Serum concentrations of dimeric inhibins, activin A, gonadotrophins and ovarian steroids during the menstrual cycle in older women

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The transition from regular ovarian cyclicity to menopause is associated with a rise in the circulating concentrations of follicle stimulating hormone (FSH), despite the maintenance of serum oestradiol concentrations during the perimenopause. The aim of this study was to compare the patterns of secretion of dimeric inhibins, activin A, gonadotrophins and steroids in regularly cycling women of 40–50 years with normal and raised early follicular phase serum FSH concentrations and young women (25–33 years) during the menstrual cycle. Blood samples were taken prospectively almost daily throughout the menstrual cycle. Inhibin A is an activin A/inhibin A/inhibin B/ovary/perimenopause

transcription is not well understood. A well established endocrine finding associated with reproductive ageing is the rise of a single gonadotrophin, i.e. circulating follicle stimulating hormone (FSH) but not luteinizing hormone (LH) (Metcalf and Livesey, 1985; Lee et al., 1988; Klein et al., 1996). Raised FSH is important because it is the first measurable parameter that changes in reproductive ageing. The objective of this study is to explain the reason for this rise. Pituitary FSH production is co-regulated by inhibin and oestradiol acting via endocrine negative feedback. Changes in circulating concentrations of oestradiol that could fully account for the rise in FSH have not been observed consistently during the early stages of reproductive ageing (Metcalf and Livesey, 1985; Lee et al., 1988). It is possible that as the ovarian follicular reserve is becoming depleted, a fall in circulating concentrations of inhibins could be an important influence on the rise in FSH.

Inhibins are glycoprotein hormones consisting of two disulfide-linked subunits (α and β) linked by disulphide bridges and are secreted by the ovarian granulosa and luteal cells during the menstrual cycle. Inhibin A is an α-βA dimer and inhibin B is an α-βB dimer. Inhibins are a part of the pituitary–ovarian endocrine loop, specifically having an inhibitory effect on pituitary FSH synthesis and secretion (Muttukrishna and Knight, 1990). Pro alpha C consists of higher molecular weight inhibins containing the pro region of the alpha subunit and the biologically inert monomeric α subunit.

A study measuring FSH, LH, oestradiol and immunoreactive (ir) inhibin in women of reproductive age has shown a significant fall in concentrations of ir-inhibin with increasing age in the follicular phase (day 4–7) and luteal phase (7 days prior to the next anticipated menses) (MacNaughton et al., 1992). In that study, ir-inhibin was measured using the Monash radioimmunoassay (Robertson et al., 1985; Burger et al., 1995) which cannot distinguish between biologically active dimeric inhibin A, inhibin B and the inert monomeric alpha subunit that is in abundance in circulation.

The recent development of highly specific and sensitive enzyme immunoassays (EIA) for dimeric inhibin A (Groome et al., 1994; Muttukrishna et al., 1994), inhibin B (Groome et al., 1996), pro alpha C-containing inhibins (Groome et al., 1995) and activin A (Knight et al., 1995) has enabled us to measure the individual dimeric proteins specifically. Using these two-site EIA, cyclical patterns of inhibin A, inhibin B,
Subjects

Sixteen parous women aged 40–50 years and six women aged 25–32 years experiencing regular cycles (25–35 days) volunteered for this study. All women were healthy, had both ovaries and were not on any hormonal medication. In this longitudinal study, blood samples (5 ml) were taken at frequent intervals throughout one menstrual cycle starting from day 2/3 until day 3/4 of the next cycle. The protocol was approved by the Central Oxford Research Ethics Committee (COREC No.96.086) and written consent was obtained from subjects before enrolling for the study. Serum was separated and stored at −20°C for hormone measurements. Serum FSH concentrations on day 3 or 4 of the study cycle were used to classify the older women into two groups. The young normal FSH group constituted a third group.

Group 1: ON-FSH (older normal FSH group); day 3 FSH <8 mIU/ml (n = 10) (mean age = 43.4 years).

Group 2: R-FSH (raised FSH group); day 3 FSH >8 mIU/ml (n = 6) (mean age = 46 years).

Group 3: YN-FSH (young normal FSH group); day 3 FSH <8 mIU/ml (n = 6) (mean age = 29.6 years).

