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Adrenocorticotropic hormone elicits gonadotropin secretion in premenopausal women

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STUDY QUESTION: Does adrenocorticotropic hormone (ACTH) induce gonadotropin release in premenopausal women?

SUMMARY ANSWER: Administration of ACTH stimulates gonadotropin release, most likely by stimulation of the production of cortisol, in premenopausal women.

WHAT IS KNOWN ALREADY: In animal models, acute activation of the hypothalamic-pituitary-adrenal (HPA) axis has been shown to induce gonadotropin release in the presence of sufficiently high estrogen levels. However, it is unknown whether the HPA axis has a similar influence on gonadotropin release in humans.

STUDY DESIGN, SIZE, DURATION: This study had a mixed factorial design. A total of 60 healthy female participants participated in the experimental study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study sample comprised three distinct hormonal-based populations according to their levels of progesterone (PROG) and estradiol (E2): (i) low-PROG–low-E2, (ii) low-PROG–high-E2 and (iii) high-PROG–high-E2 women. A low dose (1 μg) of ACTH was administered to all study participants. Serum steroid and gonadotropin concentrations were measured prior to, and at 30 and 90 minutes after, intravenous ACTH administration.

MAIN RESULTS AND THE ROLE OF CHANCE: Mean serum cortisol levels increased significantly following ACTH administration in all groups ($P < 0.001$). Similarly, the serum levels of 17-OH-PROG, androstenedione, dehydroepiandrosterone and testosterone increased significantly in all groups ($P < 0.01$). The low-PROG–high-E2 and high-PROG–high-E2 groups exhibited a significant increase in LH and FSH levels ($P < 0.001$), whereas the low-PROG–low-E2 group demonstrated blunted LH and FSH responses to ACTH administration ($P < 0.05$).

LIMITATIONS, REASONS FOR CAUTION: Testing was performed during the luteal phase of the natural menstrual cycle. Testing during the follicular phase might have elicited premature, or more pronounced, LH surges in response to ACTH administration.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings suggest a novel mechanism by which the adrenal cortex functions as a mediator of gonadotropin release. These findings contribute to a greater understanding of the influence of acute stress on reproductive endocrinology.

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Introduction

Psychosocial stress is a highly significant factor predicting health outcomes and quality of life (Sapolsky, 2005). The best-studied physiological response to stress is mediated by the hypothalamic-pituitary-adrenal (HPA) axis, which can be affected by the hypothalamic-pituitary-gonadal axis, regulating metabolism and reproductive function, respectively (Viau, 2002; Handa and Weiser, 2014). Previous studies have also demonstrated that chronic persistent stress interferes with the release of hypothalamic gonadotropin-releasing hormone (GnRH), resulting in a suppression of gonadotropin levels (Brann and Mahesh, 1991; Whirledge and Cidlowski, 2013). Studies in animal models have elucidated candidate physiological mechanisms underlying the well-replicated finding of stress-induced reproductive suppression in humans (Riviera and Rivest, 1991; Tilbrook *et al.*, 2000; Wingfield and Sapolsky, 2003). The female reproductive system is powerfully modulated by stress, often leading to chronic anovulation and amenorrhea during periods of persistent stress (Warren and Perlroth, 2001). In adolescents, chronic stress has been shown to significantly delay the onset of puberty (Magner *et al.*, 1984).

Contrary to the effects of persistent stress, acute stress has been repeatedly shown to facilitate reproductive functioning by stimulating gonadotropin secretion (Brann and Mahesh, 1991; Brann *et al.*, 1991). Animal studies have yielded a candidate hormonal mechanism through which acute stressors facilitate the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Putnam *et al.*, 1991). Notably, the effect of acute stress on gonadotropin release is highly dependent upon the circulating level of estradiol (Putnam *et al.*, 1991; Puder *et al.*, 2000; Micevych *et al.*, 2008). Moreover, adrenalectomy, but not ovariectomy, abolishes the facilitation of gonadotropin release by acute stress in rodents (Mahesh and Brann, 1998; Puder *et al.*, 2000). Finally, adrenal PROG has been implicated as an important mediator of the stimulatory effect of stress on gonadotropins in the presence of an estrogen-primed environment (Putnam *et al.*, 1991; Xiao *et al.*, 1994). Taken together, widely convergent evidence in animal studies has given considerable support to the hypothesis that the facilitation of gonadotropin release by acute stress is mediated through adrenal steroids.

To date, studies regarding the effects of HPA-axis stimulation on LH release in humans have concluded that in postmenopausal women, the LH response to adrenal stimulation is highly estrogen-dependent (Puder *et al.*, 2000), and significantly potentiated by PROG (Cano and Tarín, 1998). However, it remains unknown whether gonadotropin release is facilitated by adrenal stimulation in premenopausal women and the extent to which this may be governed by ovarian function.

