SECRETAGOGUE TYPE, SEX-STEROID MILIEU, AND ABDOMINAL VISCERAL ADIPOSITY INDIVIDUALLY DETERMINE SECRETAGOGUE-STIMULATED CORTISOL SECRETION

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Abstract

Design: While androgens and estrogens control glucocorticoid secretion in animal models, how the sex-steroid milieu determines cortisol secretion in humans is less clear. To address this issue, cortisol was measured in archival sera obtained at 10-min intervals for 5 h in 42 healthy men administered double placebo, placebo and testosterone, testosterone and dutasteride (to block 5α-reductases type I and type II), or testosterone and anastrozole (to block aromatase) in a double-blind, placebo-controlled, prospectively randomized design.

Methods: Subjects received i.v. injection of saline, GHRH, GH-releasing peptide-2 (GHRP-2), somatostatin (SS), and GHRP-2/GHRH/L-arginine (triple stimulus) each on separate mornings fasting. Outcomes comprised cortisol concentrations, pulsatile cortisol secretion, and relationships with age or abdominal visceral fat (AVF).

Results: By ANCOVA, baseline (saline-infused) cortisol concentrations (nmol/l) did not differ among the sex-steroid milieus (overall mean 364 ± 14). In contrast, stimulated peak cortisol concentrations were strongly determined by secretagogue type (P < 0.001) as follows: triple stimulus (868 ± 27) > GHRP-2 (616 ± 42) > saline = SS = GHRH (grand mean 420 ± 21). After GHRP-2 injection, pulsatile cortisol secretion increased with age (R² = 0.16, P = 0.012). After the triple stimulus, pulsatile cortisol secretion correlated i) inversely with serum 5α-dihydrotestosterone (DHT) concentrations (R² = 0.53, P = 0.026) and ii) directly with computerized tomography-estimated AVF (R² = 0.11, P = 0.038).

Conclusion: Age, DHT concentrations, AVF, and secretagogue type influence pulsatile cortisol secretion at least in men. Further studies should be performed to assess ACTH secretion and native ghrelin action in defined sex-steroid milieus.

Introduction

Ghrelin, a 28-amino acid Ser³-octanoylated GH-releasing peptide (GHRP) of gastro-pancreatico-hypothalamo–pituitary–adrenal origin, and synthetic GHRPs stimulate GH, ACTH, cortisol, and prolactin secretion, and synergize with GHRH in driving GH secretion (1, 2). Unacylated ghrelin and GHRH alone do not elevate cortisol concentrations, but endogenous somatostatin (SS) may attenuate cortisol secretion induced by ghrelin/GHRP (3–5).

Pulsatile cortisol secretion, which constitutes about 85% of total daily cortisol production, is strongly determined by the availability of sex-steroid hormones in experimental animals. Sex differences in HPA regulation in laboratory species arise from both neuronal imprinting during early development and distinct actions of testosterone and estradiol (E₂) in adulthood (6–9). In particular, exposure to E₂ typically potentiates stress-induced ACTH secretion, putatively by i) attenuating negative feedback on the hypothalamus, limbic cortex, and pituitary gland (10); ii) inducing AVP, CRH, and CRH-R1 gene expression (6, 7, 11); iii) enhancing adrenal responsiveness to ACTH (12, 13); and iv) blunting homologous down-regulation of hippocampal glucocorticoid receptors. Conversely, testosterone and 5α-dihydrotestosterone (DHT) usually inhibit stress-stimulated ACTH secretion (14–17). The impact of gonadal steroids is not expressly dichotomous because E₂ can augment certain actions of DHT, whereas metabolites of DHT can oppose other effects of E₂ (14, 18). How sex steroids influence GHRP-regulated cortisol secretion is not known in any species.

No clinical analyses distinguish the capability of testosterone to act by way of distinct metabolic
products, such as DHT and E₂, or address the effect(s) of sex steroids on ghrelin/GHRP-stimulated cortisol secretion. Accordingly, the present study examines sex-steroid modulation of GHRP-stimulated cortisol secretion using existing sera obtained in a recent paradigm examining GH secretion after selective blockade of 5α-reductase and aromatase activities (4). A fixed dose of testosterone was administered concurrently to obviate potentially confounding feedback adjustments in the hypothalamo-pituitary–gonadal axis.

Methods

Inhibitors

Dutasteride (dut) was obtained from GlaxoSmithKline, and anastrozole (ana) from AstraZeneca (19, 20).

