we developed a statistically based fully auto-

mated data reduction method *(334,335)*. These technologies unveiled marked physiologic excursions in GH release over 24 h in healthy young adults *(22,258,336,337)*, and allowed uniform detection of serum GH concentrations in obese, hypothyroid, and older individuals *(83,84,258)*.

Postmenopausal women maintained interpulse serum GH concentrations as low as 0.020–0.030 μg/L in the fed awake state, and as high as 2–9 μg/L when fasting and asleep *(83,84,268)*. Oral glucose ingestion suppressed GH nadirs consistently (>95%) to <0.7 μg/L in middle-aged women and to <0.07 μg/L in men, therein highlighting another gender difference in this axis *(338)*. Intravenous somatostatin infusion (500 μg/h) and sc octreotide injection (1 μg/kg) lowered basal serum GH concentrations even more profoundly than glucose *(93,259,260)*.

Further clinical investigations evaluated the alleged impact of age, regional fat distribution, relative sarcopenia, and the sex hormone milieu on the kinetics of infused rhGH *(28,339–341)*. These analyses validated the application of deconvolution analysis without evident confounding by age, estrogenic milieu, or topography of fat accumulation.

Spontaneous and Estrogen-Driven GH Secretion in Postmenopausal Women

Oral administration of estrogen (1 mg of micronized estradiol-17β twice daily) selectively augments GH secretory burst mass and increases the mesor and amplitude of 24-h rhythmic GH release (Fig. 2). Estradiol does not alter GH pulse frequency, secretory burst duration, circadian acrophase, or the half-life of endogenous GH *(83)*. The finding of a stable GH half-life confirms our earlier kinetic inferences *(339)*, and those of Taylor et al. *(342)*. These specific GH responses to estrogen closely mimic those observed in pubertal girls, whom we studied using the same chemiluminescence assay *(82)*.

Estrogen replacement also increased the irregularity (or approximate entropy) of 24-h GH secretory profiles in postmenopausal women, akin to independent findings in middle-aged women and pubertal girls *(14,16,82,83)*. A gender contrast also persists in acute stress *(255,343)*.

The foregoing data highlight the similarity of three major quantifiable neuroendocrine features of GH secretion qualitatively in estrogen-replaced postmenopausal women and normal pubertal girls: (1) amplified GH secretory-burst mass, (2) elevated mesor and amplitude of 24-h rhythmic GH release, and (3) heightened disorderliness of subordinate (nonpulsatile) GH secretory patterns. These similarities in neuroendocrine reactivity suggest, but do not prove, that estrogen repletion postmenopausally and in normal female puberty may drive comparable feedback relationships within the GH axis.

GH Secretory Responsiveness in Older Men and Women Supplemented with Sex Hormones

Bioavailable testosterone concentrations decline with age even in premenopausal women *(344)*, and also in comparably aged men *(345)*. This decline may be important independently of gender because testosterone stimulates pulsatile GH secretion in the human (but not in the rat) conditional on its aromatization to estrogen (reviewed in refs. *1,16,86,* and *346*). In older men, 3 wk of im testosterone repletion also significantly elevated GH secretory burst mass, the mesor of the 24-h GH rhythm, and the entropy (greater irregularity) of GH release patterns *(313)*. These findings underscore the fundamental insight that GH secretory pulse amplitude (or mass), the nyctohemeral GH mesor, and the orderliness of GH release patterns are pivotal physiologic targets of both estrogen and aromatizable-androgen in aging humans of both genders *(1,16)*. Theoretical analysis of GH-axis dynamics strongly suggests that each of the aforementioned three major neuroendocrine features depends on a time-coordinated interplay among relevant secretagogues subject to feedback control. Unexpectedly, we found that the adult male GH axis operates stably for at least 2–8 wk despite marked androgen withdrawal *(347,348)*.

