Estradiol, but not testosterone, heightens cortisol-mediated negative feedback on pulsatile ACTH secretion and ACTH approximate entropy in unstressed older men and women

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Sharma AN, Aoun P, Wigham JR, Weist SM, Veldhuis JD. Estradiol, but not testosterone, heightens cortisol-mediated negative feedback on pulsatile ACTH secretion, ACTH approximate entropy, and ACTH concentrations in unstressed older men and women. Am J Physiol Regul Integr Comp Physiol 306: R627–R635, 2014. First published February 26, 2014; doi:10.1152/ajpregu.00551.2013.—How sex steroids modulate glucocorticoid feedback on the hypothalamic-pituitary-corticotrope (HPC) unit is controversial in humans. We postulated that testosterone (T) in men and estradiol (E2) in women govern unstressed cortisol-mediated negative feedback on ACTH secretion. To test this hypothesis, 24 men and 24 women age 58 ± 2.4 yr were pretreated with leuprolide and either sex steroid (E2 in women, T in men) or placebo addback. Placebo or ketoconazole (KTCZ) was administered overnight to inhibit adrenal steroidogenesis during overnight 14-h intravenous infusions of saline or cortisol in a continuous versus pulsatile manner to test for feedback differences. ACTH was measured every 10 min during the last 8 h of the infusions. The main outcome measures were mean ACTH concentrations, pulsatile ACTH secretion, and ACTH approximate entropy (ApEn). ACTH concentrations were lower in women than men (P < 0.01), and in women in the E2+ compared with E2− group under both continuous (P = 0.01) and pulsatile (P = 0.006) cortisol feedback, despite higher cortisol binding globulin and lower free cortisol levels in women than men (P < 0.01). In the combined groups, under both modes of cortisol addback, ACTH concentrations, pulsatile ACTH secretion, and ACTH secretory-burst mass correlated negatively and univariately with E2 levels (each P < 0.005). E2 also suppressed ACTH ApEn (process randomness) during continuous cortisol feedback (P = 0.004). T had no univariate effect but was a positive correlate of ACTH when assessed jointly with E2 (negative) under cortisol pulses. In conclusion, sex steroids modulate selective gender-related hypothalamic-pituitary-adrenal-axis adaptations to cortisol feedback in unstressed humans.

Adrenocorticotropic hormone; pulsatile; regulation; sex steroids; glucocorticoids

Sexual dimorphism is a hallmark of human pathophysiology. For example, women of any age are more likely to develop autoimmune disorders, whereas men are more susceptible to cardiovascular diseases (19). Women have a higher prevalence of certain psychiatric disorders, such as anxiety and depression, whereas men are more likely to participate in substance abuse (2). Stress-mediated hippocampal structures are also sexually dimorphic (29). In animals, sex-related contrasts in the hypothalamic-pituitary-adrenal (HPA) axis encompass corticotrophin-releasing hormone (CRH) drive of ACTH, ACTH feedback on the adrenal gland, and glucocorticoid negative feedback on the hypothalamo-pituitary-corticotrope (HPC) unit. Intact female rats or ovariectomized rats given estradiol (E2) maintain higher ACTH concentrations, greater ACTH-stimulated corticosterone secretion, and lesser corticosteroid-related feedback than intact male rats or orchidectomized rats given testosterone (T) (5, 42).

Sex differences may be influenced further by both species and development. For example, in the ovine fetus, exogenous E2 has no evident effect, whereas androstenedione seems to impair HPA-axis feedback (27). In humans, the role of sex steroids in HPA regulation is particularly controversial, despite the fact that life stages like infancy, puberty, and aging manifest large fluctuations in sex-steroid availability (36). Inconsistencies may arise from heterogeneous study cohorts, nonuniform subject age; variable endogenous sex-steroid concentrations, pharmacological doses and types of glucocorticoids used, single-bolus versus continuous glucocorticoid feedback models; infrequent and/or brief sampling of pulsatile ACTH secretion; and lack of gender comparisons under identical conditions (13, 31–33).

The present clinical investigation attempts to address limitations in the field by infusing cortisol rather than a synthetic corticosteroid as the negative-feedback signal; studying older subjects in whom sex differences under a gonadal-steroid clamp may be more prominent; experimentally controlling the sex-steroid milieu; sampling every 10 min for 8 h; employing equal-dose cortisol infusions in pulses or continuously so as to compare a putatively physiological (pulsatile) versus pharmacological (continuous) feedback pattern; calculating free cortisol from albumin and cortisol binding globulin (CBG) concentrations; and applying deconvolution analysis to estimate pulsatile ACTH secretion and ACTH approximate entropy (ApEn) as a model-free measure of feedback strength. Older individuals were studied since sex differences in ACTH responses may be greater and/or more consistent in older than young humans (reviewed in Ref. 37). The hypothesis was that the short-term sex-steroid milieu, rather than gender per se, governs cortisol-dependent negative feedback on mean, pulsatile, and entropic ACTH secretion in healthy men and women. This postulate predicts that experimentally clamping systemic T and E2 concentrations at high young-adult levels will influ-

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ence dynamic ACTH responses to negative feedback by the natural glucocorticoid cortisol. Moreover, the hypothesis and design allow for possible feedback differences by pulsatile and continuous cortisol addback in unstressed individuals, as recently suggested by others in animal models (15).