Hormone measurements

Inhibin A

Serum concentrations of dimeric inhibin A were measured in duplicate 50 µl aliquots as described elsewhere (Muttukrishna et al., 1994). The mean intra- and inter-assay coefficients of variation (CV) were 4.3 and 5.1% respectively. Minimum detection limit of the assay for human recombinant inhibin A [National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK] was 2 pg/ml.

Inhibin B

Serum concentrations of dimeric inhibin B were measured in 50 µl duplicates using an enzyme immunoassay as described in detail elsewhere (Groome et al., 1996). An in-house standard preparation (partially purified human follicular fluid) was standardized against human recombinant inhibin B (Genentech Inc., San Francisco, CA, USA) and was used as the assay standard. Minimum detection limit of the assay for human recombinant inhibin B was 15 pg/ml. The mean intra- and inter-assay CV were 6.2 and 7.2% respectively.

Pro alpha C

Serum concentrations of pro alpha C-containing inhibins and free pro alpha subunits were measured in duplicate 50 µl aliquots as previously described (Groome et al., 1995). The minimum detection limit of the assay for pro alpha C standard was 5 pg/ml. The mean intra- and inter-assay CV were 6.8 and 8.6% respectively.

Activin A

Serum concentrations of ‘total’ activin A were measured using an ELISA specific for ‘total’ activin A as described in detail elsewhere (Knight et al., 1996). The mean intra- and inter-assay CV were 6.5 and 7.7% respectively. The minimum detection limit of the assay for human recombinant activin A (Genentech) was 50 pg/ml.

Gonadotrophins and steroids

Serum concentrations of FSH, LH, oestradiol and progesterone were measured using Immulite-chemiluminescent assay kits (Diagnostic Products Ltd, Llanberis, Gwynedd, UK). The mean intra- and inter-assay CV were <10% for all four assays. The detection range of FSH, LH, oestradiol and progesterone were 0.1–170 mIU/ml, 0.7–400 mIU/ml, 0.073–7.32 nmol/l and 0.73–127 nmol/l respectively.

Statistical analysis

General linear model (GLM) multivariate analysis was carried out to investigate the difference in the pattern of individual hormones in the menstrual cycle between the groups. Unpaired Student’s t-tests were carried out to compare the serum concentrations of normal and raised FSH groups of women at a particular time point in the cycle. Correlation analysis was carried out to investigate the relationship between different hormones. SPSS statistical package for social sciences (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Results

In the cycles studied, cycle lengths varied from 25–35 days. All cycles were ovular (as defined by mid-luteal serum progesterone >25 nmol/l) and there was no consistent difference in length of the follicular phase in the three groups (range 10–12 days).

Circulatory pattern of inhibins and activin A in the menstrual cycle

Serum inhibin A concentrations in the YN-FSH and ON-FSH group increased steadily during the follicular phase and peaked...
Concentrations of activin A did not vary significantly between groups (Figure 2b).

Concentrations of LH and FSH in the serum from women in the ON-FSH group shared the typical pattern seen in YN-FSH women with an early follicular rise in FSH followed by a pronounced LH surge in mid-cycle. Whilst women in the R-FSH group had a similar LH surge, the pattern of FSH secretion was disordered, with fluctuations in the follicular and early luteal phases of the cycle in the R-FSH women with high early follicular phase concentrations (Figure 3).

Previously documented patterns (Muttukrishna et al., 1994) of serum oestradiol and progesterone were observed in all three groups (Figure 4). The pre-ovulatory and the luteal peaks of oestradiol in the R-FSH group (697 ± 175 and 272 ± 46.8 pmol/l respectively) were similar to the ON-FSH (719 ± 54.8 and 439 ± 13.1 pmol/l) group. Peak progesterone concentrations in the ON-FSH (56.4 ± 3.2 nmol/l) group were higher than the peak concentrations in the R-FSH group (32.5 ± 8.4 nmol/l). These differences did not reach statistical significance.

### Relationship between oestradiol, inhibins and FSH during the menstrual cycle

Classically, oestradiol, inhibins and activins have an endocrine effect on pituitary FSH secretion. Oestradiol and inhibins inhibit and activins stimulate pituitary FSH secretion. In the follicular phase, inhibin A and inhibin B were not correlated with FSH in the YN-FSH and ON-FSH groups. Oestradiol was not significantly correlated with FSH in the follicular phase in any of the three groups. However, in the R-FSH group, inhibin A \((r = -0.49\), not significant) and inhibin B \((r = -0.675\), not significant) were negatively correlated with FSH in the follicular phase, suggesting that the rise in FSH in this group of women may be due to the decrease in the negative feedback effect of inhibins on the pituitary.