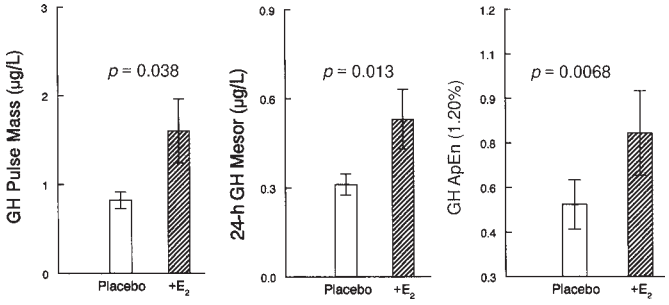

Fig. 2. Impact of short-term oral estradiol-17β (1 mg twice daily) replacement in postmenopausal women on GH secretory pulse mass **(left)**, the mesor of 24-h rhythmic GH release **(middle)**, and the approximate entropy (ApEn) of GH secretory patterns **(right)**. Data (±SEM) are from six volunteers. (Adapted with permission from ref. *83*).

Comparative Actions of Estradiol and Nonaromatizable Androgens on GH Axis

Although both aromatizable and nonaromatizable androgens stimulate high-amplitude GH pulses in the rat *(1,110,297,349)*, 5α-DHT, fluoxymesterone, and stanozolol do not consistently mimic testosterone in augmenting pulsatile GH secretion, elevating 24-h GH rhythmicity, or increasing the approximate entropy of GH release in humans *(16,312,350)*. On the other hand, we find that in young girls or women with Turner syndrome *(16,351,352)* and in boys with isolated luteinizing hormone deficiency *(312)*, administration of low-dose (100 ng/[kg·d]) estradiol or aromatizable androgen (50 mg intramuscularly once) amplifies GH pulse amplitude and increases the entropy of GH secretory patterns. These gender comparisons underscore the anticipated relevance of insights gleaned from any analysis of estrogen's neuroregulatory mechanisms to understanding the mode of action of aromatizable andro-

gens. Indeed, the same dependence of the GH axis on aromatizable androgen is evident in the domestic broiler chicken *(353,354)*.

Estradiol Selectively Augments Actions of GHRP-2 But Not GHRH in Postmenopausal Women

We have utilized a twofold strategy to examine estrogen's putative interaction with GHRH: constant 24-h iv GHRH infusions (1 μg/[kg·h]), and acute L-arginine (30 g intravenously over 30 min) pretreatment combined with a GHRH dose response analysis. The L-arginine intervention was proposed to reduce confounding by variable endogenous somatostatin tone. In the first study, estrogen supplementation stimulated pulsatile GH secretion by 1.8 to 6.2 fold, but did not enhance the stimulatory effect of a constant iv GHRH infusion on any primary parameter *(215)*. Thus, estradiol can stimulate GH secretion without directly altering the subacute (24-h) pituitary actions of GHRH. We are now finalizing our study of postmenopausal volunteers given dose-varying GHRH stimuli during concomitant L-arginine infusion.

In contrast to the data with GHRH, estrogen pretreatment does significantly augment GHRP's potency (greater pituitary sensitivity to low-dose GHRP-2) and efficacy (elevated maximal pituitary responsiveness to GHRP-2) *(239)* (*see* Fig. 3). In more prolonged studies, we combined estrogen pretreatment with a continuous GHRP-2 infusion (1 μg/[kg·h] for 24 h). Both agonists given together in 10 postmenopausal women normalized the 24-h GH acrophase to 1:45 AM, consistent with estrogen's modulation of an inferred CNS phase-shifting effect of GHRP-2; increased interpulse (basal) GH secretion by an additional twofold; elicited maximal pattern irregularity (approximate entropy) of GH secretion; and elevated plasma IGF-1 concentrations significantly across the 24-h infusion (8:00 AM to 8:00 PM) by a mean (±SEM) increment of 41 ± 9 μg/L vs the control (saline/placebo) decline of -12 ± 5.2 μg/L ($p < 0.01$). Thus, combined estrogen and GHRP-2 agonism recapitulates dynamic pubertal activation of the GH axis, as defined fourfold by an increased 24-h mesor and a physiologic GH acrophase, amplified GH secretory burst mass, elevated entropy of GH release, and increased plasma IGF-1 concentrations, Fig. 4. In longer-term (30–90 d) continuous sc GHRP-2 infusions in older men and women, there is continuing stimulation of GH pulsatility, GH approximate entropy, 24-h GH rhythmicity, and plasma IGF-1 concentrations *(170,217)*.