MATERIALS AND METHODS

Subjects

The protocol was approved by Mayo Independent Review Board and reviewed by the Food and Drug Administration for off-label use of ketoconazole (KTCZ). Women were postmenopausal, as verified by clinical history and by luteinizing hormone (LH) > 12 IU/l, follicle-stimulating hormone (FSH) > 40 IU/l, E2 < 15 pg/ml (<55 pmol/l), and T < 45 pg/ml (<165 pmol/l). Men were eugonadal based on screening values of LH, FSH, and T. Witnessed voluntary written consent was obtained before study enrollment. Participants maintained conventional work and sleeping patterns and reported no recent (within 10 days) transmeridian travel, significant weight loss or gain, intermittent psychosocial stress, substance abuse, neuropsychiatric illness, or systemic disease. A complete medical history, physical examination, and screening tests of hematological, renal, hepatic, metabolic, and endocrine function were normal.

Study Design

This was a prospectively randomized double-blinded, placebo-controlled crossover study in 24 men and 24 postmenopausal women with allowable ages 45–80 yr. Each subject received 3.75 mg im leuprolide twice 2 wk apart to induce hypogonadism followed by either sex-steroid or placebo replacement starting on the day of the second leuprolide injection (called day 1). Sex-steroid replacement schedules comprised 1) in men: T enanthate (150 mg im) or placebo (saline 1 ml im) on days 1, 8, 15, and 22 (± 1 day); and 2) in women: twice daily oral micronized E2 (1 mg) or placebo for a total of 26 days.

Infusion Protocol

The protocol required four separate overnight infusion and sampling sessions scheduled 48–72 h apart during the inclusive time window days 17–26. In Fig. 1A, participants received oral placebo or KTCZ overnight to inhibit adrenal steroidogenesis, along with superimposed intravenous infusions of saline or cortisol in a pulsatile (P) versus continuous (C) fashion. Figure 1B was as follows: 1) oral placebo at 2200, 0600, and 1200 h with continuous intravenous saline at 20 ml/h for 14 h from 2200 h until 1200 h; 2) oral KTCZ at 2200 h (800 mg for men, 600 mg for women) and 0600 h (300 mg for men, 200 mg for women) with intravenous saline for 14 h and 20 mg of oral hydrocortisone at 1200 h for safety after the last blood draw; 3) oral KTCZ twice (as in 2) along with continuous intravenous infusion of hydrocortisone hemisuccinate over 14 h (2200–1200 h) at a rate of 0.5 mg·m⁻²·h⁻¹ (total cortisol dose 7.0 mg·m⁻²·14 h⁻¹) and oral placebo at 1200 h (KTCZ/CC); and 4) oral KTCZ twice (as in 2) with pulsatile intravenous infusion of cortisol at the same total dose (7.0 mg·m⁻²·14 h⁻¹), fractionated into 10 consecutive, 10-min squarewave pulses (each 0.70 mg/m²) one every 90 min, followed by oral placebo at 1200 h (KTCZ/PC).

Blood was sampled every 10 min from 0400 to 1200 h (8 h) to provide EDTA plasma for ACTH and serum for cortisol assays.

Hormone Assays

Plasma ACTH was assayed in each 10-min sample via sensitive and specific, solid-phase immunoenzymeluminescent assay (Siemens Healthcare Diagnostics, Deerfield, IL). The detection threshold was 5 ng/l (divide by 4.5 for pmol/l). Intra-assay coefficients of variation were 6.4, 2.7, and 3.2% at 5.7, 28, and 402 ng/l, respectively. Cortisol was assayed via competitive binding immunoenzymatic assay (Beckman Coulter, Fullerton, CA). The detection limit was 0.4 μg/dl and intraassay coefficients of variations were 13, 9.4, and 6.6% at 1.6, 2.8, and 30 μg/dl, respectively. T, E2, and E1 were assayed by mass spectrometry as described (21, 30). CBG was assayed as noted earlier (28). Screening biochemical tests were performed by the Mayo Medical Laboratory.

Free cortisol concentrations were calculated as described by Mazer (18), using the latter’s literature-based kinetic constants for CBG and albumin.

Deconvolution Analysis

ACTH concentration time series were analyzed by way of a recently developed variable-waveform deconvolution method (9). The nonlinear model estimates basal secretion, a slow-phase half-life, secretory-burst mass, random effects on burst mass, and a flexible secretory-burst waveform. The rapid phase half-life was assumed to be 3.5 min, representing 37% of total decay. Units of parameters are

Fig. 1. Experimental protocol. Twenty-four healthy older men and 24 comparably aged women received 2 doses of leuprolide im 2 wk apart. Sex-steroid addback (weekly testosterone in men and daily estrogen in women) or placebo was started on the day of the second leuprolide injection (day 1) and continued for 26 days (A). Each participant underwent 4 randomly ordered overnight sampling and infusion sessions during the time window 18–26 days inclusive. Volunteers received placebo (1 session) or ketoconazole (KTCZ) (3 sessions) orally to inhibit cortisol production. Concomitantly, glucocorticoid feedback was imposed by way of a 14-h intravenous infusion of saline or midphysiological dose of cortisol in a continuous or pulsatile fashion (B). To monitor ACTH and cortisol, blood was sampled every 10 min during the last 8 h of each infusion. Time is shown in days (A) or clock hours (B).
burst frequency (number bursts per session), slow half-life (min), basal and pulsatile secretion rates (concentration units/session), mass secreted per burst (concentration units), and waveform mode (time delay to maximal secretion after burst onset, minutes) (35). Sensitivity and specificity of pulse detection are both ≥93% (17).

