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THE IMMUNOREACTIVE INHIBIN SECRETION PATTERN IN THE MIDLUTEAL PHASE: RELATIONSHIPS WITH LUTEINIZING HORMONE AND PROGESTERONE

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SUMMARY

With the development of a sensitive radioimmunoassay for inhibin, luteal phase inhibin levels have been noted to parallel progesterone levels and be acutely dependent upon luteinizing hormone (LH) stimulation. To define the midluteal secretory pattern of immunoreactive inhibin and its relationships with LH and progesterone, blood samples were obtained from five normal women every 20 min for a period of 24 h. Individual data series of LH, progesterone and inhibin were analysed for pulsatile secretion using an adaptive-threshold method. Inhibin levels exhibited a relatively frequent, low-amplitude pulsatile secretory pattern ($14{\cdot}6 \pm 6{\cdot}9$ pulses/24 h (mean ± SE), amplitude = 17% of the mean inhibin level). In contrast, LH levels demonstrated an infrequent high amplitude secretory pattern ($6{\cdot}2 \pm 0{\cdot}7$ pulses/24 h, amplitude = 139% of the mean LH level). The average progesterone pulse frequency and amplitude were intermediate to LH and inhibin ($9{\cdot}2 \pm 1{\cdot}2$ pulses/24 h, amplitude = 36% of the mean progesterone level). In addition, each individual's hormone data were analysed for coincident pulsatile secretion and cross-correlations were performed on the data, with one hormone pattern shifted relative to another by 20-min time intervals. None of the individual inhibin data series showed significant pulse coincidence when compared to the LH or progesterone data series. The cross-correlation analysis, however, revealed a significant ($P < 0{\cdot}05$) relationship in general trends between the inhibin and LH data series, and the inhibin and progesterone data series in three subjects. These findings suggest that (1)

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inhibin exhibits a relatively frequent low-amplitude pulsatile secretory pattern in comparison to LH and progesterone in the midluteal phase of the menstrual cycle, and (2) while the secretion of these three hormones is interrelated, there is not a strong coupling between secretory pulses of inhibin and either LH or progesterone pulses.

The secretory phase of the human menstrual cycle is characterized by the luteinization of granulosa and theca cells with subsequent secretion of progesterone and oestradiol from the developing corpus luteum (Ross *et al.*, 1970; Fritz & Speroff, 1982). With the development of a sensitive radioimmunoassay for inhibin, daily immunoreactive inhibin levels were noted to parallel progesterone levels suggesting that the human corpus luteum secretes inhibin (McLachlan *et al.*, 1987a, b). Isolation of the mRNA for the α and β_A subunits of inhibin, as well as both bio and immuno-reactive inhibin itself, from human corpora luteal tissue has provided further evidence that the corpus luteum is the site of inhibin production (Davis *et al.*, 1987). Recent information from McLachlan *et al.* (1989) has clarified the endocrine control of luteal inhibin production. It appears that luteal phase inhibin is LH dependent since hCG administration maintained inhibin and progesterone secretion in women rendered hypogonadotrophic by a GnRH antagonist. Previous studies from our group and others (Filicori *et al.*, 1984; Veldhuis *et al.*, 1988; Soules *et al.*, 1988) have described LH-entrained progesterone secretion from the corpus luteum.

While inhibin has been demonstrated to be a secretory product of the corpus luteum, the human secretory pattern has not yet been determined. Recent findings by McNeilly and Baird (1989) have demonstrated pulsatile inhibin release from preovulatory follicle(s) in the ewe. We hypothesized that inhibin would be secreted by the corpus luteum in an intermittent (pulsatile) manner and perhaps would be coupled with LH and/or progesterone secretion. To test this hypothesis, frequent blood samples for immunoreactive levels of LH, progesterone and inhibin were obtained in normal women, for a period of 24 h, in the midluteal phase of their menstrual cycle and their respective secretion patterns were analysed.

MATERIALS AND METHODS

Subjects

Five women volunteers (age range: 23–33 years) were studied during the course of a spontaneous menstrual cycle for the assessment of corpus luteum function. These women were also subjects in a previous report (Soules *et al.*, 1988). All women had a history of normal menstrual cycles and biphasic basal body temperature charts before entering the study, were within 10% of ideal body weight (range: 90–107%, Metropolitan Life Tables, 1980) and not taking any medications. During the midluteal phase of the study cycle, each subject was admitted to the General Clinical Research Center of the University of Washington for a period of 24 h. All subjects were studied between 7 and 10 days after the LH surge as determined from daily venous blood samples obtained between 0800 and 1000 h. During each admission, venous blood samples (5 ml) were obtained every 20 min through a heparinized indwelling intravenous line. Serum was separated, frozen at

−20°C, and stored until analysis for immunoreactive LH, progesterone and inhibin. This study was approved by the University of Washington Human Subjects Committee and each subject gave her informed consent prior to study.