Estrogen Does Not Alter Efficacy of Exogenous Somatostatin's Inhibition of GH Secretion

In view of the key role played by somatostatin in regulating pulsatile GH secretion, we have tested the ability of estrogen replacement to limit the inhibitory effects of a dose-varying 3-h constant iv infusion of somatostatin-14 (0, 30, 100, and 300 μg/[1.73 m^2·h]) in postmenopausal women *(260)*. Doses in excess of 30 μg/(1.73m^2·h) all achieved near-maximal but equivalent absolute and percentage suppression of GH secretion in estrogen-sufficient and -deficient conditions; that is, estradiol did not alter somatostatin's inhibitory efficacy *(260)* (Fig. 5). However, this experiment does not assess estrogenic control of endogenously secreted somatostatin or define possible sensitivity changes. We are now extending these analyses by using lower doses of somatostatin (0, 3, 10, and 30 μg/[1.73 m^2·h]) to evaluate any impact of estrogen on pituitary sensitivity.

Impact of Infused rhIGF-1 on GH Secretion in Postmenopausal Women

In an initial appraisal of IGF-1 feedback, we have undertaken continuous 6-h iv infusions of saline vs rhIGF-1 (5 μg/[kg·h]) in six estrogen-deficient and estrogen-replaced women *(355)*. This dose of rhIGF-1 suppressed spontaneous and GHRH-stimulated GH secretion minimally, but equally, in the two conditions. Accordingly, we are also using a higher dose of rhIGF-1 (10 μg/[kg·h]) to suppress GH secretion near maximally. This analysis should corroborate or refute the hypothesis that estradiol repletion modulates IGF-1's feedback efficacy. In this regard, whereas Berman et al. *(226)* showed that a systemic IGF-1 infusion (10 μg/[kg·h]) suppresses GH secretion less effectively in young women than men, we cannot necessarily predict this outcome in the postmenopausal estrogen-treated vs estrogen-withdrawn individual. Indeed, another IGF-1 infusion study disclosed similar IGF-1 inhibitory efficacy in older (sex steroid–deficient) and young adults *(235)*.

Clinical interpretation of these experiments is hampered by the pharmacologic threefold physiologic plasma-free IGF-1 concentrations achieved by a 10 μg/(kg·h) infusion dose of rhIGF-1 *(235,355,356)*. To obviate this confound, we believe that an alternative investigative strategy will be needed, wherein one selectively mutes the endogenous IGF-1 feedback signal. We are using a novel potent rhGH-receptor antagonist to deplete IGF-1 nonsteroidally and thereby test whether reduced circulating IGF-1 concentrations contribute to estrogen's stimulation of GH secretion.

Preliminary Somatostatin Rebound Analysis

To explore the impact of estradiol replacement on "somatostatin-induced rebound" GH secretion in postmenopausal women, we infused iv somatostatin continuously for 3 h to block the secretion (but not synthesis) of GH *(260)*. Stopping the somatostatin infusion triggered acute GH release. Our preliminary analysis confirms the recent report by degli Uberti et al. *(225)* that rebound GH release is markedly attenuated in hormone-unreplaced older individuals. Because the 60-min post-somatostatin infusion interval that we analyzed may not have captured the full magnitude of rebound GH secretion (*see* Fig. 5), we are now extending the rebound phase of this analysis to 150 min.

