Changes in pituitary sensitivity to GnRH in estrogen-treated post-menopausal women: evidence that gonadotrophin surge attenuating factor plays a physiological role

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BACKGROUND: The purpose of this study was to investigate changes in pituitary response to GnRH in post-menopausal women during substitution treatment with exogenous estrogen and progesterone. METHODS: Seven healthy post-menopausal women (group 1) were treated with various doses of E2 valerate for 43 days, so as the serum concentrations of E2 mimicked those of a follicular (FP-1), a luteal (LP) and a second follicular (FP-2) phase. During the LP, progesterone was also administered. The 30 min response of LH (ΔLH) and FSH (ΔFSH) to GnRH (10 μg i.v.) (pituitary sensitivity) was investigated every 24 h in group 1 and also in seven normally cycling women (group 2) during a spontaneous (control) follicular phase (FP). Based on the hormone profiles, day 32 in group 1 (FP-2) corresponded to day 2 in the spontaneous FP of group 2. RESULTS: Basal FSH concentrations were significantly higher in FP-2 than in the control FP (P<0.05), while basal LH concentrations were similar in the two phases with higher values in FP-2 towards the end of the experiment (corresponding to days 10 and 11, P<0.05). However, an LH surge was seen only in the control FP. ΔFSH values remained stable in both phases and increased only in the control FP on days 12 and 13. ΔLH values remained stable in the control FP and only increased on days 12 (P<0.05) and 13 (P<0.05), but in FP-2, ΔLH values increased earlier (corresponding to day 7, P<0.05). CONCLUSIONS: The present study demonstrates for the first time that in the absence of ovarian function, follicular phase E2 concentrations sensitize the pituitary to GnRH at an earlier stage (corresponding to the midfollicular phase) than in the normal menstrual cycle (late follicular phase). It is suggested that during the early to midfollicular phase the ovaries produce a gonadotrophin surge attenuating factor (GnSAF) that antagonizes the pituitary-sensitizing effect of E2 to GnRH.

Key words: FSH/LH/GnRH/gonadotrophin surge attenuating factor/pituitary

Introduction

It has been established that during the follicular phase of the normal menstrual cycle estradiol (E2) mediates the ovarian mechanism that controls basal gonadotrophin secretion, while at midcycle a positive feedback effect occurs (Messinis and Templeton, 1988; Ordog et al., 1998). It is generally accepted that both E2 and GnRH are important for the expression of the positive feedback effect and the occurrence of the endogenous LH surge (Hoff et al., 1977; Liu and Yen, 1983; Miyake et al., 1983; Moenter et al., 1990). E2 sensitizes the pituitary to GnRH possibly through an increase in GnRH receptors (Laws et al., 1990) and this results in the augmentation of both the releasable and the reserve pools of the gonadotrophs (Lasley et al., 1975). In addition, E2 affects GnRH secretion via a multimodal regulation of the activity of GnRH neurons (Herbison, 1998).

An assessment of the two gonadotrophin pools can be achieved in normal women with the i.v. administration of two submaximal doses of GnRH 2 h apart (Wang et al., 1976). The 30 min point in response to the first injection represents the releasable pool or the pituitary sensitivity, while the whole area under the stimulation curve represents the second pool or the pituitary reserve. Although it would be expected that the enhancement in the pituitary sensitivity to GnRH during the follicular phase of the normal cycle would be a continuous process, recent studies have demonstrated with the use of the model of the two GnRH pulses that a significant increase in pituitary sensitivity takes place only in
the late follicular phase, i.e. during the pre-ovulatory period (Messinis et al., 1994, 1998). It is reasonable therefore to hypothesize that during the greater part of the follicular phase an unknown factor counteracts the sensitizing effect of E2 on the pituitary.