Therefore, the aim of this study was to determine the influence of acute adrenocortical stimulation by administration of a low dose of ACTH on the release of LH and FSH in women with a normal menstrual cycle. In addition, we sought to explore the modulatory effect of estrogen and progestin on adrenal facilitation of gonadotropin release by administering a low dose of ACTH in three distinct healthy populations: (i) women having a natural menstrual cycle, (ii) women taking oral contraceptive pills (a combination of estrogen/progestin) and (iii) women using a progestin-releasing intrauterine device (IUD). Combination estrogen/progestin contraceptives have previously been shown to inhibit ovarian function (Amy and Tripathi, 2009; Benagiano *et al.*, 2009). In comparison, the progestin-releasing IUD has been suggested to, only partly and only during the first year, inhibit ovarian function, leaving circulating estradiol within the normal range for women of reproductive age (Barbosa *et al.*, 1990; Lähteenmäki *et al.*, 2000). Considering that levonorgestrel-releasing IUD (LNG-IUD) does not generally excrete sufficient amounts of progesterone to suppress the hypothalamic ovarian axis (Apter *et al.*, 2014), and given the possibility of a difference in gonadotropin release between young premenopausal women in the preovulatory versus postovulatory phase, we reclassified the groups based on PROG level and ovulatory phase. This study design provided us with the opportunity to compare the effects of acute stimulation of the adrenal cortex on gonadotropin release under conditions of intact ovarian function at different cycle phases, as well as in a setting of complete ovarian suppression. In addition, given previous studies reporting an association of hormonal contraceptives with emotional lability, anxiety and depression (Sanders *et al.*, 2001; Oinonen and Mazmanian, 2002), we performed structured assessments of the psychological affective state of our study participants in order to evaluate potential confounding effects.

Subjects and Methods

Subjects

An a priori power analysis was performed at 80% power with a significance threshold of 0.05 in order to determine the cohort sample size. The power analysis indicated that a total sample size of 60 would provide confidence to detect differences of at least medium effect size between conditions. A total of 60 healthy women of reproductive age participated in this study (mean 22.83, SD 3.12, range 18–30 years). Participants were recruited through local advertisements and provided with monetary compensation (€50) for their participation. Hormonal contraceptive use was determined based on a structured questionnaire during the initial telephone screening and reconfirmed on the day of testing. Women were considered eligible for the study only if they met one of the following inclusion criteria for continuous hormonal contraceptive use for at least the previous 4 months: (i) oral monophasic combined preparation containing ethinylestradiol (EE) 0.03 and 0.15 mg levonorgestrel (Ethinylestradiol/levonorgestrel, Microgynon® 30 [EE30/LNG group; $N = 20$]), (ii) progestin-only LNG-releasing IUD 0.02 mg/24 hours (Mirena®; Bayer [LNG-IUD group; $N = 20$]) or (iii) absence of any hormonal contraceptives and having a regular menstrual cycle length between 23 and 35 days (naturally cycling [NC] group; $N = 20$). The duration of LNG-IUD use ranged from 16 to 28 months. All participants had a normal menstrual cycle length between 26 and 29 days. Exclusion criteria were a history of clinically significant psychiatric, neurologic, endocrine or medical illness (including alcohol or drug dependence, asthma, allergies, cardiovascular disease, endometriosis, polycystic ovary disease or gynecologic infection), body mass index (BMI) < 19 or >26 kg/m^2, atypical sleep pattern, the use of any prescription medication other than hormonal contraceptives within the previous 4 months and pregnancy or lactation within the previous 12 months. Women in the EE30/LNG group were tested during the active pill weeks. NC women were tested in the luteal phase of the menstrual cycle, between days 20 and 27 of their cycle.

The study was conducted according to the declaration of Helsinki and was approved by the Medical Ethical Research Committee of the Erasmus MC, University Medical Center Rotterdam. All subjects provided written informed consent for their participation.

Psychological assessment

To examine the possibility that responses to ACTH administration could be confounded by differences in affect regulation between the contraceptive groups, all participants completed the Positive and Negative Affect Scale (PANAS), a well-validated questionnaire for measuring general, positive and negative affective states (Watson et al., 1988). Each of the 20 items is rated on a five-point Likert scale ranging from 1 (very slightly or not at all) to 5 (extremely). The PANAS has been established to have high reliability (positive affect scale: Cornbach's $\alpha = 0.89$, negative affect scale: $\alpha = 0.85$) (Watson et al., 1988).

ACTH administration

Participants abstained from smoking, alcohol, caffeinated beverages and physical exercise on the day of testing. There were no other dietary restrictions. Testing was conducted between 13.00 and 16.00 hours. The testing procedure began with a general medical examination to reconfirm the subject's physical and mental health status. An intravenous catheter was inserted either into the antecubital or the medial cubital vein to obtain serial blood samples. The intravenous catheter was flushed with normal saline immediately after each blood sampling time point. Following an initial 30-min rest period, baseline venous blood samples were obtained for steroid and protein hormone assessments (cortisol, 17-hydroxyprogesterone [17-OH-PROG], PROG, testosterone, dehydroepiandrosterone [DHEA], androstenedione, dehydroepiandrosterone sulfate [DHEAS] and estradiol [E2]), globulin levels (corticosteroid binding globulin [CBG], sex hormone binding globulin [SHBG], LH and FSH). Immediately following withdrawal of the baseline venous blood samples, a 1 μg intravenous bolus of 1–24 ACTH (Synacthen®; Novartis, Basel, Switzerland) was administered. Additional blood samples were obtained at 30 and 90 minutes following ACTH administration. Subjects were asked to sit quietly in a semi-recumbent position throughout the entire procedure. No adverse events were reported.