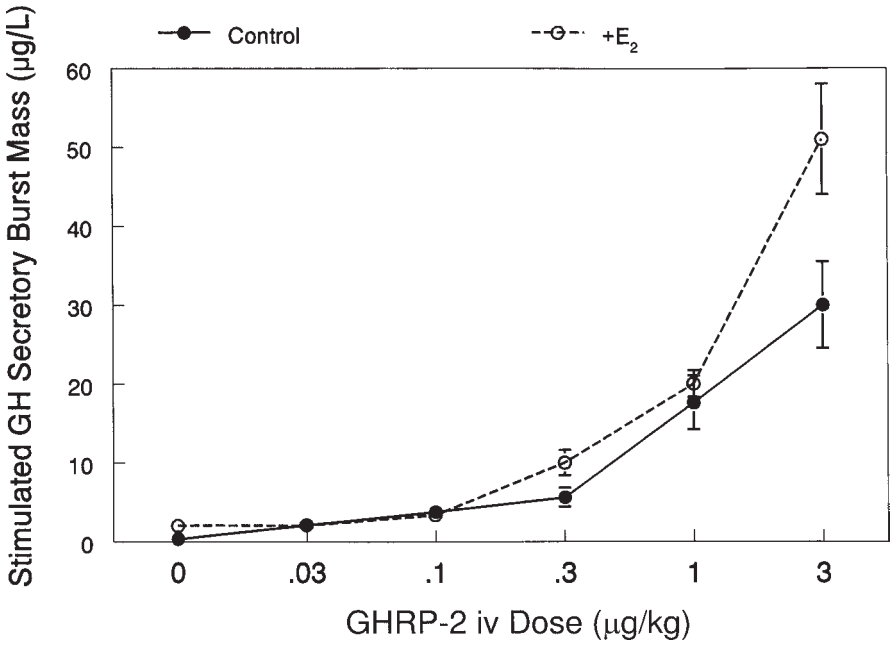

Fig. 3. Estradiol enhances the dose-dependent stimulatory actions of bolus iv GHRP-2 infusion in nine postmenopausal women. A log-linear regression is applied to relate doses of GHRP-2 (0, 0.03, 0.1, 0.3, and 1.0 μg/kg) to the GH secretory response. (Adapted with permission from ref. *239*).

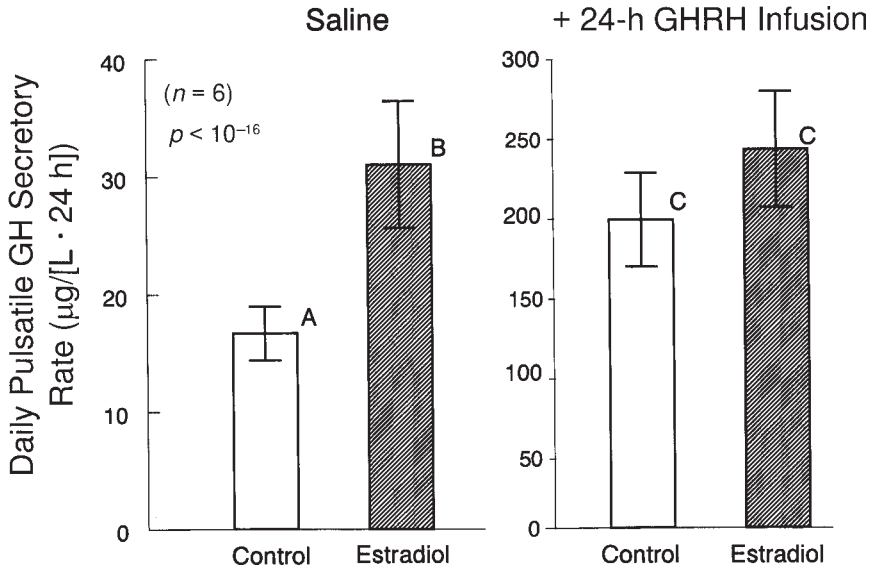

Fig. 4. Short-term estrogen replacement augments 24-h GH secretion stimulated by a constant iv infusion of GHRP-2 (1 μg/[kg·h]) vs saline (control) in 10 healthy postmenopausal women studied on four randomly ordered occasions. *p* values reflect the results of nested repeated-measures analysis of variance. (Adapted with permission from ref. *215*).