**ApEn and Cross-ApEn Analysis**

ApEn is a model-free and scale-independent regularity measure that provides a barometer of feedback changes (38). ApEn comprises a family of two-parameter statistics defined by ApEn (m, r), where m is run length and r is de facto tolerance width. Parameters m = 1 and r = 20% are validated for neurohormone profiles. ApEn is distinct from, but complementary to, conventional pulse detection, with higher ApEn denoting greater disorderliness (less pattern regularity).

**Statistical Analysis**

The 8-h model-free mean plasma ACTH concentration was the primary endpoint. The effects of gender (2 factors), sex-steroid add-back (4 conditions), and specific cortisol-feedback interventions (4 types) on the (natural logarithm of) ACTH levels were determined by three-way analysis of covariance (ANCOVA) in a 2 × 4 × 4 block design. ACTH measured under placebo-saline served as the covariate. Means were compared post hoc using Tukey’s Honestly Significantly Different (HSD) test.

Univariate linear regression was utilized to explore the possible relation between T or E2 and ACTH dynamics. Multivariate (2 independent variables, T and E2) regressions were assessed stepwise (MATLAB version 7, Natik, MA).

Experiment-wise P ≤ 0.05 was considered significant. Linear-regression critical P value was ≤0.01, given testing of four variables. Data are presented as means ± SE and also median (range), where specified.

**RESULTS**

Table 1 in the text summarizes baseline subject characteristics obtained before any treatment. FSH, LH, T, E2, and estrone values confirmed postmenopausal status in women. LH, FSH, and T were normal for age in men (Table 1). Gender differences were evident for baseline E2, T, estrone, IGF-I (lower in women), and LH, FSH, and sex hormone-binding globulin (lower in men). Age, body mass index, and 0800 h (single sample) morning cortisol concentration were unaffected by sex.

Comparisons of sex-steroid concentrations measured during the four inpatient study visits are given in Table 2. ANOVA showed that mean T concentrations in men who received leuprolide plus T addback (783 ± 98 ng/dl) were higher than those given leuprolide and placebo (13 ± 2.6 ng/dl; P < 0.001). This was also true for mean E2 levels in women given leuprolide plus E2 versus leuprolide plus placebo addback (viz., 92 ± 12 vs. 9.8 ± 0.1 pg/ml; P < 0.001). Among men who received T addback and women who received T addback, mean T (men) and E2 (women) concentrations were comparable in all four study arms. There were no effects on leuprolide-suppressed T and E2 levels when KTCZ and/or cortisol were administered.

Oral KTCZ, compared with oral placebo, without glucocorticoid addback served as a positive control. KTCZ plus saline reduced mean 8-h cortisol concentrations from 10.2 ± 0.4 to 7.5 ± 0.5 μg/dl (P < 0.001) in the combined group (men and women, N = 48). Concomitantly, mean ACTH concentrations rose more than threefold from 19.1 ± 1.2 to 65.4 ± 5.4 ng/l (P < 0.001), thus verifying feedback withdrawal. Mean total cortisol levels are given by subgroup in Table 3, with no differences by gender or sex-steroid milieu. However, Table 4 showed that CBG concentrations were higher and free cortisol

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**Table 1. Subject characteristics at screening before any interventions**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>T+ (n = 12)</th>
<th>T− (n = 12)</th>
<th>E2+ (n = 10)</th>
<th>E2− (n = 14)</th>
<th>P Value (1-Way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, yr</strong></td>
<td>59 ± 3</td>
<td>55 ± 2.1</td>
<td>60 ± 2.4</td>
<td>58 ± 2.2</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>BMI, kg/m²</strong></td>
<td>28 ± 1.4</td>
<td>28 ± 1.2</td>
<td>29 ± 1.4</td>
<td>25 ± 1.2</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>TSH, mU/l</strong></td>
<td>2.2 ± 0.2</td>
<td>2.7 ± 0.5</td>
<td>2.2 ± 0.4</td>
<td>2 ± 0.2</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>FSH, IU/l</strong></td>
<td>6.2 ± 0.9</td>
<td>6.9 ± 1</td>
<td>93 ± 8.8</td>
<td>90 ± 6.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>LH, IU/l</strong></td>
<td>3.4 ± 0.5</td>
<td>3.6 ± 0.9</td>
<td>30 ± 4.3</td>
<td>27 ± 2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Estradiol, pmol/l</strong></td>
<td>102 ± 9.2</td>
<td>101 ± 9.1</td>
<td>35 ± 2.2</td>
<td>42 ± 5.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Estrone, pmol/l</strong></td>
<td>143 ± 9.6</td>
<td>124 ± 10.7</td>
<td>84 ± 10</td>
<td>88 ± 12</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Testosterone, nmol/l</strong></td>
<td>19 ± 2</td>
<td>18 ± 1.4</td>
<td>0.55 ± 0.06</td>
<td>0.53 ± 0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Cortisol, μg/dl</strong></td>
<td>13.2 ± 0.6</td>
<td>12.3 ± 0.8</td>
<td>11.8 ± 1</td>
<td>11.7 ± 0.6</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Albumin, g/l</strong></td>
<td>43 ± 1</td>
<td>45 ± 1</td>
<td>44 ± 0.3</td>
<td>44 ± 0.1</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>Growth Hormone, μg/L</strong></td>
<td>0.7 ± 0.3</td>
<td>0.1 ± 0.02</td>
<td>0.7 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>Insulin-like growth factor-I, μg/l</strong></td>
<td>174 ± 22.1</td>
<td>170 ± 23.9</td>
<td>102 ± 11.4</td>
<td>115 ± 2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Proctolin, pmol/l</strong></td>
<td>330 ± 21.7</td>
<td>291 ± 17.4</td>
<td>265 ± 21.7</td>
<td>296 ± 26.1</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Sex-hormone binding globulin, nmol/l</strong></td>
<td>35 ± 4.5</td>
<td>34 ± 4.3</td>
<td>46 ± 7.5</td>
<td>60 ± 9.5</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Data are means ± SE, n = number of subjects. BMI, body mass index; TSH, thyroid-stimulating hormone; FSH, follicular stimulating hormone; LH, luteinizing hormone; T, testosterone; E, estradiol. To convert cortisol to SI units (nmol/l) multiply by 27.6. P values are for gender differences.