Hormone assays

Serum samples from each subject were analysed within a single assay for each hormone. LH was measured by double-antibody radioimmunoassay (RIA) with reagents supplied by the National Institutes of Health using the LER-907 reference preparation as the standard (Midgley, 1966). The sensitivity of the LH assay was 6 μg/l at a volume of 200 μl; intra and inter-assay coefficients of variation (CV) were 5·5 and 8·4%, respectively.

Serum progesterone was measured by RIA with reagents from Diagnostic Products Corporation (Los Angeles, CA). This assay had a cross-reactivity of 0·3% with 17α-OH progesterone and <0·01% with testosterone, oestradiol, pregnenolone and cortisol (data supplied by producer). Sensitivity in our laboratory was <1·6 nmol/l at a serum volume of 100 μl; intra and inter-assay CV were 9·0 and 12·0%, respectively. External quality control for the progesterone assay used the World Health Organization programme, which involves monthly measurements of unknown samples; no significant bias (>10%) was detected, and variabilities were comparable to those detected for internal quality controls.

Serum inhibin was measured by the methods of Robertson *et al.* (1988b), in a heterologous double-antibody RIA based on purified 31 kDa bovine follicular fluid inhibin. The rabbit antiserum (As 1989) was raised to 31 kDa bovine inhibin and ^{125}I-labelled 31 kDa bovine inhibin was used as a tracer. Cross-reactivity of transforming growth factor-beta, bovine activin A, bovine Mullerian inhibitory substance and free subunits of inhibin obtained following reduction and alkylation of 31 kDa bovine inhibin was <1% in this assay. The inhibin RIA standard was derived from pooled serum (obtained from women undergoing ovulation induction) and was calibrated against a partially purified human follicular fluid preparation using the techniques previously described (McLachlan *et al.*, 1987b). Assay sensitivity was 100 U/l at a serum volume of 200 μl in duplicate. Intra-assay CVs in the upper, mid, and lower regions of the standard curve were 10·7, 2·8 and 6·0%, respectively. The interassay CV, as derived from the repeated measurement of multiple dilutions of serum obtained from normal women during the midluteal phase, was 10·9% from 10 assays.

Recently, an α-subunit-derived dimeric protein (termed pro-α-C) has been isolated from bovine follicular fluid (Robertson *et al.*, 1989). This substance cross-reacts in this inhibin RIA, but shows no inhibin-like bioactivity. The α-subunit isolated by Knight *et al.* (1989), however, does not cross-react in the present assay (D. deKretser, personal communication). Studies of the ratio of bioactivity to immunoreactivity in women (Robertson *et al.*, 1988b) and rats (Robertson *et al.*, 1988a) suggest that any interference of non-bioactive substances in the present RIA is small.

Statistical analysis

The existence of ultradian fluctuations in inhibin secretion was verified before the data were subjected to pulse analysis. We first performed a quadratic regression on each data series using every assay replicate in that series. The values predicted by the regression were

then subtracted from each replicate to provide a set of residuals from which slow trends in the data had been removed. These residuals were subjected to a one-way analysis of variance to ascertain whether there were any statistically significant, rapid variations in the data series. This analysis indicated that ultradian variations were present in the inhibin data series from each of the five women.

Pulse analysis was performed using an adaptive-threshold method, the DC 3 pulse detector, previously described and evaluated (Clifton *et al.*, 1988). A pulse was defined as an increase from local minimum to local maximum that was greater than a threshold value, which was determined in an iterative manner (Clifton, 1987). To examine the interrelationships between hormones we used both cross-correlation and coincidence analysis. Cross-correlation analysis determines whether two hormones tend to rise and fall either in unison with each other, or with some delay between the two. Both slow trends and rapid changes in hormone levels are accounted for by cross-correlation. Coincidence analysis determines whether pulses in one hormone series tend to occur at either the same time or at some fixed interval before or after pulses in another corresponding hormone data series.

Cross-correlations were calculated with no time shift or by shifting one data series various intervals (+20 min each interval) relative to the other data series. For comparisons between the LH and the inhibin data series, only a discrete range of cross-correlations (0–120 min) were considered plausible to account for possible LH-dependent release of inhibin from the corpus luteum. For comparisons between the progesterone and inhibin data series, only correlations at no time shift and ±20 min (one time interval) were considered.