Maximal postsomatostatin rebound release of GH is antagonized by GHRH antiserum in the rat *(291)*. However, a biosynthetic GHRH-receptor antagonist does not impede postsomatostatin-induced rebound GH secretion in the human *(180)*. The latter clinical observation, if confirmed, allows the exciting postulate that postsomatostatin-induced rebound GH secretion will provide a barometer of the activity of endogenous non-GHRH secretagogues of GH.

Acute GH Autofeedback Restraint of GHRH, GHRP-2, and Exercise-Stimulated GH Secretion in Postmenopausal Women

To document GH autofeedback first in young adults, we initially tested the dose-dependent ability of a 6-min iv pulse of rhGH (1, 3, and 10 μg/kg) to suppress spontaneous and exercise-stimulated GH secretion in young men and women *(357)*. These doses of rhGH consistently inhibited

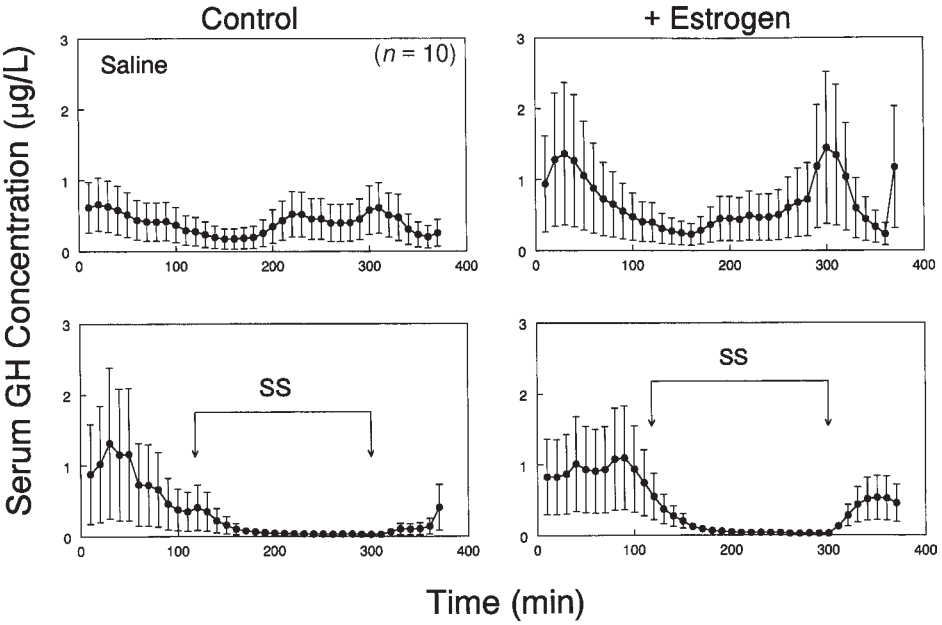

Fig. 5. Lack of impact of at least 1 wk of oral estradiol-17β pretreatment on the inhibitory effect of a 3-h constant iv infusion of somatostatin (SS) (300 μg/[1.73 m^2·h]) on serum GH concentrations in 10 fasting postmenopausal women sampled in randomly ordered estrogen-deficient vs estradiol-replaced sessions. Serum GH concentrations were assayed by chemiluminescence (sensitivity of 0.005 μg/L of 22-KDa rhGH). Blood samples were withdrawn every 10 min for 6 h (2 h basal, 3 h SS infusion, and 1 h rebound). Data are expressed as mean ± SEM. (Adapted with permission from ref. *260*).