Sample collection

Blood samples were collected using Vacutainer® tubes, immediately placed on ice upon collection and centrifuged at 4 °C for 10 minutes at 3000*g* within 1 hour of collection. The resulting serum was aliquoted prior to storage at −80 °C.

Hormone determinations

With the exception of estradiol, steroid hormones were measured using the liquid chromatography-tandem mass spectrometry method with the CHS™ MSMS Steroids Kit (Perkin Elmer, Turku, Finland). The Steroids Kit uses a combined solvent extraction and protein precipitation method with acetonitrile containing the deuterated internal standards $^2H_5$-androstenedione, $^2H_3$-cortisol , $^2H_8$–17-OH-PROG, $^2H_6$-DHEA, $^2H_9$-PROG and $^2H_5$-testosterone. The internal standards undergo processing identical to the analytes. Chromatographic separation was performed on a Waters® (Milford, MA, USA) Acquity™ UPLC HSS T3 1.8 μm column (diameter 1 mm, length 10 cm) with acetonitrile/MeOH gradient and in-line filters with 0.2 μm frits. A Waters® XEVO-TQ-S system equipped with an electrospray ionization (ESI) source operating in the electrospray positive mode was used, except for DHEAS (negative ESI). Multiple reaction monitoring was applied for the detection of the analytes using both quantifiers and qualifiers.

The lower limits of quantification for androstenedione, cortisol, DHEA, DHEAS, PROG, 17-OH-PROG and testosterone were 0.20, 2.57, 2.2, 24.7, 0.13, 0.10 and 0.07 nmol/l, respectively. During the LC-step of the steroid assay, PROG and 17-OH-PROG were completely separated, thereby removing the possibility of cross-reactivity in this assay. Estradiol was measured by the Coat-A-Count radioimmunoassay of Siemens Healthcare Diagnostics Products (Los Angeles, CA, USA). Intra- and inter-assay coefficients of variation for the steroid assays were <7.0 and <8.0% for androstenedione, <6 and <6% for cortisol, <7 and <8% for DHEA, <8 and <13% for DHEAS, <6 and <7% for PROG, <6 and <6% for 17-OH-PROG, <6 and <9% for testosterone and <5 and <7% for estradiol. LH, FSH and SHBG concentrations were measured using the Siemens Immulite XPi system. Serum CBG concentrations were determined by radioimmunoassay (DRG Instruments GmbH, Marburg, Germany). Intra- and inter-assay coefficients of variation were <4 and <7% for LH, <3 and 6% for FSH, <4 and <5% for SHBG and <9 and <11% for CBG.

Data analysis

Given the influence of menstrual phase (preovulatory versus postovulatory) on gonadotropin release, participants from the natural cycling and LNG-IUD groups were classified based on PROG level. Women with PROG concentrations above 5 nmol/l were classified in the high-PROG/high-E2 group ($n = 12$) and women with a lower PROG concentration in the low-PROG/high-E2 group ($n = 28$). Estradiol levels in these two groups were not different. Women using EE30/LNG were designated as low-PROG/low-E2 ($n = 20$).

Statistical analyses were conducted using the SPSS statistical software package (IBM SPSS Statistics, Version 21). Results are expressed as means ± SEM, unless otherwise specified. Data per parameter were tested for normality of distribution and homogeneity of variance using Kolmogorov–Smirnov and Levene's tests. In six patients, one of the hormone measurements was not possible to quantify due to interfering peaks in the chromatogram (PROG: $n = 1$; E2: $n = 1$; androstenedione: $n = 4$). To meet the normality assumption, where necessary, hormonal data were logarithmically transformed. After log-transformation, the data were normally distributed. In order to examine group differences in demographic characteristics and affect, chi-squared tests and one-way analyses of variance (ANOVAs) were conducted. To analyze hormone profiles in response to ACTH administration, ANOVAs were performed with a repeated-measure factor Time (baseline, +30 minutes, +90 minutes), between-subject factor Group (low-PROG–low-E2, low-PROG–high-E2, high-PROG–high-E2) and the interaction effect of Time x Group. Post hoc analyses, where necessary, were performed using Bonferroni multiple means comparisons. To reduce the possibility of a Type I error when analyzing steroid reactivity, statistical significance for these tests was defined at the more stringent threshold of $P < 0.01$. In order to check for potentially confounding effects of age, BMI and PANAS scores on the steroid and gonadotropin responses, these parameters were first evaluated separately in a set of ANOVAs for repeated measures. Age, BMI and PANAS scores did not yield significant main or interaction effects in relation to the steroid or gonadotropin responses. Therefore, these variables were not included as covariates in subsequent analyses.

Since EE influences levels of CBG, which binds cortisol with high affinity, CBG concentrations were included as covariates in analyses of cortisol concentrations. For general linear models, *F*-values, degrees of freedom and *P*-values were corrected by the Greenhouse-Geisser procedure whenever the assumption of sphericity was violated. Effect sizes were calculated by partial eta squared (η^2). *P*-values <0.01 were considered to be statistically significant.