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**Table 2. Sex steroids during infusion studies**

<table>
<thead>
<tr>
<th>Groups</th>
<th>PI/Sal</th>
<th>KTCZ/Sal</th>
<th>KTCZ/CC</th>
<th>KTCZ/PC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone, ng/dl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men-PI</td>
<td>13.0 ± 2.6</td>
<td>7.1 ± 0.2</td>
<td>7.1 ± 0.2</td>
<td>6.9 ± 0</td>
</tr>
<tr>
<td>Men-T</td>
<td>783 ± 98</td>
<td>786 ± 92</td>
<td>722 ± 127</td>
<td>837 ± 72</td>
</tr>
<tr>
<td>Women-PI</td>
<td>12 ± 1.5</td>
<td>9.6 ± 1.3</td>
<td>8.1 ± 0.8</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>Women-E2</td>
<td>13 ± 1.7</td>
<td>10 ± 1.2</td>
<td>7.6 ± 0.3</td>
<td>11 ± 2.7</td>
</tr>
</tbody>
</table>

| **Estradiol, pg/ml** |         |          |         |         |
| Men-PI | < 5     | < 5      | < 5     | < 5     |
| Men-T  | 42 ± 6.8 | 35 ± 5.7 | 26 ± 3.5 | 32 ± 2.4 |
| Women-PI | 9.8 ± 0.1 | 9.9 ± 0.01 | 9.8 ± 0.1 | 9.8 ± 0.1 |
| Women-E2 | 92 ± 12 | 105 ± 10 | 98 ± 9   | 92 ± 8   |

Data are means ± SE measured by mass spectrometry. KTCZ, ketocortisol; CC, continuous cortisone; PC, pulsatile cortisone. As marked in the PI/Sal column, different (unshared) alphabetic superscripts denote significant row differences (P < 0.01). All patients received leuprolide with either placebo (PI) or sex-steroid (T or E2) addback. Multiply T by 0.0347 and E2 by 3.67 for nmol/l and pmol/l units.
lower in women (with or without E2 addback) than in men (with or without T addback).

**Impact of Gender and Sex Steroids**

**Mean ACTH concentration.** The 10-min patterns of ACTH increase (means ± SE) are depicted separately for men and women in all four interventions in Fig. 2.

Gender and sex-steroid addback did not influence control placebo-saline ACTH concentrations (see Fig. 2, top left plots in men and women).

Based on ANCOVA ($P < 10^{-5}$ overall), gender ($P < 0.001$), sex steroids ($P = 0.02$), and cortisol addback ($P < 0.001$) all determined mean ACTH concentrations along with two interaction terms (gender-infusion: $P = 0.005$, and sex-steroid-infusion: $P = 0.0016$; Fig. 3). Post hoc analyses showed that women, compared with men, had lower mean ACTH concentrations in the KTCZ/CC arm ($17 ± 2.3$ vs. $29 ± 2.1$ ng/l: $P = 0.001$) and the KTCZ/PC arm ($18 ± 2.1$ vs. $29 ± 2.7$ ng/l: $P = 0.006$). This occurred at comparable total cortisol (Table 3) and despite lower free cortisol concentrations, suggesting that women have a greater cortisol-mediated feedback inhibition than men. Further analyses revealed that E2 addback, compared with placebo addback, in women lowered mean ACTH concentration in the KTCZ/PC arms ($12 ± 3$ vs. $22 ± 2.5$ ng/l: $P = 0.046$). E2 addback did not influence mean ACTH concentration in the placebo-saline (intact axis) or KTCZ-saline (open-loop axis) arms ($P = 0.084$ trend KTCZ/CC). In men, there was no effect of T addback on mean ACTH concentration in any of the four treatment arms (Figs. 2 and 3). Gender by sex-steroid interactions were due to a lower mean ACTH concentration in the E2 addback group in women than in both the T and placebo addback groups of men, specifically under KTCZ/CC (12 ± 2.9 ng/l in women vs. 27 ± 3 and 30 ± 3.5 ng/l: $P = 0.001$ and <0.001, respectively in men), and under KTCZ/PC (12 ± 3 ng/l in women vs. 30 ± 4.3 and 29 ± 3.7 ng/l: $P = 0.001$ for both in men). There were no gender differences in mean ACTH concentrations in the hypogonadal sex steroid-unreplaced leuprolide treatment plus placebo addback groups.