### Discussion

This longitudinal study reports the cyclical pattern of circulating concentrations of biologically active dimeric inhibins and activin A throughout the menstrual cycle in older women. Circulating concentrations of dimeric inhibins during the menstrual cycle in the ON-FSH group of women in this study are similar to the concentrations in the YN-FSH group (Table I). However, in the R-FSH group, the patterns of inhibin secretion were markedly different. This is the first study to compare the concentrations of dimeric inhibins and activin A in older women with normal and raised day 3 FSH concentrations.

A reduction in the circulating concentrations of dimeric inhibins is not unexpected in R-FSH women, as it is well established that dimeric inhibins are undetectable in post-menopausal women. Serum dimeric inhibin B is regarded as a measure of ovarian reserve and follicle number as it is mainly secreted by pre-antral follicles (Klein et al., 1996), whereas inhibin A is mainly produced by the dominant follicle. In a previously reported study, the authors had investigated the secretion of inhibin A by granulosa-luteal cells obtained at oocyte retrieval from IVF patients with raised day 3 serum

### Table I. Comparison of peak concentrations of hormones in young normal follicle stimulating hormone (FSH) and older normal FSH groups of women.

<table>
<thead>
<tr>
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<th>Follicular phase peak</th>
<th>Luteal phase peak</th>
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<tr>
<td>Inhibin A (pg/ml) (25–32 years)</td>
<td>42.96 ± 5.8</td>
<td>54.66 ± 19</td>
</tr>
<tr>
<td>Inhibin A (pg/ml) (40–50 years)</td>
<td>39.1 ± 4.6</td>
<td>82.63 ± 18.79</td>
</tr>
<tr>
<td>Inhibin B (pg/ml) (25–32 years)</td>
<td>207 ± 27.4</td>
<td>–</td>
</tr>
<tr>
<td>Inhibin B (pg/ml) (40–50 years)</td>
<td>171 ± 13</td>
<td>–</td>
</tr>
<tr>
<td>Pro alpha C (pg/ml) (25–32 years)</td>
<td>143.15 ± 16.3</td>
<td>294.4 ± 10.3</td>
</tr>
<tr>
<td>Pro alpha C (pg/ml) (40–50 years)</td>
<td>343 ± 41</td>
<td>525 ± 19.7</td>
</tr>
<tr>
<td>FSH (IU/l) (25–32 years)</td>
<td>4.1 ± 1.1</td>
<td>–</td>
</tr>
<tr>
<td>FSH (IU/l) (40–50 years)</td>
<td>5.98 ± 0.4</td>
<td>–</td>
</tr>
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(42.96 ± 5.8 pg/ml; 39.1 ± 4.6 pg/ml respectively; Table I) prior to the pre-ovulatory LH surge (Figure 1a). Concentrations fell immediately after ovulation and then rose steadily during the luteal phase forming a peak (54.66 ± 19 pg/ml; 82.63 ± 18.8 pg/ml respectively; Table I) 4–6 days after the LH surge. Inhibin A concentrations fell in the late luteal phase before luteolysis along with oestradiol and progesterone. In the R-FSH group, concentrations of inhibin A were significantly lower \((P = 0.001)\) than in the ON-FSH group. Whilst there were no distinct pre-ovulatory (30 ± 6.9 pg/ml) and mid-luteal peaks (27 ± 10 pg/ml) of inhibin A in the R-FSH group the concentrations were higher during this period of the cycle (Figure 1a).

The cyclical pattern and serum concentrations of inhibin B were similar in both YN-FSH and ON-FSH groups. Serum concentrations of inhibin B rose in the early follicular phase, reaching a peak (207 ± 27.4 pg/ml; 172.1 ± 12.6 pg/ml respectively; Table I) around day 5 of the cycle. Inhibin B concentrations fell after day 5, and had a small peak at the time of the LH surge and declined gradually during the luteal phase. A similar cyclical pattern of inhibin-B was observed in the R-FSH group. However, inhibin B concentrations were significantly lower with an attenuated early follicular phase peak (108 ± 15 pg/ml) in the R-FSH group (Figure 1b).