Results

Subject characteristics, and baseline ACTH and binding globulin levels

The groups did not differ significantly in age or BMI (Table I). No significant differences were found in positive or negative affect scores on the PANAS, indicating comparable baseline affective states between the study groups (Table I). The study groups were also similar in their baseline ACTH levels (Table I). Importantly, however, the low-PROG–low-E2 group exhibited significantly higher baseline CBG ($P < 0.001$) and SHBG levels ($P < 0.001$), due to the stimulating effect of the synthetic estrogen in the oral contraceptive (Table I).

Effect of ACTH administration on gonadotropin release

ACTH administration resulted in significant time-dependent changes of LH and FSH levels in all groups (low-PROG–high-E2 and high-PROG–high-E2, $P < 0.001$; low-PROG–low-E2, $P < 0.05$). The groups differed significantly regarding LH levels, with the low-PROG–low-E2 group displaying overall lower LH concentrations ($P < 0.001$; post hoc: low-PROG–high-E2 = high-PROG–high-E2 > low-PROG–low-E2; Fig. 1a). No significant Group × Time interaction effect was observed. The FSH levels differed significantly between the study groups ($P < 0.001$). A significant Group x Time interaction was observed ($P < 0.05$; post hoc: low-PROG–high-E2 > high-PROG–high-E2 > low-PROG–low-E2; Fig. 1b); the EE30/LNG group displayed a blunted FSH response to ACTH administration ($P < 0.01$).

Effects of ACTH administration on the steroid profile

ACTH administration resulted in significant time-dependent changes in the levels of cortisol, 17-OH-PROG, PROG, testosterone, DHEA, DHEAS and androstenedione ($P < 0.001$ for each group × steroid combination), all displaying significant increases at 30 minutes after ACTH administration ($P < 0.01$ for each group × steroid combination). With regard to E2, a significant increase was observed 90 minutes after ACTH administration in the low-PROG–high-E2 and high-PROG–high-E2 groups ($P < 0.001$ for each group), but no change was found in the low-PROG–low-E2 group.

Cortisol

The study groups differed significantly with regard to total serum cortisol levels. Women using oral contraceptives (low-PROG–low-E2 group) exhibited significantly higher mean total cortisol levels, compared to the low-PROG–high-E2 and high-PROG–high-E2 groups ($P < 0.001$; Fig. 2a). However, after controlling for CBG levels, no significant group or interaction effect remained, confirming the influence of CBG on cortisol levels.

Progesterone

The study groups differed significantly regarding PROG levels, with the high-PROG–high-E2 group showing higher overall PROG than the low-PROG–high-E2 and low-PROG–low-E2 groups ($P < 0.001$). ACTH administration induced a significant increase in PROG in the low-PROG–high-E2 and low-PROG–low-E2 groups, but not in the high-PROG–high-E2 group ($P < 0.001$; Fig. 2b).

17-OH-PROG

17-OH-PROG levels differed significantly between the study groups at baseline, +30 and +90 minutes post-ACTH administration ($P < 0.001$; post hoc: high-PROG–high-E2 > low-PROG–high-E2 > low-PROG–low-E2). Furthermore, a significant Group × Time interaction effect was observed ($P < 0.001$), with the low-PROG–low-E2 group displaying relatively higher 17-OH-PROG increases at 30 minutes post-ACTH administration compared to the high-PROG–high-E2 and low-PROG–high-E2 groups (Table II).

Table I Subject characteristics, affect state and baseline globulin and ACTH levels of the experimental groups.

	low-PROG/ high-E2 ($n = 28$)	high-PROG/ high-E2 ($n = 12$)	low-PROG/ low-E2 ($n = 20$)
Age, mean (SD), years	23.04 (3.26)	23.42 (4.64)	22.2 (1.47)
BMI, mean (SD), kg/m^2	22.16 (2.11)	21.87 (1.30)	22.53 (2.89)
PANAS			
Positive affect scale, mean (SD), score	28.14 (5.82)	28.58 (3.87)	29.45 (6.89)
Negative affect scale, mean (SD), score	13.75 (3.23)	13.17 (2.73)	12.00 (2.15)
SHBG, mean (SD), µg/ml	25.76 (8.53)	26.41 (8.94)	50.94 (14.69)[a]
ACTH, mean (SD), µg/ml	3.40 (1.37)	2.03 (1.07)	3.07 (3.35)
CBG, mean (SD), µg/ml	52.91 (8.60)	57.08 (6.11)	120.99 (22.11)[a]

PANAS, positive affect and negative affect scale; CBG, corticosteroid binding globulin; SHGB, sex hormone binding globulin; ACTH, adrenocorticotropic hormone.
[a]One-way analysis of variance between three experimental groups, $P < 0.001$.