**Deconvolution estimates of ACTH secretion.** The mechanisms mediating the ACTH-concentration effects of gender, sex steroid, and cortisol treatment were appraised next (Fig. 4). Compared with men, women had a significantly lower ACTH secretory-burst mass in the KTCZ/CC and KTCZ/PC arms (24 ± 4 vs. 35 ± 2.3 ng/l: $P = 0.009$, and 24 ± 3.5 vs. 39 ± 3.5 ng/l: $P = 0.002$, respectively). Lower ACTH burst mass resulted in lower pulsatile ACTH secretion in the KTC/CC and KTC/PC arms in women than men (167 ± 30 vs. 271 ± 20 ng/l·h$^{−1}$·8 h$^{−1}$: $P = 0.01$ and 160 ± 24 vs. 294 ± 30 ng/l·h$^{−1}$·8 h$^{−1}$: $P = 0.001$, respectively). More particularly, women who received E2 addback, compared with women receiving placebo, had 1) markedly lower ACTH burst mass in the KTCZ/CC (89 ± 33 vs. 223 ± 40.6 ng/l: $P = 0.01$) and KTCZ/PC arms (13 ± 4.7 vs. 31 ± 4.2 ng/ml: $P = 0.002$); 2) somewhat decreased basal ACTH secretion in the KTCZ/CC

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**Table 3. Total cortisol concentrations during the four infusion schedules separately in men and women**

<table>
<thead>
<tr>
<th></th>
<th>Placebo/Saline</th>
<th>KTCZ/Saline</th>
<th>KTCZ/CC</th>
<th>KTCZ/PC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Men</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No testosterone ($n = 12$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cortisol, µg/dl</td>
<td>10.6 ± 0.5</td>
<td>7.4 ± 0.5</td>
<td>10.1 ± 0.3</td>
<td>9.3 ± 0.5</td>
</tr>
<tr>
<td>Median, range</td>
<td>9.8 (8.8–4.6)</td>
<td>7.4 (3–10.1)</td>
<td>10.1 (8.4–11.8)</td>
<td>9.1 (5.5–11.9)</td>
</tr>
<tr>
<td>+Testosterone ($n = 12$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cortisol, µg/dl</td>
<td>10.1 ± 0.3</td>
<td>8.3 ± 0.3</td>
<td>10 ± 0.4</td>
<td>9.8 ± 0.4</td>
</tr>
<tr>
<td>Median, range</td>
<td>10.2 (8.3–11.5)</td>
<td>8.6 (6.6–9.8)</td>
<td>10.5 (7.5–12.3)</td>
<td>9.7 (7.5–12.1)</td>
</tr>
<tr>
<td><strong>Women</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Estrogen ($n = 14$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cortisol, µg/dl</td>
<td>9.8 ± 0.3</td>
<td>7.5 ± 0.5</td>
<td>10.3 ± 0.3</td>
<td>9.8 ± 0.4</td>
</tr>
<tr>
<td>Median, range</td>
<td>9.5 (8.7–12.4)</td>
<td>7.3 (4.6–11.1)</td>
<td>10 (8.3–12.8)</td>
<td>9.5 (7.5–13.4)</td>
</tr>
<tr>
<td>+Estrogen ($n = 10$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cortisol, µg/dl</td>
<td>10.5 ± 0.5</td>
<td>8.7 ± 0.5</td>
<td>13 ± 0.7</td>
<td>12 ± 0.6</td>
</tr>
<tr>
<td>Median, range</td>
<td>10.5 (8.7–13.2)</td>
<td>8.9 (6.6–11.5)</td>
<td>13 (9.3–16.2)</td>
<td>11.3 (9.7–15.2)</td>
</tr>
</tbody>
</table>

Data are means ± SE or median (range); $n$, number of subjects. Multiply by 27.6 for nmol/l units.

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**Table 4. CBG is higher and free cortisol is lower in women than men**