Coincidence analysis was performed by statistical criteria derived from Monte Carlo computer simulations (Moore, 1969; Clifton *et al.*, 1988). The Monte Carlo technique recognizes that coincident pulses could occur by chance alone, dependent upon the number of confirmed pulses within the two data series. Pulses from two hormonal series were considered coincident if an identified pulse from one series occurred simultaneously or was within one data interval (±20 min) of an identified pulse in the second series. Additionally, in the LH–inhibin comparisons, the inhibin series was shifted from one to six time intervals (up to 2 h) after its respective LH level prior to the examination for coupling of pulses. Correlation between hormone data series and the coincidence of distinct pulses was considered significant at the $\alpha = 0{\cdot}05$ level.

RESULTS

During the 24-h period sampled, LH, progesterone and inhibin each exhibited an intermittent (pulsatile) secretory pattern. An example of the variability present for each hormone in a single individual (Fig. 1) and group findings (Table 1) are shown. For these five subjects, LH pulses were relatively infrequent with a large pulse amplitude (group mean (±SE) LH pulse frequency = 6·2 ± 0·7/24 h, group mean amplitude = 139% above mean LH level). In contrast, inhibin pulses were more frequent with a small pulse amplitude (group mean inhibin pulse frequency = 14·6 ± 6·9/24 h, group mean amplitude = 17% above mean inhibin level). For progesterone, average pulse frequency and amplitude were intermediate to LH and inhibin (9·2 ± 1·2/24 h and 36% above the group mean progesterone level, respectively). For the study group, there were no significant correlations between the five individual 24-h mean levels of LH-progesterone ($r = -0{\cdot}46$, $P = 0{\cdot}43$), LH-inhibin ($r = -0{\cdot}54$, $P = 0{\cdot}34$), or progesterone-inhibin ($r = 0{\cdot}65$, $P = 0{\cdot}23$).