Up to now, a gonadotrophin surge attenuating factor (GnSAF) has been proposed as a substance that is produced by ovaries undergoing ovulation induction and which attenuates the endogenous LH surge by reducing the pituitary response to GnRH (Messinis and Templeton, 1989, 1991a). Although data regarding the characterization of this substance are conflicting (Tio et al., 1994; Danforth and Cheng, 1995; Pappa et al., 1999; Fowler et al., 2002), higher bioactivity of GnSAF has been detected in small growing follicles than in pre-ovulatory follicles (Fowler et al., 2001) as well as in the circulation of women during the early and midfollicular phase compared to the late follicular phase of the normal menstrual cycle (Martinez et al., 2002). It is possible therefore that GnSAF in the early to midfollicular phase of the cycle antagonizes the sensitizing effect of E2 on the pituitary.

The present study was undertaken to test this hypothesis by assessing the pituitary response to GnRH in women with inactive ovaries, such as after menopause, during substitution treatment with exogenous estrogen and progesterone that simulates the hormone profile of the normal menstrual cycle.

Materials and methods

Patients

Fourteen healthy women volunteered for the study and gave written informed consent. Seven of the women aged 55–70 years were post-menopausal (group 1), while the remaining seven aged 21–27 years were normally cycling women and were used as controls (group 2). Their clinical and hormonal characteristics are shown in Table I. The study was approved by the local ethics committee. Before their inclusion in the study, all women were thoroughly investigated with history interview, physical and gynaecological examination and Pap smears. Women in group 1 were further evaluated by serum tumour markers, transvaginal sonography and mammography. The exclusion criteria were: history of serious endocrine or other diseases (cardiovascular, liver, renal, malignancy, etc.), hysterectomy plus bilateral or unilateral ovariectomy, HRT, endocrinopathies and medications that might interact with the endocrine function. Various endocrinopathies were excluded based on specific blood tests, including evaluation of thyroid and adrenal function. All 14 women had normal serum prolactin levels.

In group 1, the women after priming for 1 week with E2 valerate (E2V) per os at the dose of 1 mg per day (Divina tablets, 2 mg estradiol valerate; Organon, Greece) were then treated with various doses of E2V for 43 days plus progesterone (Utrogestan capsules, 100 mg progesterone; Faran, Greece) for 13 days (Figure 1), so as the serum concentrations of these steroids mimicked those of a follicular phase (FP-1, days 1–15) and a luteal phase (LP, days 16–28) followed by a second follicular phase (FP-2, days 29–43). The simulating regimens (Figure 1) were chosen after testing various doses of these drugs in pilot experiments performed in two volunteer women before the onset of the study. During the FP-1, E2V was given daily per os as follows: 2 mg on days 1–3, 3 mg on days 4–6, 4 mg on days 7–9, 6 mg on days 10–12, 8 mg on days 13 and 14 and 2 mg on day 15. During the LP, the daily dose of E2V was 2 mg on days 16–19 and the same on days 26–28, and 3 mg on days 20–25. Progesterone was administered intravaginally at the following doses: 100 mg on days 16 and 28, 200 mg on days 17 and 27 and 300 mg on days 18–26. During the FP-2, the FP-1 simulated regimen of E2 was used again on days 29–43 (Figure 1).

In both groups, the response of LH and FSH to GnRH (09:00 h) was investigated in each woman as follows: in group 1, every 24 h during the two simulated follicular phases (FP-1 and FP-2) and every 72 h during the LP starting on day 1 before the onset of E2V treatment; and in group 2, every 24 h during the follicular phase of a spontaneous menstrual cycle (days 2 to 13) (Figure 1). Each time, the dose of GnRH (Relefact LH-RH, 0.1 mg/ml; Hoechst, Germany) was 10 μg i.v. Blood samples in relation to each GnRH injection (time 0) were obtained at −15, 0 and 30 min. The 30 min point was chosen because at that time a maximal response to GnRH is obtained both in pre- and post-menopausal women, representing pituitary sensitivity (Lasley et al., 1975; Wang et al., 1976; Messinis and Templeton, 1991b). In all blood samples, LH and FSH were measured. The response of LH and FSH to GnRH was calculated as the net increase at 30 min (ΔLH and ΔFSH respectively) above their basal value (mean of the values at −15 and 0 min) in order to assess the absolute capacity of the gonadotrophs and therefore changes in pituitary sensitivity.