<table>
<thead>
<tr>
<th></th>
<th>CBG, µg/ml</th>
<th>Albumin, g/dl</th>
<th>Cortisol, µg/dl</th>
<th>Free Cortisol, µg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Men</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>24 ± 0.70†</td>
<td>4.4 ± 0.083</td>
<td>10.6 ± 0.53</td>
<td>0.73 ± 0.11†</td>
</tr>
<tr>
<td>Testosterone</td>
<td>23 ± 1.2†</td>
<td>4.3 ± 0.082</td>
<td>10.1 ± 0.32</td>
<td>0.72 ± 0.089†</td>
</tr>
<tr>
<td><strong>Women</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>29 ± 0.95‡</td>
<td>4.4 ± 0.080</td>
<td>9.8 ± 0.34</td>
<td>0.50 ± 0.051‡</td>
</tr>
<tr>
<td>Estradiol</td>
<td>28 ± 1.1‡</td>
<td>4.4 ± 0.091</td>
<td>10.5 ± 0.5</td>
<td>0.58 ± 0.084‡</td>
</tr>
<tr>
<td>$P$ value (ANOVA)*</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Date are means ± SE. *Comparisons are made among means within columns, wherein different superscripts identify different means at $P < 0.05$ by Tukey’s test. NS, $P > 0.05$. Data are from the placebo/saline control session. Assumed $K$, for cortisol-cortisol binding globulin (CBG) was $5.23 	imes 10^5$ l/mol and $2.7 	imes 10^5$ l/mol for albumin.
arm (313 ± 43 vs. 517 ± 53 ng·l⁻¹·h⁻¹; P = 0.02); and 3) reduced basal and pulsatile ACTH secretory rates in the KTCZ/PC arm (basal: 240 ± 57 vs. 407 ± 54 ng·l⁻¹·h⁻¹; P = 0.015; and pulsatile: 92 ± 35 vs. 208 ± 25 ng·l⁻¹·h⁻¹; P = 0.008, respectively). Men, given T versus placebo, had similar ACTH pulse number, burst mass, and total secretion when matched by feedback intervention.

**ACTH ApEn.** ApEn, a regularity metric, was calculated as a model-free (probabilistic) measure of feedback changes (38). ACTH ApEn was significantly reduced in the KTCZ-saline and the KTCZ/PC groups (all 48 subjects) compared with placebo-saline (0.84 ± 0.02 and 0.80 ± 0.03 vs. 0.95 ± 0.03; P = 0.017, respectively). ApEn in the KTCZ/PC arm was intermediate. Men had an overall higher ACTH ApEn than women, independently of infusion arm (0.97 ± 0.02 vs. 0.84 ± 0.02: P = 0.01). Post hoc analysis did not identify any interaction between gender and infusion type (P = 0.86).

**Regression Analysis**

The effects of sex steroids in modulating HPA axis feedback were explored first by univariate linear regression analysis. Mean ACTH concentrations, deconvolved ACTH secretion rates, and ACTH ApEn served as dependent variables in the regression analysis. Men had higher ACTH ApEn than women, independently of infusion arm (0.97 ± 0.02 vs. 0.84 ± 0.02: P = 0.01). Post hoc analysis did not identify any interaction between gender and infusion type (P = 0.86).
combined group (men and women, n = 48), while E2 or T concentrations were the independent variable. E2 and T were obtained at the start of each inpatient study visit. Because of multiple tests, protected P < 0.01 was construed as significant.

In the combined KTCZ/CC arm, E2 concentrations univariately negatively predicted mean ACTH concentration (R = −0.41, P = 0.004), ACTH burst mass (R = −0.43, P = 0.003), and pulsatile ACTH secretion (R = −0.42, P = 0.004). In the KTCZ/PC arm, E2 concentration negatively forecast mean ACTH concentration (R = −0.42; P = 0.002), mean ACTH burst mass (R = −0.45; P = 0.001), and pulsatile ACTH secretion (R = −0.43; P = 0.002); Table 5. Mean ACTH concentrations also correlated negatively with E2 levels during both continuous (P < 0.01) and pulsatile (P < 0.01) cortisol feedback, albeit when assessed in women (n = 24) only. ACTH ApEn varied inversely with E2 levels in the overall groups (n = 48) under placebo-saline (R = −0.37, P = 0.010) and KTCZ/CC setting (R = −0.41, P < 0.004). There was a trend-level effect of E2 on ApEn under KTCZ-saline (P = 0.08) or KTCZ/PC (P = 0.10). No univariate T effects were seen whether regression analysis was applied to all 48 subjects or was limited to men (n = 24).

By stepwise linear regression in the combined male/female groups (n = 48), T was a significant positive joint correlate with E2 of ACTH concentration (P = 0.008, R = +0.37), pulsatile ACTH secretion (P = 0.005, R = +0.39), and ACTH secretory-burst mass (P = 0.06, R = +0.37) during pulsatile cortisol delivery. The corresponding joint contributions of E2 concentrations were P = 0.006, R = −0.39 (ACTH concentration), P = 0.002, R = −0.44 (pulsatile ACTH secretion), and P = 0.001, R = −0.46 (ACTH secretory-burst mass); Table 6. Thus T and E2 had opposite associations with pulsatile and mean ACTH release under pulsatile cortisol addback. This was not evident under continuous cortisol addback.