Pro alpha C exhibited a similar cyclical pattern to inhibin A. Concentrations rose steadily in the follicular phase forming a peak prior to ovulation and in the mid-luteal phase of the cycle. The cyclical pattern of pro alpha C was similar in all three groups. However, the pre-ovulatory and mid-luteal peaks in the ON-FSH group (343 ± 41.4 and 525 ± 19.7 pg/ml respectively) were observed to be higher than the R-FSH (205 ± 39.5 and 383 ± 79.5 pg/ml respectively) (Figure 2a). Compared to the pro alpha C in the YN-FSH group the ON-FSH group tended to have higher follicular (343 ± 41.4 and 143.15 ± 16.3 pg/ml respectively) and luteal peak concentrations (525 ± 19.7 and 294.4 ± 10.3 pg/ml respectively) (Table I). However, the apparent difference did not reach statistical significance.

The cyclical pattern of serum activin A was as previously documented (Muttukrishna et al., 1996) in all three groups.
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Figure 1. Circulating concentrations of inhibins during the menstrual cycle in older women. Mean ± SEM serum concentrations of (a) inhibin A and (b) inhibin B in older women with normal (n = 10, ○) and raised (n = 6, ●) day 3 serum follicle stimulating hormone (FSH) during the menstrual cycle. Day 0 = day of luteinizing hormone (LH) surge. Student’s t-tests *P < 0.05, **P < 0.01, ***P < 0.001 compared to the normal FSH group. Circulatory pattern of inhibin A and inhibin B in young control women are presented as insets of (a) and (b) respectively.

FSH (>8 mIU/ml) and normal day 3 serum FSH (<8 mIU/ml) (Seifer et al., 1996). It was reported that luteinized granulosa cells cultured in vitro from patients with raised FSH secreted lower concentrations of inhibin A compared to cells obtained from patients with normal FSH. The finding in the current study that the follicular phase peak of inhibin A was significantly lower in the older women with raised FSH is in agreement with that in-vitro study.

Activin A concentrations were similar in all three groups of women during the follicular phase. In the luteal phase, concentrations of activin A in the R-FSH group were elevated compared to the ON-FSH group. As activin A has a stimulatory effect on pituitary FSH production (Muttukrishna and Knight, 1991), activin A could have an endocrine effect on the pituitary that contributes to the early follicular phase increase in FSH secretion of the following cycle.

It has been reported that (ir) inhibin concentrations decrease with increasing age and an inverse relationship was found between FSH and ir-inhibin (MacNaughton et al., 1992). However, the reported studies used the Monash radioimmunoassay (Robertson et al., 1985; Burger et al., 1995) that measured both dimeric inhibins and inert monomeric alpha subunit, rendering it non-specific for biologically active dimers. It has recently been reported (Burger et al., 1998) that inhibin A concentrations fall substantially with no significant change in inhibin A and oestradiol in the early follicular phase in the early peri-menopausal phase of the menopausal transition. These observations were made on a single sample taken from women between days 3–8. The inhibin A and inhibin B data of the current study are consistent with the above report at that given time point.

The present study showed that the absolute concentrations
Inhibins and activin A in the menstrual cycle of older women

Figure 2. Serum concentrations of activin A during the menstrual cycle of older women. Mean ± SEM serum concentrations of (a) pro alpha C-containing inhibins and (b) ‘total’ activin A in older women with normal (n = 10, ○) and raised (n = 6, ●) day 3 serum FSH during the menstrual cycle. Day 0 = day of LH surge. Circulatory patterns of pro alpha C-containing inhibins and activin A in young control women are presented as insets.

of inhibin A and inhibin B are similar in YN-FSH (25–32 years) (Table I) and in ON-FSH (40–50 years) women. This is the first study to compare the concentrations of inhibins in young controls with older women with normal FSH, and suggests that inhibin secretion is maintained at a relatively consistent amount throughout the cycle despite the steady decline in ovarian follicle number that occurs throughout reproductive life (Faddy and Gosden, 1996).

It has been reported (Klein et al., 1996) that older women with elevated early follicular phase concentrations of FSH (day 3 FSH >8 mIU/ml) have significantly lower concentrations of inhibin B, with no difference in the concentration of inhibin A and oestradiol compared to younger women (20–25 years, day 3 FSH <8 mIU/ml). This study was restricted to women with raised FSH concentrations during the early follicular phase. Consistent with the reported study, the present study found that circulating concentrations of dimeric inhibin B were significantly lower in women of 40–50 years with raised FSH. However, it has been shown by Welt et al. (Welt et al., 1999) and in this study that inhibin A concentrations during the FP peak and luteal phase peak were also significantly lower in the R-FSH group of women. The data presented here suggest that a reduction in the number and quality of the follicles in perimenopause results in a decrease in circulating concentrations of both inhibins A and B resulting in the rise in FSH seen at all stages of the cycle in R-FSH women.