Hormone assays

FSH, LH and E2 were measured in serum using a Chemiluminescent Microparticle Immunoassay (Architect FSH, Architect LH and Architect Estradiol respectively; Abbott Laboratories). The results are expressed as IU/l for FSH and LH, and as pmol/l for E2. Serum progesterone was measured using a Microparticle Enzyme Immunoassay (AxSYM Progesterone, Abbott Laboratories, USA). The results are expressed as nmol/l. The lower limits of detection for FSH, LH, E2 and progesterone were 0.05 IU/l, 0.07 IU/l, 66.06 pmol/l, and 0.636 nmol/l respectively. Inter- and intra-assay coefficients of variation were 3.0 and 3.4%, 2.1 and 3.3%, 4.4 and 6.2%, and 5.9 and 6.7% respectively.

### Table I. Clinical and hormonal parameters of the post-menopausal women before their treatment (day −7) (group 1) and the pre-menopausal women on day 2 of their cycle (group 2)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1</th>
<th>Group 2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SEM</td>
<td>Range</td>
<td>Mean ± SEM</td>
<td>Range</td>
</tr>
<tr>
<td>Chronological age (years)</td>
<td>60.8 ± 2.0</td>
<td>55–70</td>
<td>23.7 ± 0.7</td>
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<tr>
<td>Post-menopausal age (years)</td>
<td>10.4 ± 2.4</td>
<td>5–20</td>
<td>23.8 ± 0.8</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.4 ± 0.8</td>
<td>22.6–29.1</td>
<td>5.64 ± 0.1</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>41.6 ± 6.4</td>
<td>17.9–62.3</td>
<td>6.32 ± 0.2</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>91.5 ± 13.4</td>
<td>47.5–129.3</td>
<td>211.3 ± 14.4</td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>217.6 ± 22.2</td>
<td>124.8–297.3</td>
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</tbody>
</table>
Statistical analysis

In FP-2 (group 1) and in the follicular phase of group 2, the pattern of the time effect in the two groups was investigated using a generalized linear model for repeated measures (Crowder and Hand, 1990; Dafopoulos et al., 2004) followed by contrast testing (Mead, 1988). The model has the following form: \( \text{(women x group)}_{ik} + (\text{time})_{j} + (\text{time x group})_{jk} + (\text{error})_{ijk} \), where \( y \) is the parameter of interest, the \( (\text{women x group}) \) is the interaction between women (\( i = 1–7 \)) and groups (\( k = 1–2 \)), time is the time effect (\( j = 1–12 \)) and (\( \text{time x group} \) is the interaction between times and groups. When the \( (\text{time x group}) \) interaction is significant then the pattern of time differences is different for the two groups. The significance of the time effect and the \( (\text{time x group}) \) interaction was assessed using the Huynh and Feldt (1976) adjustment of degrees of freedom. In the contrast testing, individual time point means or groups of means are compared using the error mean square of the model. For FP-1, LP and FP-2, the differences between the baseline (first time point or first day of each phase) and the consequent time points for each parameter measured were tested by fitting a generalized linear model for repeated measurements followed by contrast testing. The model has the following form: \( (y)_{ij} = (\text{women})_{i} + (\text{time})_{j} + (\text{error})_{ij} \), where \( y \) is the parameter of interest. The significance of the time effect was assessed using the Huynh and Feldt adjustment. The contrast testing is based on the error mean square of the model. For comparison of the various corresponding days between the two groups, Student’s \( t \)-test was used. The statistical analysis was performed using the SAS r6.12 software.

Results

Figure 2 shows that the pattern of changes in serum levels of \( \text{E}_2 \) and progesterone that were achieved after the exogenous administration of hormones was similar to that seen in a spontaneous menstrual cycle, although there may be some differences in actual values. Basal FSH and LH concentrations declined during treatment with the steroids (\( P < 0.05 \)). \( \Delta \text{FSH} \) values also declined gradually (\( P < 0.01 \)), while \( \Delta \text{LH} \) values did not change significantly during the FP-1, decreased during the LP (\( P < 0.05 \)) and increased gradually during the FP-2 (\( P < 0.01 \)) (Figure 2).