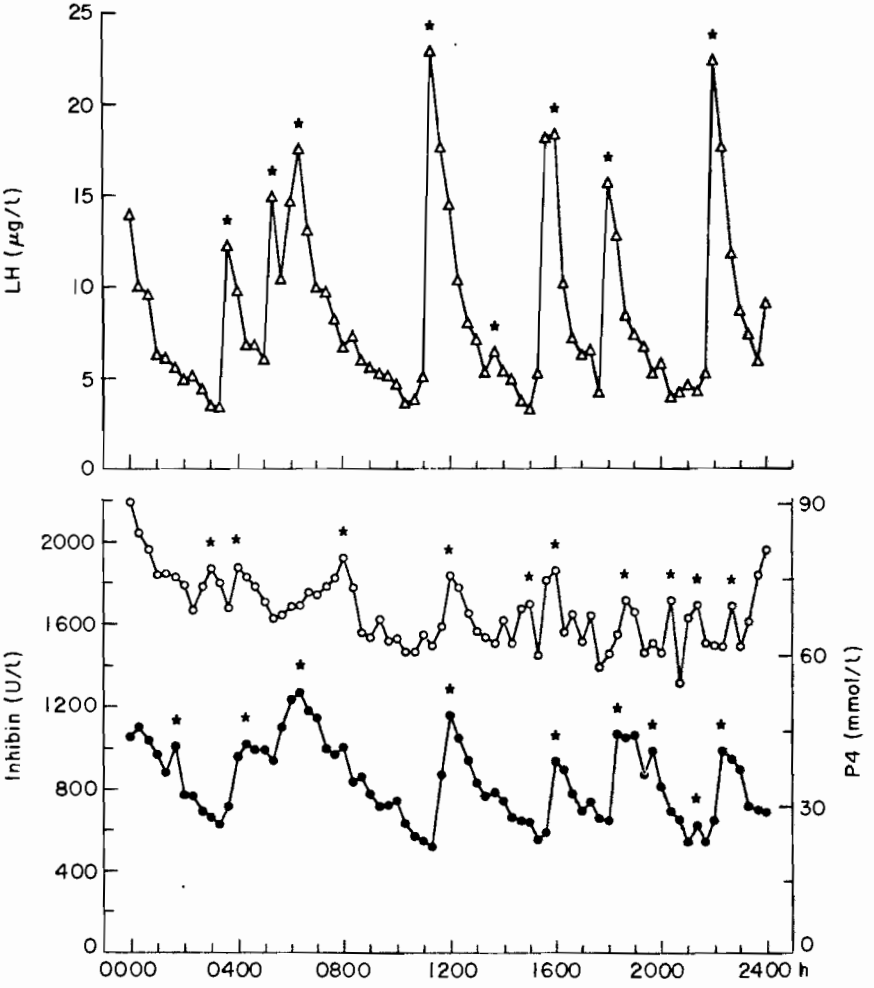

Fig. 1. Immunoreactive levels of △, LH, ○, inhibin and ●, progesterone (P4) for subject E, 10 days after the LH surge. *Points identified as pulse peaks. (SI unit conversion for P4: 1 ng/ml = 3·18 nmol/l).

The inhibin and LH data series were significantly cross-correlated in three of the five subjects (Fig. 2a), with maximal correlation at different shiftings of the inhibin data series (time lags: 0–120 min). Luteal production of progesterone and inhibin were significantly cross-correlated in three of the five subjects at no time lag or ±20 min (Fig. 2b).

When the LH–inhibin and progesterone–inhibin data sets were evaluated for coincident pulses, there was no evidence for a significant coupling between the hormones at any time lag (Table 2).

DISCUSSION

In this study, midluteal immunoreactive inhibin levels, like LH and progesterone, demonstrated a pulsatile pattern. While an intermittent pattern is the usual mode of

Table 1. Pulse analysis results, 24 h midluteal admission

Subject	LH			Progesterone			Inhibin		
	Pulses /24 h	Mean pulse amplitude (μg/l)	Mean LH (μg/l)	Pulses /24 h	Mean pulse amplitude (nmol/l)	Mean prog. (nmol/l)	Pulses /24 h	Mean pulse amplitude (U/l)	Mean inhibin (U/l)
A	7	12·9	10·0	12	21·9	76·0	21	412	1918
B	7	13·5	14·9	11	12·1	44·5	8	485	2089
C	5	42·8	30·7	5	18·8	29·9	11	129	1202
D	4	26·4	12·9	9	12·1	33·4	23	108	1252
E	8	11·3	8·5	9	14·0	36·3	10	281	1667
$\bar{x}\pm$SEM	6·2±0·7	21·4±6·0	15·4±4·0	9·2±1·2	15·8±2·0	44·0±8·4	14·6±6·9	283±75	1626±394

Progesterone conversion factor: 1 ng/ml = 3·18 nmol/l.

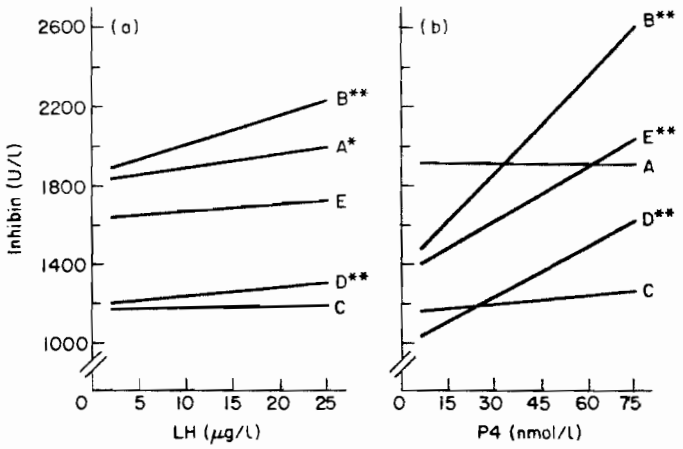

Fig. 2. Cross-correlation analysis of inhibin and both a, LH and b, progesterone levels. Three of the five women demonstrated a significant relationship (*$P<0.05$, **$P<0.01$). Values of r: a: A, 0·25; B, 0·35; C, 0·18; D, 0·44; E, 0·11; b: A, −0·01; B, 0·50; C, 0·11; D, 0·63; E, 0·43.

secretion for hormones, patterns differ between various hormones and endocrine organs. For example, the luteal steroid secretion pattern (e.g. progesterone) appears to combine a relatively rapid LH-entrained pattern upon a more prolonged pattern over several hours (Soules *et al.*, 1988). A luteal peptide secretion pattern (e.g. inhibin), however, could very well differ from the steroid pattern. In this study, we found the inhibin secretory pattern to be relatively more rapid and of lower amplitude in comparison to progesterone.