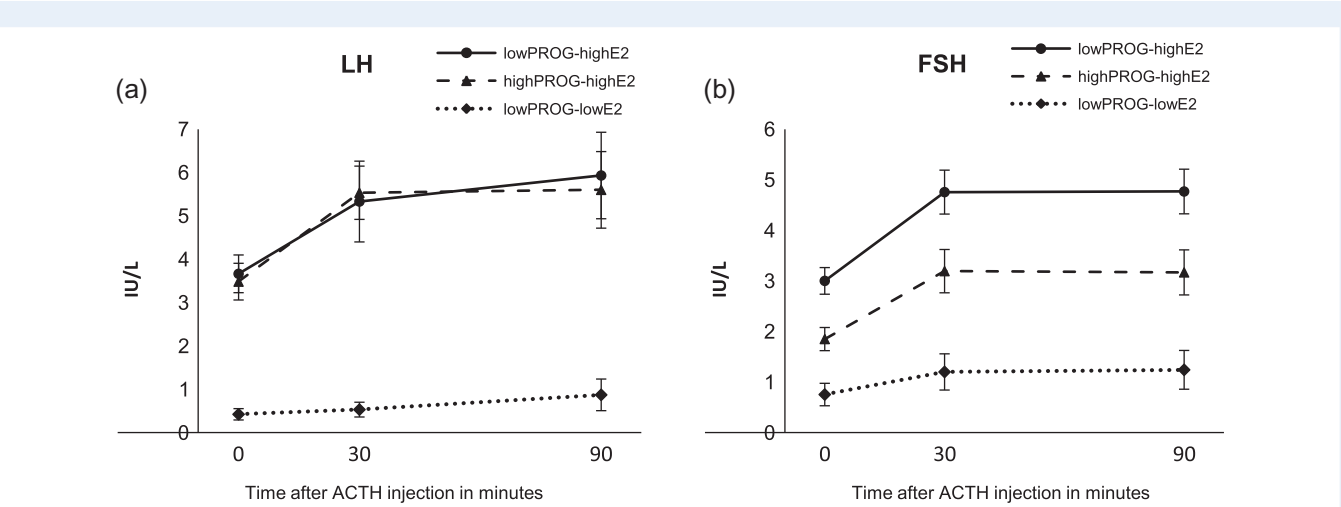

Figure 1 Untransformed raw LH (**a**) and FSH (**b**) mean ± SEM values at baseline, +30 and +90 minutes after administration of low-dose (1 μg) adrenocorticotropic hormone (ACTH). ACTH was administered in three experimental hormonal-based populations: low-PROG–low-E2, low-PROG–high-E2 and high-PROG–high-E2. ACTH administration resulted in significant time-dependent changes of LH and FSH levels in all groups (low-PROG–high-E2 and high-PROG–high-E2, $P < 0.001$; low-PROG–low-E2, $P < 0.05$). (a) The groups differed significantly regarding LH levels, with the low-PROG–low-E2 group displaying overall lower LH concentrations ($P < 0.001$). No significant interaction effects were found. (b) The FSH levels differed significantly between the study groups ($P < 0.001$, post hoc: low-PROG–high-E2 > high-PROG–high-E2 > low-PROG–low-E2). A significant Group × Time interaction was observed ($P < 0.02$); the low-PROG–low-E2 group displayed a blunted FSH response to ACTH administration ($P < 0.01$).

Androstenedione

Androstenedione levels differed significantly between the groups ($P < 0.001$; post hoc: high-PROG–high-E2 = low-PROG–high-E2 > low-PROG–low-E2), with the low-PROG–low-E2 group displaying overall lower androstenedione levels, compared to the high-PROG–high-E2 and low-PROG–high-E2 groups (Table II). No significant Group × Time interaction effect was observed.

Dehydroepiandrosterone

The study groups differed significantly in DHEA concentrations at baseline, and +30 and +90 minutes post-ACTH administration ($P < 0.001$; post hoc: high-PROG–high-E2 = low-PROG–high-E2 > low-PROG–low-E2; Table II). No significant Group × Time interaction effect was observed.

Dehydroepiandrosterone sulfate

DHEAS levels differed significantly between the study groups ($P < 0.01$; post hoc: high-PROG–high-E2 = low-PROG–high-E2 > low-PROG–low-E2), with the high-PROG–high-E2 and low-PROG–high-E2 groups displaying higher overall levels when compared to the low-PROG–low-E2 group (Table II). No significant Group × Time interaction effect was observed.

Testosterone

The low-PROG–low-E2 group exhibited overall lower testosterone levels, compared to the high-PROG–high-E2 and low-PROG–high-E2 groups ($P < 0.001$; Table II). A significant Group × Time interaction effect demonstrated a larger increase of testosterone levels following ACTH administration in the low-PROG–low-E2 group, compared to the high-PROG–high-E2 and low-PROG–high-E2 groups ($P < 0.01$).

Estradiol

E2 levels were significantly different between the study groups: the low-PROG–low-E2 group had lower E2 levels than the high-PROG–high-E2 and low-PROG–high-E2 groups ($P < 0.001$). No differences were observed in E2 levels between the high-PROG–high-E2 and low-PROG–high-E2 groups. ACTH administration induced a significant increase of E2 in the high-PROG–high-E2 and low-PROG–high-E2 groups, but not in low-PROG–low-E2 users ($P < 0.001$; post hoc: NC = LNG-IUD > EE30/LNG; Fig. 2c).

Discussion

The aim of our study was to examine the influence of acute adrenal cortex stimulation on gonadotropin release in three groups of premenopausal women distinguished by the different levels of PROG and estradiol: high-PROG–high-E2, low-PROG–high-E2 and low-PROG–low-E2. Basal hormone levels differed between groups on the basis of cycle phase (PROG and 17-OH-PROG in the high-PROG–high-E2 and low-PROG–high-E2 groups), and on the basis of suppression of LH and FSH in the female group using oral contraceptives, with low-PROG–low-E2 leading to suppression of the ovarian component of the production of androgens and estradiol.