2-h time-lagged spontaneous, but not exercise-stimulated, GH pulsatility in this age group *(358)*. Therefore, we have used a single 10 μg/kg rhGH pulse to test GH's feedback restraint of spontaneous and GHRH- and GHRP-2-stimulated GH secretion in postmenopausal women *(239,359)*. Data obtained in nine volunteers document consistent GH autofeedback that is more marked for GHRH than GHRP-2 in the estrogen-unreplaced female (Fig. 6). This provides an experimental foundation for assessing the hypothesis that estrogen will ameliorate GH's feedback inhibition of GHRP and GHRH stimulation. Comparing responses to the two secretagogues will be of particular interest, because in young adults GHRH and GHRP show unequal susceptibility to either GH or IGF-1-mediated autoinhibition *(1,186,233,234,237,271,360–362)*. However, how age and estrogen govern such differential autofeedback susceptibility of the two secretagogues still remains unknown.

Conclusion

Clinical studies almost three decades ago (2,3,5–7,10,302) established that estrogen acts as a dominant positive regulator, and age as a major negative determinant, of daily GH secretion (reviewed in refs. *1, 31, 35,* and *38*). However, the neuroendocrine mechanisms by which sex steroids govern GH output in the human have remained elusive. Continuing basic science advances in the biology of somatostatin, GHRH, and GHRPs now provide a broader investigative foundation to address this knowledge deficit in more innovative and insightful ways. Indeed, compelling recent developments in GHRP chemistry, receptorology, and physiology create a unique and timely basis for more informative clinical studies. Given the cardinal role of estrogen (or aromatizable androgen) in sustaining GH secretion throughout the human lifetime, further clinical studies should yield unique insights into the mechanisms by which sex-steroid hormones regulate the GH-IGF-1 axis in health and disease.

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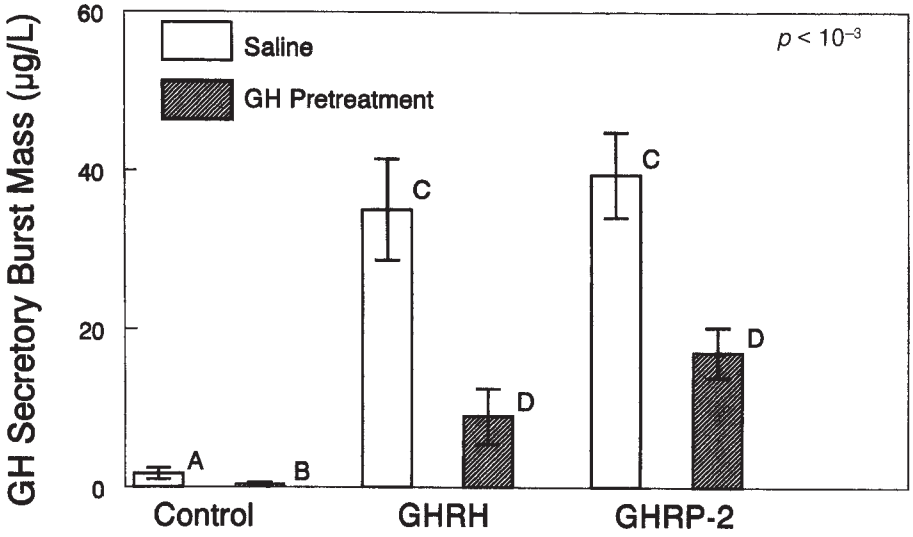

Fig. 6. rhGH (infused as a 10 µg/kg iv pulse over 6 min) represses basal, GHRH, and GHRP-2-stimulated GH secretion in nine healthy nonestrogen-replaced postmenopausal women. (Adapted with permission from ref. *359*).

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