Although the half-life of inhibin has not yet been determined in humans, we believe the inhibin secretion pattern is probably due to pulsatile release with a rapid initial phase of clearance, followed by a slower second phase. In the rat, inhibin has been shown to have two distinct phases of clearance: a rapid initial component ($T_{\frac{1}{2}}=15$ min) and a slower second component ($T_{\frac{1}{2}}=20$ h), (Robertson *et al.*, 1988a). If human inhibin half-time is

Table 2. Coincident pulses between the LH, progesterone and inhibin data series

Subject	Coincident pulses (±20 min), no shifting		
	LH–P4	LH–inhibin	P4–inhibin
A	2 (6)	7 (>)*	10 (12)
B	3 (6)	2 (5)	5 (7)
C	1 (3)	1 (5)	2 (5)
D	1 (4)	4 (>)*	8 (>)*
E	4 (6)	2 (6)	6 (7)

P4 Progesterone.

( ) Number of coincident pulses to achieve statistical significance.

* The probability is greater than 0·05 that all pulses will be coincident, even when the pulse generators are independent.

similar and it is secreted in pulses, a low-amplitude discrete secretory pattern overlying a more prolonged secretion pattern would be expected. A potential confounding variable is that immunoreactive, not bioactive, precursor forms of inhibin (e.g. pro-α-C) may circulate in serum and contribute to the observed secretion pattern. Whether pro-α-C or other cross-reacting substances even occur in human beings, and if so, whether they circulate in significant quantity, is unknown. Further studies will be necessary to verify and clarify these findings.

Alternatively, the fluctuations seen in inhibin levels may be due to sharp alterations in the hormone's metabolic clearance rate (MCR), abrupt changes in the volume of distribution (V_d) of the hormone, or a combination of these factors. Although episodic secretion of inhibin is an appealing explanation for the observed variability, significant postprandial increases in the MCR of progesterone have been reported (Nakajima & Gibson, 1989). The effects of food and other physiological events on the MCR of inhibin and LH are at present unknown. Rapid changes in the V_d of circulating inhibin could contribute, as well, to the variability in the inhibin levels. This is unlikely, however, since all subjects remained in an inactive recumbent position during the study period. We favour episodic secretion as the major factor for the observed variability in inhibin levels in this study.

Although daily luteal inhibin levels parallel progesterone levels (McLachlan *et al.*, 1987a, b), the results of this study indicate only a loose association between inhibin and progesterone secretory patterns. Analysis of the intermittent hormone pulses did not suggest a coupled secretion pattern on a minute-to-minute basis, nor significant coincident secretion. Even despite the relatively high frequency of inhibin pulses and the potential for false positive coincident secretion with LH or progesterone, relatively few LH–inhibin and progesterone–inhibin pulses were coincident (Table 2). These findings are in agreement with McNeilly and Baird (1989) who reported pulsatile inhibin release from the granulosa cells of preovulatory follicle(s) in the ewe, unrelated to pulses of LH or oestradiol. Further study by the same group failed to find an association between

secretory bursts of inhibin and endogenously and exogenously administered LH (Campbell *et al.*, 1989). The absence of coupling between inhibin and progesterone seen in this present report may be due to differences in the synthesis and subsequent release of a peptide (e.g. inhibin) compared with a steroid (e.g. progesterone). Alternatively, distinct subgroups of luteal cells may be responsible for inhibin and progesterone secretion. A previous study (Ohara *et al.*, 1987) has demonstrated that progesterone emanates from both the large (>20 μm diameter, granulosa-derived) and small (5–20 μm diameter, theca-derived) luteal cells. At present, it is unknown whether inhibin originates from the large luteal cells and/or the small luteal cells. Besides different cell types of origin for inhibin and progesterone, other possible differences could also include: additional control systems besides LH, autocrine and paracrine relationships (e.g. activin), and modulation by growth factors.

Collectively, our findings further support the existence of a different mechanism for LH-dependent luteal inhibin production compared to progesterone. It is possible that the cell(s) responsible for inhibin production may be more sensitive to and/or have a prolonged receptor affinity for LH and require only infrequent stimulation for episodic inhibin secretion. This concept would explain the acute dependency of inhibin upon LH stimulation (McLachlan *et al.*, 1989), yet would account for the lack of a clear association between individual inhibin and progesterone secretory patterns. It is possible that an immunoreactive precursor form of inhibin (e.g. pro-α-C) may not be under LH control. Alternatively, the luteal cell(s) responsible for progesterone and inhibin production may have post-receptor differences and/or translational differences accounting for their secretory patterns. These findings and hypotheses underscore the complexity of the many possible physiological mechanisms controlling luteal hormone secretion.

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