Table 5. Univariate correlations of ACTH parameters with either E2 or T in men and women combined

<table>
<thead>
<tr>
<th>Condition</th>
<th>Independent Variable</th>
<th>R Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KTCZ/CC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTH conc</td>
<td>E2</td>
<td>−0.41</td>
<td>0.004</td>
</tr>
<tr>
<td>Pulsatile ACTH conc</td>
<td>E2</td>
<td>−0.42</td>
<td>0.004</td>
</tr>
<tr>
<td>ACTH burst mass</td>
<td>E2</td>
<td>−0.43</td>
<td>0.003</td>
</tr>
<tr>
<td>ACTH ApEn</td>
<td>E2</td>
<td>−0.41</td>
<td>0.004</td>
</tr>
<tr>
<td>KTCZ/PC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ACTH conc</td>
<td>E2</td>
<td>−0.42</td>
<td>0.003</td>
</tr>
<tr>
<td>Pulsatile ACTH conc</td>
<td>E2</td>
<td>−0.43</td>
<td>0.002</td>
</tr>
<tr>
<td>ACTH burst mass</td>
<td>E2</td>
<td>−0.45</td>
<td>0.001</td>
</tr>
<tr>
<td>ACTH ApEn</td>
<td>E2</td>
<td>−0.24</td>
<td>0.10</td>
</tr>
</tbody>
</table>

n = 48 (24 men, 24 women) subjects. There were no significant (P > 0.01) univariate correlations with T. CC denotes continuous and PC pulsatile cortisol addback under KTCZ.
Table 6. Multivariate correlations of ACTH parameters with both E2 and T

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>R Value</th>
<th>P Value</th>
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<tr>
<td>KTCZ/CC</td>
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</tr>
<tr>
<td>ACTH concentration</td>
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<td>-0.08</td>
</tr>
<tr>
<td></td>
<td>E2</td>
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</tr>
<tr>
<td>Pulsatile ACTH secretion</td>
<td>T</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>-0.45</td>
</tr>
<tr>
<td>ACTH burst mass</td>
<td>T</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>-0.45</td>
</tr>
<tr>
<td>ACTH ApEn</td>
<td>T</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>-0.51</td>
</tr>
<tr>
<td>KTCZ/PC</td>
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<td></td>
</tr>
<tr>
<td>ACTH concentration</td>
<td>T</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>E2</td>
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<tr>
<td>Pulsatile ACTH secretion</td>
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<tr>
<td></td>
<td>E2</td>
<td>-0.44</td>
</tr>
<tr>
<td>ACTH burst mass</td>
<td>T</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>-0.46</td>
</tr>
<tr>
<td>ACTH ApEn</td>
<td>T</td>
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</tr>
<tr>
<td></td>
<td>E2</td>
<td>-0.05</td>
</tr>
</tbody>
</table>

n = 48 (24 men, 24 women) subjects. ApEn, approximate entropy.

Impact of Feedback Type (Continuous vs. Pulsatile Cortisol) on ACTH Measures

In the absence of regression on sex steroids, mean ACTH concentration, pulsatile ACTH secretion, and ACTH ApEn did not differ between KTCZ/CC and KTCZ/PC. However, pulsatile cortisol infusion was more effective than continuous cortisol infusion in suppressing pulsatile ACTH secretion as a function of E2 levels in women: viz., KTCZ/PC arm (R = -0.55, P = 0.006) vs. KTCZ/CC arm (R = -0.39, P = 0.059). This was due to greater suppression of ACTH secretory-burst mass by pulsatile cortisol feedback under higher E2 levels in women.

DISCUSSION

Experiments in animals have identified reproducible effects of gender and/or sex steroids on the regulation of the hypothalamic-corticotrope-adrenal axis (4, 5, 37, 41, 42). On the other hand, clinical investigations have been sparse and conflicting (6, 11, 31, 32). Inconsistencies may reflect interstudy differences in age, type of stressor, sampling paradigm, and dose, type, and pattern of glucocorticoid administration. Type of glucocorticoid may be relevant, given protein-binding, kinetic, and brain-uptake differences between synthetic and natural glucocorticoids (7, 12, 22). Pattern may also be important since no clinical investigations of negative feedback have yet attempted to emulate a physiological train of cortisol pulses (34). Whatever the bases for existing discrepancies, earlier clinical assessments suggested increased feedback sensitivity in women (10), no gender difference (8), and decreased feedback sensitivity in women (23, 40). By including both pulsatile and continuous intravenous cortisol feedback under both low and high systemic E2 (in women) and T (in men) concentrations, the present analyses demonstrate strong inhibitory effects of E2 but not T on ACTH concentrations, pulsatile ACTH secretion, and ACTH ApEn (secretory-pattern orderliness) in healthy older adults. Since total cortisol concentrations under the intravenous cortisol clamps were comparable in the men and women, and calculated free cortisol levels were actually lower in women, a straightforward hypothesis is that E2 may mediate enhanced (free) cortisol feedback on ACTH secretion in women. Conversely, in the combined groups (men and women, n = 48) during pulsatile cortisol addback, T concentration was a positive correlate along with E2 as a negative correlate of ACTH concentration and pulsatile ACTH secretion. This would suggest a stimulatory effect of T or its metabolites especially during pulsatile cortisol feedback, distinct from the inhibitory effect of E2 under both pulsatile and continuous feedback.

In the present investigation, cortisol-mediated ACTH suppression was greatest in women who received E2 addback compared with women without E2 addback, as well compared with men with or without T addback. The inferably suppressive effect of E2 versus placebo on ACTH secretion was confirmed by E2 concentration-dependent inhibition of ACTH secretion both in the combined cohort (48 adults) and in the women’s cohort alone (n = 24). The measures of heightened cortisol feedback included ACTH secretory-burst mass, pulsatile, basal, and total ACTH secretion, as well as ACTH ApEn. The importance of these end points of cortisol feedback is that ACTH secretory-burst mass and pulsatile ACTH secretion denote amplitude control (35); basal ACTH secretion largely signifies constitutive ACTH release (35); and ApEn quantifies ACTH secretory regularity, a validated barometer of feedback adaptations (38). Thus E2 seems to potentiate cortisol feedback or reduce forward ACTH drive. The experimental model used here does not discriminate between these two principal possibilities.