Steroid-dependent regulation of gonadotropin release has been shown to involve a complex interaction with estrogen, as observed in studies of estrogen-replacement therapy in postmenopausal women, in which activation of the HPA axis resulted in gonadotropin release only in the presence of sufficient levels of circulating estrogen (Puder *et al.*, 2000). In our study, estrogens were present in all study groups: endogenous estradiol in the high-PROG–high-E2 and low-PROG–high-E2 groups, and EE in the low-PROG–low-E2 group. Further

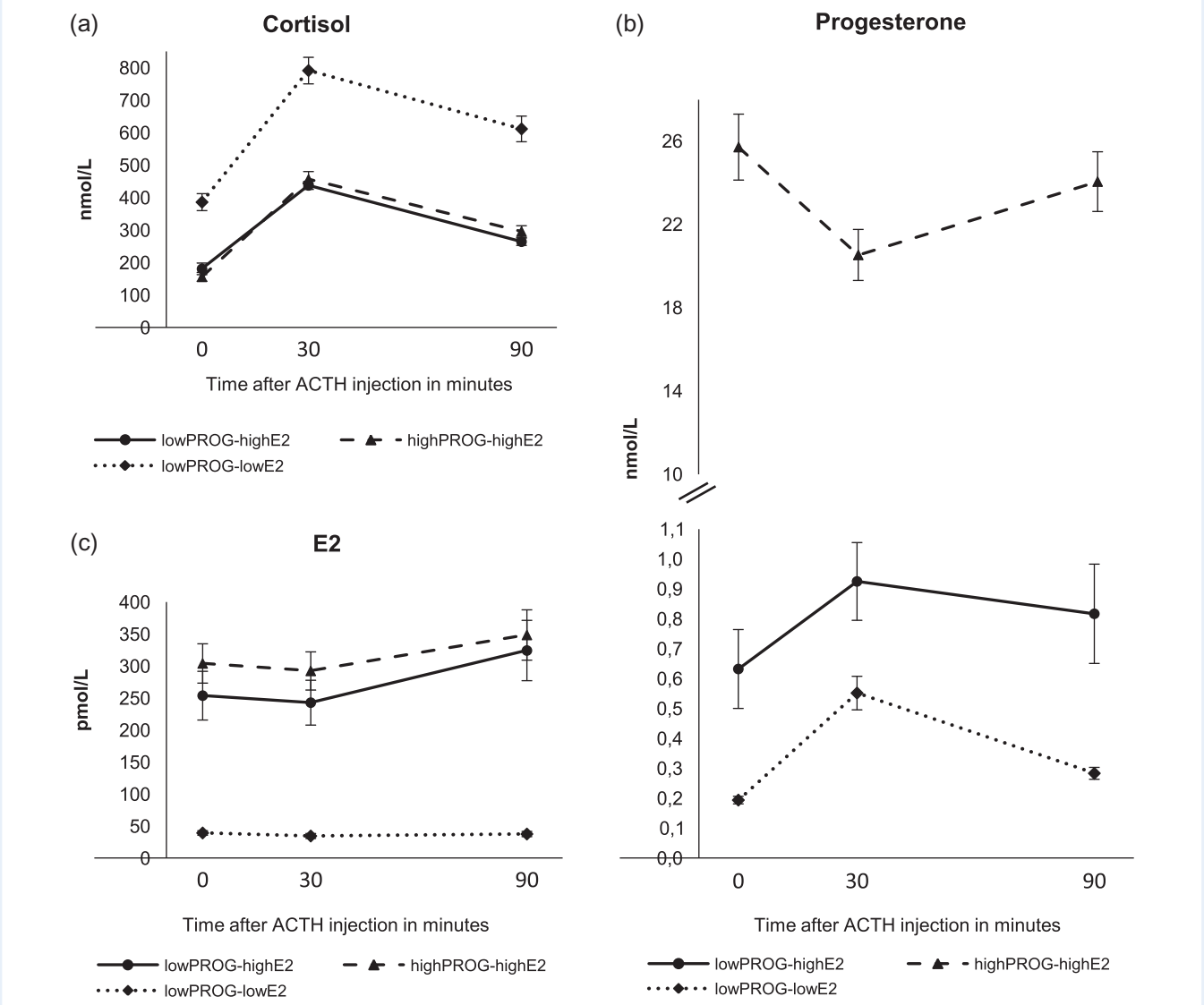

Figure 2 Untransformed raw cortisol (**a**), progesterone (**b**) and estradiol (**c**) mean ± SEM values at baseline, +30 and +90 minutes after administration of low-dose (1 μg) ACTH. ACTH was administered in three experimental hormonal-based populations: low-PROG–low-E2, low-PROG–high-E2 and high-PROG–high-E2. (a) The study groups differed significantly with regard to total serum cortisol levels. Women using EE30/LNG (low-PROG–low-E2) exhibited significantly higher mean total cortisol levels, compared to the low-PROG–high-E2 and high-PROG–high-E2 groups ($P < 0.001$). However, after controlling for CBG levels, no significant group or interaction effect remained. (b) ACTH administration induced a significant increase in PROG in the low-PROG–high-E2 and low-PROG–low-E2 groups, an effect that was not observed in the high-PROG–high-E2 group ($P < 0.001$). (c) The low-PROG–low-E2 group had lower E2 levels than the low-PROG–high-E2 and high-PROG–high-E2 groups ($P < 0.001$). ACTH administration induced a significant increase in E2 in the low-PROG–high-E2 and high-PROG–high-E2 groups, but not in the low-PROG–low-E2 group ($P < 0.001$).