Subjects

The protocol and subjects were described recently in relation to GH measurements (4). The same sera were used and submitted to cortisol assay instead. Plasma was not available to allow ACTH measurements. Procedures were approved by the Mayo Institutional Review Board (IRB) according to the Declaration of Helsinki. Volunteers underwent medical screening as outpatients, including history and physical examination. Inclusion criteria were normal hepatic, renal, hematological, metabolic, and endocrine function (TSH, prolactin, LH, FSH, and total testosterone). Reasons for exclusion were recent use of neuroactive medications, glucocorticoids or sex hormones, diabetes mellitus, systemic disease, acute illness, drug or alcohol abuse, anemia, history of heart failure, prostatic disease, or unwillingness to provide written informed consent.

Diet

A standardized meal (10 kcal/kg of 50% carbohydrate, 20% protein, and 30% fat) was served at 1800 h on the night before the study, after which, subjects remained fasting during sampling and infusions.

Body composition measurement

Computerized tomography (CT) of the abdomen at L3–4 was used to quantify the cross-sectional area (cm²) of visceral adipose tissue as described (21).

Hormone assays

Serum cortisol concentrations were determined by a solid-phase competitive chemiluminescent enzyme immunoassay, IMMULITE 2000 (Siemens Healthcare Diagnostics Inc. (Deerfield, IL 60015, USA)). Assay sensitivity is 5.6 nmol/l with intra-assay precision of 7.5–9.8% at cortisol concentrations of 134–1064 nmol/l.

E₂, testosterone, and DHT were measured by tandem liquid chromatography–ion-spray mass spectrometry exactly as described (4).

Clinical protocol

The study was described fully earlier when GH was measured (4). Briefly, the design was prospective, double-blind, randomized, and placebo-controlled. Subjects received i) three injections of double placebo (pl/pl) comprising i.m. saline 1.0 ml weekly, and oral placebo once daily for 21 days; ii) three injections of testosterone enanthate 200 mg i.m. weekly and oral placebo (testosterone/pl); iii) testosterone enanthate i.m. and oral dut 1 mg once daily for 21 days (testosterone/dut); and iv) testosterone enanthate i.m. and oral ana 2 mg once daily for 21 days (testosterone/ana).

Infusion and sampling sessions occurred in the morning fasting in a prospectively randomized order on any four nonconsecutive mornings within the 15-day window defined by days 9–24 following the first pl or testosterone injection (day 1). Subjects received i.v. saline (30 ml/h) from 0800 to 0930 h to establish a baseline, and then, they received the following (separately): i) SS-14 (5 μg/m² per h continuously) from 1000 to 1300 h; ii) GHRH (0.15 μg/kg bolus) at 1000 h; iii) GHRP-2 (3.0 μg/kg bolus) at 1000 h; and iv) L-arginine (30 gm i.v. from 0930 to 1000 h) followed by a combined bolus of GHRP-2 and GHRH (described above) at 1000 h, referred to as a triple stimulus. Blood was obtained concurrently (1.5 ml every 10 min) for 5 h, beginning at 0800 h in subjects who were awake.

Deconvolution analysis

Cortisol concentration time series were analyzed using an automated Matlab program (22). First, this detrends the data and normalizes the concentrations to the unit interval [0, 1]. Secondly, multiple potential pulse-time sets, each containing one fewer burst, are created by a smoothing process (a nonlinear adaptation of the heat diffusion equation). Lastly, a maximum-likelihood estimation method calculates all secretion and elimination rates simultaneously for each candidate pulse-time set. Cortisol kinetics were fixed to correspond to a rapid half-life of 2.8 min and slow half-life of 56 min representing 63% of total decay (23). Model selection was performed using the Akaike information criterion penalized for pulse number. The parameters (and units) are basal and pulsatile cortisol secretion rates (nmol/l per h), secretory-burst mass (nmol/l), and secretory-burst shape (mode of waveform or time delay in minimal to maximal secretion after burst onset).
**Statistical assessment**

The primary hypothesis was that sex-steroid milieu determines pulsatile cortisol secretory responses to GHRP-2 and the triple stimulus, but not to saline, SS, or GHRH. This postulate was addressed by two-way ANCOVA (using the mean 90-min prestimulus saline-infused cortisol concentration as a covariate) in a 4 × 4 factorial design: four sex-steroid interventions (categorical variables) and four secretagogues (repeated measures within a subject). Baseline (prestimulus) data were assessed by two-way ANOVA. Data were log transformed to limit dispersion of residual variance. Post hoc comparisons were made by Tukey’s honestly significant difference test. Based on a sample size of 37 subjects, statistical power exceeded 90% to detect a 30% effect of sex-hormone milieu by unpaired Student’s t-test at $P \leq 0.01$ on the least-expected contrast of a $1.5 \pm 0.13$ (s.d.)-fold greater stimulatory effect of GHRP-2 under testosterone than placebo. A corollary postulate was that systemic testosterone, DHT, or E$_2$ concentrations and abdominal visceral fat (AVF) determine GHRP-2-stimulated cortisol secretion. This postulate was addressed by stepwise forward-selection multivariate linear regression analysis at $\alpha = 0.05$ ($n=42$ subjects) using Systat Version 11.0 (San Jose, CA, USA). The dependent variable was pulsatile cortisol secretion (summed burst mass) after stimulation.