Greater ACTH suppression by cortisol in women given E2 differs diametrically from most data obtained in animal studies. The mechanisms remain unknown in humans. However, E2 may increase 11-β-hydroxysteroid dehydrogenase type 1, leading to conversion of cortisone to cortisol, which in turn inhibits ACTH through negative feedback (20). Estrogen administration in ovariectomized rats mutes glucocorticoid negative feedback, resulting in higher ACTH levels, whether corticosterone or a synthetic glucocorticoid is administered by single bolus injection to test fast feedback via nongenomic mechanisms (25) or infused continuously to test slow feedback via nuclear glucocorticoid receptors (4). In animals both neonatal and adult sex-steroid exposure can alter sexual dimorphism of ACTH regulation (24). Mechanistically, E2 and propylpyrazololetriol, a selective estrogen receptor α-agonist, both impair dexamethasone’s suppression of c-fos activation in the paraventricular nucleus (39).

In other clinical studies, obese premenopausal women exhibited greater percentage inhibition of ACTH and cortisol concentrations compared with obese men during low-dose (1 mg) dexamethasone suppression (23). In two reports, E2 add-back in postmenopausal women, compared with no addback, blunted the cortisol response to a combined dexamethasone suppression/CRH stimulation test, implying greater ACTH suppression (14, 16). Dexamethasone suppressed cortisol levels in premenopausal women more during the early follicular (low E2) than the midluteal (high E2, high progesterone) phase of the menstrual cycle (1). The present investigation extends these outcomes by showing that E2 potentiates both continuous and pulsatile intravenous cortisol-feedback effects on specific ACTH outflow mechanisms, viz., pulsatile, basal, and entropic modes of ACTH secretion. Conversely, when considered to-
together with E2 levels, T concentrations in men and women are positive correlates of both mean and pulsatile ACTH secretion, at least during pulsatile cortisol inhibition.

Additional investigation will be required to elucidate the extent to which observed sex differences in cortisol feedback modulate an individual’s response to classical stressors (24). In one study, postmenopausal women under low E2 exhibited less ACTH suppression by a feedback signal than estrogen-sufficient premenopausal women and manifested higher acute HPA stress responses (3). Nonetheless, the degrees to which experimental cortisol feedback-associated sex differences in unstimulated humans apply to pathophysiological states (26) and/or to classical stress responses, cardiovascular diseases, diabetes, and hypertension (33) remain to be clarified.

Caveats include the relatively limited size of the study sample (48 adults in total) and a limited blood-sampling duration (8 h). The latter restricts piecewise assessment of ACTH responses. Thus the present inferences require confirmation in larger cohorts sampled over 24 h. Additionally, the conclusions of this study apply to healthy older adults and not necessarily to children, adolescents, premenopausal women, chronically stressed individuals, or ill patients. Other limitations include: 1) possible extra-adrenal effects of KTCZ; however, exogenous cortisol normalized ACTH concentrations otherwise stimulated by KTCZ administration; 2) possible diurnal or seasonal effects (26, 40); 3) unknown effects of CBG per se on feedback by cortisol; and 4) uncertainty regarding long-term gonadal-steroid effects on HPA axis regulation.

In conclusion, estradiol strongly heightens feedback suppression of the hypothalamic-pituitary-corticotrope unit by systemically clamped cortisol, when inhibition is quantified by mean ACTH concentrations, pulsatile ACTH secretion, and ACTH ApEn. In contrast, inerfare T effects are positive when covaired with E2 effects under the same conditions. Pulsatile cortisol infusion was more inhibitory of pulsatile ACTH secretion than continuous cortisol infusion, specifically as a function of E2 concentrations in postmenopausal women. Further studies will be needed in selected pathophysioologies to further generalize sex differences in cortisol feedback.

Perspectives and Significance

The present combined models of sex-steroid and cortisol feedback clamp unmask consistently negative effects of E2 in older humans. E2 modulates mean, pulsatile, basal, and enotropic measures of ACTH secretion under both continuous and pulsatile cortisol feedback. When considered together with E2 under pulsatile cortisol feedback, T opposed several of the main negative effects of E2. Two major new questions are raised by these data. Specifically, how do sex steroids modulate the mechanisms by which 1) continuous cortisol delivery especially inhibits basal ACTH secretion and ACTH ApEn, and 2) pulsatile cortisol feedback especially suppresses ACTH concentrations and pulsatile ACTH secretion. Recent investigations of pulsatile versus continuous glucocorticoid effects upon the expression of central-nervous-system genes (15) provides a possible experimental framework for studying these questions in animal models as well.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.N.S., P.A., J.R.W., and S.M.W. performed experiments; A.N.S. and J.D.V. analyzed data; A.N.S. and J.D.V. interpreted results of experiments; A.N.S. and J.D.V. prepared figures; A.N.S. and J.D.V. drafted manuscript; A.N.S., P.A., J.R.W., S.M.W., and J.D.V. approved final version of manuscript; J.D.V. conception and design of research; J.D.V. edited and revised manuscript.

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