evidence that adrenal steroid secretion is associated with gonadotropin release comes from animal studies in which adrenalectomy and pretreatment with RU486, which has antiglucocorticoid and anti-PROG activities, each abolished stress-induced gonadotropin release (Putnam *et al.*, 1991; Xiao *et al.*, 1994, Zalanyi, 2001). Similar to the results of human studies, the stimulatory effect of ACTH was observed only in estrogen-primed rats, consistent with the essential requirement of adequate estradiol (Putnam *et al.*, 1991; Mahesh and Brann, 1998).

In our data, a significant increase in ACTH-induced gonadotropin levels was observed in all groups. Among women with low levels of PROG, ACTH administration led to increased PROG in the presence of normal estradiol levels. This permissive hormonal context is comparable to that in the beginning of the midcycle peak of LH and FSH (Hoff *et al.*, 1983). Earlier research has established that estradiol and PROG influence the induction of the midcycle gonadotropin surge (Young and Jaffe, 1976; Terasawa *et al.*, 1980). In our study, adrenal stimulation by ACTH caused a near doubling of the relatively low-PROG levels in the low-PROG–high-E2 and low-PROG–low-E2 groups. However, in the high-PROG–high-E2 group, in which estradiol levels were comparable to those in the low-PROG–high-E2 group, a similar increase in gonadotropin levels was observed in the absence of

Table II Adrenal steroid levels in response to ACTH stimulation in the experimental groups.

	low-PROG/high-E2	high-PROG/high-E2	low-PROG/low-E2	*P*-value within group	*P*-value between group
Cortisol (nmol/l)	*n* = 28	*n* = 12	*n* = 20		
Baseline	182.58 (89.16)	158.82 (41.55)	386.60 (116.99)	<0.001	<0.001
30 minutes	435.73 (61.28)	456.86 (77.89)	791.81 (183.24)		
90 minutes	261.26 (56.25)	295.48 (65.88)	612.08 (176.64)		
Progesterone (nmol/l)	*n* = 28	*n* = 11	*n* = 20		
Baseline	0.63 (0.50)	25.71 (16.88)	0.19 (0.06)	<0.001	<0.001
30 minutes	0.93 (0.68)	20.53 (12.77)	0.55 (0.25)		
90 minutes	0.81 (0.78)	24.06 (14.45)	0.28 (0.09)		
17-OH Progesterone (nmol/l)	*n* = 28	*n* = 12	*n* = 20		
Baseline	1.25 (0.76)	4.35 (1.89)	0.19 (0.13)	<0.001	<0.001
30 minutes	2.39 (0.98)	5.79 (2.69)	1.49 (0.71)		
90 minutes	1.41 (0.69)	4.38 (1.90)	0.39 (0.21)		
Androstenedione (nmol/l)	*n* = 27	*n* = 12	*n* = 17		
Baseline	4.01 (1.70)	3.90 (1.48)	1.73 (0.71)	<0.001	<0.001
30 minutes	5.48 (2.27)	5.51 (1.33)	2.61 (0.84)		
90 minutes	4.16 (1.61)	4.01 (1.20)	1.92 (0.63)		
DHEA (nmol/l)	*n* = 28	*n* = 12	*n* = 20		
Baseline	21.50 (10.43)	16.84 (5.48)	11.18 (4.91)	<0.001	<0.001
30 minutes	43.73 (15.95)	45.31 (12.90)	24.52 (10.73)		
90 minutes	21.25 (8.57)	20.52 (7.48)	11.85 (3.87)		
DHEAS (μmol/l)	*n* = 28	*n* = 12	*n* = 20		
Baseline	5.15 (2.43)	6.06 (2.73)	4.10 (1.69)	<0.01	0.02
30 minutes	5.34 (2.53)	5.87 (2.16)	4.21 (1.68)		
90 minutes	5.15 (2.29)	5.97 (2.31)	4.06 (1.66)		
Testosterone (nmol/l)	*n* = 28	*n* = 12	*n* = 20		
Baseline	0.97 (0.36)	1.04 (0.45)	0.55 (0.18)	<0.001	<0.001
30 minutes	1.11 (0.36)	1.13 (0.36)	0.70 (0.23)		
90 minutes	1.08 (0.40)	1.10 (0.41)	0.58 (0.17)		
E2 (pmol/l)	*n* = 27	*n* = 12	*n* = 20		
Baseline	253.96 (197.80)	304.27 (106.38)	39.44 (15.97)	<0.001	<0.001
30 minutes	243.00 (181.95)	292.58 (103.17)	34.71 (17.47)		
90 minutes	324.45 (244.79)	348.72 (136.16)	37.80 (18.49)		

Data are presented as mean ± SD. DHEA, dehydroepiandrosterone; DHEAS, DHEA sulfate.

increased PROG levels. Therefore, the analogous ACTH effects on gonadotropin release in the high-PROG–high-E2 and low-PROG–high-E2 groups suggests that PROG is unlikely to be mediating the increase in LH and FSH.