**Results**

As reported for GH measurements in this study (4), subject characteristics in the four groups did not differ. After pharmacological testosterone administration, testosterone, E$_2$, and DHT concentrations rose by 2.8-, 1.9-, and 2.6-fold respectively compared with pl/pl. Combined testosterone/dut administration did not affect E$_2$ or testosterone concentrations, but it reduced DHT concentrations by 89% compared with testosterone/pl and by 70% compared with pl/pl (both $P < 0.001$). Combined testosterone/ana treatment did not alter DHT or testosterone concentrations, but it decreased E$_2$ values by 86% compared with testosterone/pl, and by 74 and 89% compared with pl/pl and testosterone/dut respectively (each $P < 0.001$). Individual sex-steroid concentrations were reported by Veldhuis et al. (4).

Cortisol concentration profiles obtained by 10-min sampling over 5 h are depicted before (baseline 1.5 h) and after (3.5 h) i.v. injection of GHRP-2 and the triple stimulus. Profiles for saline, GHRH, and SS are not shown, since they exerted no stimulatory or inhibitory effect. Baseline (prestimulus) cortisol concentrations averaged $364 \pm 14$ nmol/l, consistent with unstressed morning values, and were unaffected by sex-steroid milieu (Fig. 1).

Two-way ANCOVA demonstrated that secretagogue type but not sex hormone condition was a strong determinant of poststimulus peak cortisol concentrations (overall $P < 0.001$, covariate $P < 0.005$, interaction $P > 0.3$; Fig. 2). The descending rank order of stimulated peak cortisol concentrations (nmol/l) was triple stimulus $> GHRH-2 > GHRP-2 >$ saline $= GHRH = SS$ (grand mean 420 ± 21). The rank order of triple stimulus $> GHRP-2 >$ saline $= GHRH = SS$ was also independent of sex-steroid milieu when estimated as incremental (peak minus mean baseline) cortisol responses in the 42 subjects (Fig. 3).

Two-way ANCOVA of pulsatile cortisol secretion disclosed no significant effect of the baseline covariate ($P = 0.19$) or inhibitor treatment group (pl/pl, testosterone/pl, testosterone/dut, and testosterone/ana) ($P = 0.71$). However, secretagogue type strongly determined pulsatile cortisol secretion (overall $P < 0.001$, secretagogue $P < 0.001$, interaction $P = 0.84$; Table 1). The descending rank order of pulsatile cortisol secretion was triple stimulus $> GHRP-2 >$ saline $= GHRH = SS$, thus confirming peak and incremental cortisol concentration data.

Exploratory linear regression analysis showed that age was a positive correlate of GHRP-2-driven
pulsatile cortisol secretion ($R^2 = 0.16, P = 0.012$). In addition, the concentration of DHT in the testosterone/dut group was a negative determinant of triple stimulus-induced pulsatile cortisol secretion ($R^2 = 0.53, P = 0.026$). Conversely, AVF was a positive correlate of triple secretagogue action on this endpoint ($R^2 = 0.11, P = 0.038$).

Cortisol secretory-burst mode (time delay from onset of a burst to maximal secretion) was not influenced by sex-steroid milieu or by secretagogue. The mean mode was $18 \pm 1.2$ min.

**Discussion**

This study evaluates how sex-steroid milieu controls the stimulation of cortisol secretion by classical GH secretagogues in healthy men. The pharmacological paradigm exploited here yielded a wide range of testosterone, $E_2$, and DHT concentrations that were measured by tandem ion-spray mass spectrometry (4). During administration of ana and dut (19, 20), $E_2$ concentrations were lowered by 86% and DHT concentrations by 89% respectively. Under these conditions, systemic DHT concentrations (negatively) and age and AVF (positively) determined GH secretagogue-stimulated pulsatile cortisol secretion. $E_2$ and testosterone levels did not correlate with cortisol output. The selectivity of sex-steroid, secretagogue, age, and AVF associations adds a further dimension to understanding the acute regulation of cortisol (and putatively ACTH) secretion.