Figure 3 shows the comparison between the simulated FP-2 in group 1 and the control follicular phase (group 2) regarding serum \( \text{E}_2 \), progesterone, FSH, LH, \( \Delta \text{FSH} \) and \( \Delta \text{LH} \) concentrations. Based on the pattern of decline of progesterone concentrations in group 1, we considered that day 32 in the simulated FP-2 corresponded to day 2 of the control cycles. On day 32, the levels of progesterone were similar to those on day 2 in the control group but during the next days and up to day 43 the levels declined further in group 1 and were significantly lower in the FP-2 than in the control group (\( P < 0.05 \)). Serum \( \text{E}_2 \) concentrations showed a similar pattern of increase in the two groups, although in actual values initially they were significantly higher in the simulated FP-2 (\( P < 0.001 \)). Eventually, however, \( \text{E}_2 \) values achieved similar levels, as there was no significant difference between days 41, 42 and 43 in group 1 and the corresponding days 11, 12 and 13 in group 2.

Basal FSH concentrations were higher in the simulated FP-2 than in the control follicular phase (\( P < 0.05 \)) (Figure 3). For FSH, the (time x group) interaction was significant (\( P < 0.05 \)), indicating that FSH values decreased. Basal LH concentrations did not differ significantly between the simulated FP-2 (day 32) and the control follicular phase (day 2). For LH the (time x group) interaction was significant (\( P < 0.01 \)). For group 1, the control testing showed that basal LH concentrations increased in FP-2, and on days 40 and 41 (corresponding to days 10–11) the values were
significantly higher than in the control follicular phase (Figure 3, Table II). For group 2, basal LH values increased on day 13 corresponding to an LH surge that was seen only in that phase.

For ΔFSH, there was significant (time × group) interaction \( (P = 0.05) \), therefore the time effect has a different pattern for the two groups (Figure 3, Table II). For group 1, ΔFSH remained constant in FP-2, while for group 2 the time effect was due to the large value of day 13 \( (P < 0.01) \).

ΔLH values did not differ significantly between the simulated FP-2 (day 32) and the control follicular phase (day 2). For ΔLH, there was significant (time × group) interaction \( (P < 0.01) \), indicating that the pattern of time effect is different for the two groups (Figure 3, Table II). In particular for group 1, the contrasts of the baseline (day 32) versus day 37 and beyond were significant \( (P < 0.05) \) (the contrast between baseline and day 43 was significant, \( P < 0.01) \). For group 2, the contrast of the baseline (day 2) was significant from days

Figure 2. Serum concentrations (mean ± SEM) of basal estradiol, progesterone, FSH, LH, ΔFSH and ΔLH in group 1 during the whole experimental period.
and 13 (P < 0.05). Therefore, in group 1 there was an earlier increase in ΔLH values than in the control group (Figure 4).

All women had withdrawal bleeding 3 days after the cessation of progesterone administration.

**Discussion**

The present study is the first that has investigated changes in pituitary response to GnRH in women with non-functioning ovaries during a simulated follicular phase. The results demonstrate that the LH response was enhanced at an earlier
stage in the simulated than in the natural follicular phase. In order to achieve conditions that were closer to those in the normal follicular phase the women were treated successively with ovarian steroids for 5 weeks before the onset of the comparison procedure. That the steroids given were biologically active was evident from the significant decline in basal concentrations of FSH and LH and their responses to GnRH.

To attain levels of E2 and progesterone exactly the same as those in the normal menstrual cycle is difficult; however, the levels that were eventually achieved in this study were within the normal range for a spontaneous cycle (Messinis and Templeton, 1988). There were only two differences as compared to the control follicular phase, i.e. higher E2 concentrations at the beginning of FP-2 and lower progesterone values during the same phase. These differences could not account for the differential response of the pituitary in the two groups for the following reasons: previous data in normally cycling women seem also to provide some support for the GnSAF hypothesis, although their primary endpoint was the regulation of LH pulse frequency by gonadal steroids (Nippoldt et al., 1989; McCartney et al., 2002). In experiments in which E2 concentrations were maintained at the midluteal level, although progesterone concentrations were low the increase in LH pulse frequency that was seen for 2 weeks was not accompanied by a similar increase in FSH response. This is consistent with the notion that GnSAF controls GnRH-induced LH but not FSH secretion (Fowler and Templeton, 1996).