Alternative mechanisms to explain the ACTH-induced release of LH and FSH might involve the influence of 17-OH-PROG, androgens, estradiol or cortisol. In our study, the relative effect of ACTH on circulating levels of 17-OH-PROG was even larger than observed for PROG, in accordance with previous studies (De Geyter *et al.*, 2002). Elevated levels of 17-OH-PROG are in line with earlier reports showing that peripheral levels of 17-OH-PROG during the luteal phase of the cycle are higher than those during the follicular phase (Aedo *et al.*, 1981). It has recently been described that 17-OH-PROG may have glucocorticoid activity due to its binding to the glucocorticoid receptor (GR) and its ability to transactivate the GR *in vitro* (Pijnenburg-Kleizen *et al.*, 2015). However, considering that 17-OH-PROG binds weakly to the GR and is a less potent agonist of GR than cortisol, it is unlikely that the observed gonadotropin increase in our study is mediated by 17-OH-PROG (Pijnenburg-Kleizen *et al.*, 2015). Furthermore, although earlier research in rhesus monkeys has suggested that 17-OH-PROG may facilitate the onset of LH surges (Schenken *et al.*, 1985), the stimulating effect of 17-OH-PROG on LH release was not found in humans (Leyendecker *et al.*, 1976). This makes it unlikely that the increase of 17-OH-PROG caused the surge of gonadotropins.

Regarding the influence of increased levels of androgens and estradiol in the ACTH-induced release of gonadotropins, it is very unlikely that these steroids function prominently since only suppressive effects of androgens have been described in patients with androgen-

producing tumors (Gabrilove *et al.*, 1981; Jarabak and Talerman, 1983) or in rats (Clayton, 1993). Moreover, the increase in estradiol levels was detectable only 90 minutes after the administration of ACTH, whereas the surge of LH and FSH was already evident after 30 minutes.

Taken together, we believe that cortisol is the most parsimonious mediator of the increased levels of LH and FSH after ACTH injection. This is in accordance with the results of experiments in rats, in which glucocorticoids have been shown to affect gonadotropin release via receptor-mediated mechanisms (Briski, 1996), and for which GR activity has been shown to modulate LH through both pituitary and neuroendocrine mechanisms following exposure to stress (Siegel *et al.*, 1981; Armario *et al.*, 1986; Lopez-Calderon *et al.*, 1990).

This study has several limitations. Because this is a secondary data analysis, examining the impact of acute adrenal stimulation on gonadotropin release was not the primary goal when designing the original study. Therefore, women having a natural menstrual cycle were tested during their luteal phase. Testing during the follicular phase of the menstrual cycle might have elicited premature, or more pronounced, LH surges in response to ACTH administration. However, reclassification of our data based on different PROG levels, despite similar estradiol concentrations, did not change the findings. Additionally, women were not randomly assigned to the study, but were recruited based on their use of contraceptives. However, the groups were very similar for all known confounding variables, including general medical health, age, BMI, and affective state.

While it is likely that the increase in gonadotropins observed in this study is due to a mediating effect of cortisol, it is also possible that administration of ACTH might have resulted in downstream adaptations to corticotropin-releasing hormone through a secondary feedback loop. However, the low-dose (1 μg) ACTH stimulation test has been well documented to be more physiological and sensitive than, for example, higher dose (250 μg or 100 μg) ACTH stimulation tests. The 1 μg low-dose administration results in a maximal serum ACTH concentration of 200 ng/l, which is of a similar order of magnitude to that observed in venous blood samples from the sinus petrosus inferior (W. W. de Herder, unpublished results). Therefore, it seems unlikely that a 1 μg dose of ACTH directly affects pituitary function additionally because the extensive literature of investigations using the same low-dose ACTH formulation has never previously reported direct alteration of pituitary function. Furthermore, we acknowledge the lack of prolactin measurements that might have provided better insight into the stress-induced gonadotropin release. However, considering that prolactin is released from the anterior pituitary and our focus was on the effects of adrenal stimulation, we considered the effect of prolactin to be negligible.

In conclusion, our data are the first to demonstrate that acute stimulation of adrenal steroid production, most likely cortisol, mediates enhanced gonadotropin release in healthy premenopausal women. More generally, these findings contribute to an improved understanding of the influence of acute stress on reproductive endocrinology.

Authors' roles

J.A., J.H.M.T., and S.A.K. conceived of the study. J.A. and M.T. recruited the study participants. M.T. administered the ACTH and collected blood samples. E.F.C.vR. and F.H.dJ. assisted in the interpretation of the data. Y.B.dR. performed serum steroid and gonadotropin measurements. J.A. performed the statistical analyses. J. A., J.H.M.T., F.H.dJ. and S.A.K. wrote the manuscript. All authors edited the manuscript for content and approved the final draft.

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Conflicts of interest

None of the authors reports any competing financial interest or conflict with the research described in the manuscript.

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