The secretory pattern of oestradiol during the cycle remains similar in both the N-FSH and R-FSH groups, indicating that the elevations in FSH seen throughout the cycle in the R-FSH group were not the result of alterations in the secretion of
Figure 3. Circulatory pattern of FSH and LH during the menstrual cycle of older women. Mean ± SEM serum concentrations of (a) FSH and (b) LH in older women with normal (n = 10, ○) and raised (n = 6, ●) day 3 serum FSH during the menstrual cycle. Day 0 = day of the LH surge. Student’s t-tests **P < 0.01, ***P < 0.001 compared to the normal FSH group. Circulatory pattern of FSH and LH in young control women are presented as insets.

oestradiol by the dominant follicle and the corpus luteum. The aim of this work was to study concentrations of inhibins in ovular women entering menopause, hence ovulatory patterns of oestradiol and progesterone were documented in all cases. However, in the R-FSH group, this ovular pattern was maintained against a background of disordered and generally elevated concentrations of FSH.

Correlation analysis suggested that during the follicular phase, FSH had a negative relationship to inhibin A (r = -0.49, not significant) and inhibin B (r = -0.67, not significant) in the R-FSH group. This confirms previous observations (Santoro et al., 1999) and suggests that both dimeric inhibins (A and B) may influence the rise in early follicular phase FSH. However, consistent with a previous study (Schipper et al., 1998) there was no relationship between inhibin A and/or inhibin B and FSH in the follicular phase of the YN-FSH and ON-FSH group of women, suggesting that in women with normal FSH, other factor(s) may be mainly responsible for the FSH regulation.

A recent study has investigated the predictive value of measurement of inhibin A and B in IVF cycles (Hall et al., 1999). The data showed that concentrations of day 1–4 inhibin A and or inhibin B were not better than age and number of oocytes in predicting the IVF outcome, although lower FSH and higher inhibin B were associated with greater chance of pregnancy. However, during ovarian stimulation, higher
Inhibins and activin A in the menstrual cycle of older women

Figure 4. Circulatory pattern of oestradiol and progesterone during the menstrual cycle of older women. Mean ± SEM serum concentrations of (a) oestradiol and (b) progesterone in older women with normal (n = 10, ○) and raised (n = 6, ●) day 3 serum FSH during the menstrual cycle. Day 0 = day of LH surge. Circulatory patterns of oestradiol and progesterone in young control women are presented as insets.

concentrations of inhibin A and B in serum were associated with successful IVF. The lack of relationship between day 1–4 concentrations of inhibins and IVF outcome (Hall et al., 1999) may be because inhibin A concentrations are very low and inhibin B concentrations steadily rise at this time of the cycle. Therefore, measurement of inhibin B around day 5–7 (the time of early follicular phase peak) would be more precise and useful in predicting IVF outcome.

Activin A concentrations in the late luteal phase of the raised FSH group are marginally higher until luteolysis. This suggests that higher concentrations of activin A in the late luteal phase may have an endocrine effect in increasing pituitary FSH production. However, further detailed studies with more subjects should be carried out during luteo-follicular transition to confirm this endocrine effect of activin A on pituitary FSH production.

Pro alpha C is mainly a luteal product and it appeared to show a positive relationship to progesterone in the ON-FSH (r = 0.5, not significant) and R-FSH (r = 0.65, not significant) groups of women. However, it is interesting to note that pro alpha C concentrations in YN-FSH group are considerably lower (Table I) than in the ON-FSH group, suggesting that pro alpha C concentrations increase with age. The similar pro alpha C-containing inhibin concentrations throughout the cycle in the ON-FSH and R-FSH groups of women suggest that the corpus luteum function with more subjects should be carried out during luteo-follicular transition to confirm this endocrine effect of activin A on pituitary FSH production.

In summary, this study has shown that inhibin B concentra-
tions are significantly altered in the follicular phase and inhibit A concentrations are significantly altered in the follicular and luteal phase of older women during the early stage of perimenopause with no significant alterations in oestradiol. The rise in FSH in the older R-FSH group of women may be explained by the decrease in the negative feedback on the pituitary due to declining inhibin A and B production by the ovaries.

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