Although testosterone and $E_2$ are principally inhibitory and facilitative of cortisol secretion respectively in animals, this dichotomy is not so evident in humans (24). For example, men exhibit larger ACTH pulses and greater ACTH/cortisol responses to certain stressors than premenopausal women (25–29). Nonetheless, transdermal $E_2$ administration in young men augmented psychosocial stress-induced ACTH release and suppressed cortisol secretion (28). Conversely, estrogen supplementation in postmenopausal women reduced ACTH responses to CRH (25, 26). In one study, testosterone concentrations in obese women correlated positively with AVP/CRH-stimulated ACTH release (27), whereas the reverse applied to CRH’s stimulation in men (30). Here, using GHRP-2 as an HPA axis stimulus, age was a positive correlate of cortisol secretion ($P = 0.012$), at least in men. Using a triple stimulus that contained GHRP-2, DHT concentrations and the body composition marker, AVF, were respectively negative and positive correlates of pulsatile cortisol secretion. Earlier discrepant outcomes might have arisen variously from age-related, body fat, and/or sex-steroid effects on hypothalamo-pituitary regulatory mechanisms. Such mechanisms could be subserved by...
the relative abundance of aromatase and 5α-reductase activity; specific actions of E2, DHT, and testosterone at different neuroanatomical sites; and selective expression of AR, ER-α, and ER-β in the brain, corticotropes, and adrenal cortex (9, 15, 16, 31, 32). In particular, ER-α is expressed in corticotropes and CRH and GHRH neurons, AR in AVP and SS neurons, and ER-β in oxytocin and AVP neurons as well as in the pituitary gland (2, 24). A further confounding point is that DHT acting via AR and its metabolite, 5α-androstane-3β,17β-diol, acting via ER-β, can inhibit AVP and ACTH release in the rodent (14, 33). Indeed, we found that under combined type I/II 5α-reductase blockade, DHT concentrations (negatively) predicted 53% of the intersubject variability in peak triple secretagogue-stimulated cortisol secretion.

Several clinical investigations have imposed a constant sex-steroid milieu during gonadal suppression to assess corticotropic axis outflow. None used GH secretagogues (25, 28, 34). In the current study, a major finding was the marked capability of a triple stimulus comprising GHRP/GHRH/L-arginine to stimulate corticotropin release. Indeed, peak cortisol concentrations averaged 868 ± 27 nmol/l after the triple stimulus, and 616 ± 42 after the administration of GHRP-2 alone compared with a baseline mean of 364 ± 14 nmol/l. Although incremental (peak minus baseline) and pulsatile cortisol secretion followed the same pattern (Fig. 3 and Table 1), direct measurement of cortisol clearance is needed to test whether unexpected changes in elimination rates affected concentration values.

GHRP has the capability to induce the release of ACTH secretagogues, such as CRH, AVP, or both, from hypothalamic tissue in vitro and animals in vivo (35–39). In rats, DHT inhibits the AVP promoter, whereas estrogen stimulates the CRH promoter (40). In humans, however, the mechanisms mediating GHRP’s stimulation of ACTH and cortisol secretion are less clear (41–43). If androgens inhibited AVP and/or CRH secretion or stimulated SS secretion in men as they do in animals (2, 8, 44), this might explain the strong inverse correlation (R = −0.728) observed here between DHT concentrations and cortisol secretion induced by a triple stimulus.

Potentiation between l-arginine and GHRP-2 acting centrally to release ACTH and cognate secretagogues offers the simplest explanation for the powerful effect of the triple stimulus on pulsatile cortisol secretion. This is because GHRH has no consistent discernible effect on cortisol levels acutely (present data and (45)). However, SS withdrawal and l-arginine infusion can potentiate ghrelin/GHRP-stimulated cortisol secretion (5, 38). Whereas further investigations will be needed to test this interpretation, the remarkable effects of the triple stimulus on cortisol secretion should be noted.

AVF was estimated by CT scan. Correlational analysis disclosed a positive association between AVF and pulsatile cortisol secretion induced by the triple stimulus, and likewise, between age and the pulsatile cortisol secretory response to GHRP-2. On the other hand, both age and AVF suppress GH secretion (2, 4), pointing to quite different regulatory mechanisms compared with those supervising cortisol secretion. The most obvious point of cortisol regulation would be via ACTH release (2). Other possible explanations are that GHRP/ghrelin peptide and/or receptors are expressed by human and rat adrenal cells, allowing for potentially direct regulation of ACTH-dependent cortisol secretion by secretagogues (46–48). Less likely would be direct adrenal effects of l-arginine, which in some studies augments adrenal glucocorticoid secretion directly (49, 50).

Caveats include the possibility that lower doses of GHRP or natural ghrelin as well as more prolonged (compared with those in 3 weeks) changes in testosterone, DHT, and E2 concentrations may affect the corticotropic axis differently. In addition, future studies should also estimate ACTH, CBG, and free cortisol concentrations to appraise regulatory mechanisms further.

In conclusion, age, systemic DHT concentrations, abdominal visceral adiposity, and type of peptidyl effector determine cortisol secretion acutely in healthy older men.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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