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that was attenuated as compared to the midcycle LH surge, while the FSH surge was normal (Taylor et al., 1995). Although the authors of that study explained their results on the basis of a missing ovarian factor in the midfollicular phase that is required for the normal midcycle surge, they could also easily accept that the attenuation was related to the production of GnSAF by the ovaries at that stage of the cycle.

In terms of basal gonadotrophin secretion, serum FSH values were suppressed during the experimental period, while basal LH values in FP-2 increased gradually and significantly. The inability of E2 to maintain very low levels of LH in the absence of ovarian function indicates that during the follicular phase of the natural cycle this steroid is not the only mediator of the ovarian negative effect on LH secretion. Since in this study serum progesterone concentrations in FP-2 were very low, it is possible that during the normal follicular phase progesterone even at low levels contributes to the ovarian suppressing effect on basal LH secretion. These data demonstrate the differential control of FSH and LH by ovarian steroids as has been suggested previously (Messinis et al., 2002; McNeilly et al., 2003). Whether inhibin might have an effect on LH secretion is rather unlikely, as administration of inhibin A to rhesus monkeys in the early follicular phase suppressed FSH but not LH levels (Molskness et al., 1996).

Another interesting finding in the present study is the failure of a positive feedback mechanism to induce an endogenous LH surge either in FP-1 or in FP-2 despite the fact that serum E2 concentrations had exceeded the appropriate threshold level for several days. These data are in disagreement with a previous study in which four out of five post-menopausal women treated with exogenous estrogen and progesterone to simulate the natural menstrual cycle displayed an endogenous LH surge (Lutjen et al., 1986). Although this difference is difficult to explain, in that study all five women had premature menopause and were younger (<37 years old) than in the present study. It is possible that the pituitary behaves in a different way in older compared with younger post-menopausal women. Since, however, post-menopausal women aged >50 years can express a positive feedback effect when treated with high doses of estrogen even for only 3 days (Liu and Yen, 1983), it may be that in these women the pituitary is less sensitive to the gradual than to the abrupt increase in serum E2 concentrations. Nevertheless, we cannot exclude the possibility that in our study attenuated LH surges occurred during the experimental period that were missed due to the blood sampling frequency or that an LH surge would occur at a later stage.

The lack of an LH surge was noted despite the earlier sensitization of the pituitary to GnRH by E2. This indicates that at a specific point during the normal menstrual cycle the E2-enhanced pituitary sensitivity to GnRH is further augmented by an ovarian factor that plays a key role in timing the onset of the midcycle LH surge. Progesterone may be such a factor since this steroid advances and augments the E2 positive feedback effect experimentally (Liu and Yen, 1983; Messinis and Templeton, 1990), while the progesterone antagonist mifepristone blocks the endogenous LH surge in normal women despite the presence of high E2 concentrations (Messinis et al., 1997). In that respect, a small rise in serum progesterone levels in the late follicular phase (Roseff et al., 1989) may not be necessary for the occurrence of the midcycle LH surge.

The following hypothesis is supported by the results of the present study. During the normal menstrual cycle, E2 sensitizes the pituitary to GnRH, an action that in the early to midfollicular phase is counteracted by GnSAF. In the late follicular phase, the sensitizing effect of E2 is facilitated both by a decrease in the production of GnSAF and by the synergistic action of E2 and progesterone. With these hormonal interactions the secretion of LH up to the midfollicular phase is maintained at a low level and is only markedly enhanced at midcycle when high amounts of this hormone are required for the ovulatory events. In the context of this hypothesis, progesterone secreted in small amounts during the follicular phase of the cycle plays an important physiological role both through the negative and the positive feedback mechanisms.

In conclusion, the present study provides new insights into the ovarian control of LH secretion during the normal menstrual cycle. The results demonstrate for the first time that E2 sensitizes the pituitary to GnRH at an earlier stage in women with inactive ovaries than in the follicular phase of the natural cycle. It is suggested that during the normal follicular phase the ovaries control LH secretion not only through E2 but also via the participation of GnSAF and progesterone.

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K.Dafopoulos et al.


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