EFFECT OF THE NORETHISTERONE MINIPILL ON THE PLASMA LEVELS OF BIOLOGICALLY AND IMMUNOLOGICALLY ACTIVE LUTEINIZING HORMONE IN WOMEN

By

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ABSTRACT

The relationship between biologically and immunologically active luteinizing hormone (LH) in plasma was investigated daily throughout a menstrual cycle in 29 women before and during the second month of the oral administration of norethisterone (Mini-Pe®, Astra-Syntex) in a daily dose of 0.3 mg. The biological activity was determined by an in vitro bioassay and the immunological activity by a radioimmunoassay method. A characteristic midcycle surge of LH was observed with both assay methods in all 29 control cycles. Of the 29 treatment cycles studied with both assay methods, 10 cycles exhibited a similar LH pattern, although the levels of both activities were significantly depressed ($P < 0.001$) during all phases of the cycle. The biological to immunological (B/I) ratios of plasma LH between the treatment and control cycles were not significantly different.

Of the remaining 19 treatment cycles, in which no "midcycle" LH peak was evidenced, the levels of both activities in the treatment periods were significantly lower ($P < 0.05$) than in the follicular phase and significantly higher than in the luteal phase ($P < 0.05$) of the control cycles. The overall B/I ratios were significantly lower in the treatment periods than in the control cycles ($P < 0.001$). However, the influence of this difference on the general pattern of immunological activity in comparison with that of biological activity was relatively small.

It is concluded that the irregular patterns of immunological hLH activity which are observed after treatment with low doses of norethisterone are similar to those of the biological activity.

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The continuous administration to normally menstruating women of oral contraceptives containing microdoses of progestogens frequently results in abnormal plasma and urinary patterns of LH activity throughout the menstrual cycle, sometimes in contrast to the seemingly normal patterns of plasma oestradiol and progesterone (for reviews consult Population Reports (1975). Moghissi (1976) and Fotherby (1977)).

In a recently completed large scale study, in which plasma levels of immunoreactive LH, oestradiol and progesterone were analysed daily in 43 women throughout a control cycle and then during the second month of the daily oral administration of 300 µg of norethisterone, an unexpected dichotomy was frequently observed between the profile of the plasma levels of LH, on the one hand, and the levels of oestradiol and progesterone, on the other hand (Landgren et al., in press). In some cases a marked LH surge was observed in the absence of any rise in progesterone levels (Landgren & Diczfalusy 1978), whereas in other cases the lack of any midcycle surge of LH was associated with normal “ovulatory” profiles of progesterone, 17-hydroxy-progesterone, 20α-dihydroprogesterone and oestradiol (Diczfalusy 1978). These findings raise the question as to whether the plasma levels of LH assayed by radioimmunoassay procedures in women taking norethisterone minipills are truly reflecting the biologically active hormone present in the circulation.

A study of the daily levels of LH was therefore undertaken in 29 women, using in parallel an in vitro bioassay and a radioimmunoassay technique during a pre-treatment (control) cycle and during the second treatment cycle with daily oral doses of 300 µg of the norethisterone minipill.

MATERIAL AND METHODS

Abbreviations. – B: biological activity, I: immunological activity, NET: norethisterone (17α-ethynyl-17β-hydroxy-4-oestren-3-one), RIA: radioimmunoassay.

International reference preparations. – Human pituitary gonadotrophin (1st IRP of Human Pituitary Gonadotrophins (FSH and LH/ICSH)) for bioassay (code No. 69/104) and human pituitary luteinizing hormone (LH/ICSH) 1st IRP for immunoassay (code No. 68/40) were provided by the National Institute of Biological Standards and Control, London. Norethisterone (NET) was a commercially available preparation (Mini-Pe®, placed at the disposal of this laboratory by the Astra-Syntex Co).

Clinical material. – The 29 healthy women who volunteered for the study were part of a larger group studied in an investigation of the pharmacodynamic effects of NET, the details of which are to be reported elsewhere (Landgren et al., in press). These 29 women were in the fertile age (mean age 29 years, range 19 to 39 years) with a history of regular cycles and exhibiting normal haemoglobin values. The criteria used to define these cycles as normal have been previously described (Guerrero et al. 1976; Aedo et al. 1976).
Blood was collected daily throughout the control cycles from all 29 women and
from the same women for one cycle during the second month of the administration of
the "minipill" (300 µg NET/day orally). In addition, blood was collected daily through¬
out the sixth treatment cycle from 5 of the 29 subjects. The plasma was stored at
−20°C prior to assay.

The average length of the control cycles was 28 days (range 24–32). Due to bleeding
irregularities it was not possible to establish the cycle length in the treatment cycles.
Of the second month treatment cycles, of which bleeding records were available in 27
subjects, the bleeding patterns were seemingly unambiguous in 15 cycles with the
number of days of blood loss (corresponding to menstrual bleeding) ranging from 1 to
7 days (average 4.6 days). In the remaining 12 treatment cycles, spotting or bleeding
was observed at various times throughout the collection period.

**Plasma progesterone.** – The plasma levels of progesterone were determined through¬
out the menstrual cycle in all cycles by a rapid radioimmunoassay (RIA) procedure
(Aso et al. 1975). In a recently completed study (Landgren et al., submitted) of the
daily hormone levels of 100 normally menstruating women it was observed that 95 %
of the normal ovulatory cycles were associated with plasma progesterone levels above
5.0 ng/ml (16 nmol/l) for at least 5 days. These values have been used in this study
to characterize a "normal" ovulatory cycle. The patterns of progesterone which were
lower than normal showed either no change in progesterone levels (<2 ng/ml)
throughout the collection period (characterized as "suppressed") or exhibited an inter¬
mediate, incomplete response (>2 < 5 ng/ml; characterized as "partially suppressed").

**In vitro bioassay method.** – An in vitro bioassay method for measuring LH activity
was used (Van Damme et al. 1974). The method is based on the production of
testosterone by Leydig cell preparations from mouse testes incubated in the presence
of graded doses of LH. It yields valid potency estimates when applied to plasma
samples obtained from any part of the menstrual cycle (Romani et al. 1976). The LH
activity of the plasma samples investigated in this study was determined at one dose
level against a linearized standard response line, using the 69/104 preparation as
standard (Robertson et al. 1978). The within-assay variation as assessed by the coef¬
ficient of variation of duplicate measurements from 400 estimates was 4.6%. The
between-assay variation as assessed on the basis of the reproducibility of the potency
of a plasma pool, assayed concurrently with the unknowns, was 3.8% (n = 26).

**Radioimmunoassay (RIA) procedure.** – An hLH-RIA procedure using the Kabi hLH
kit (Kabi Diagnostica, Stockholm) was used. Non-equilibrium assay conditions were
employed with the 69/104 preparation as standard. Further details of the assay are
presented elsewhere (Robertson & Diczfalusy 1977). The immunoactivity in the plasma
samples was determined at one dose level against a linearized standard response line.
The 68/40 preparation, while it is the officially recommended and by now preferred
standard preparation for the radioimmunoassay of hLH in this laboratory, could not
be employed in this assay system¹), since the logit log-dose response line was non-linear
over the whole working range (Robertson & Diczfalusy 1977). This was in contrast to

¹) The assay reported in this paper were conducted in 1976–1977. Using another RIA-
system with improved reagents, in recent studies the 68/40 reference preparation and
a great variety of plasma samples yield parallel dose-effect lines (Robertson et al.,
submitted).
the response line of the 69/104 preparation, which was linear over 6–8 doses (64–256-fold dilution). The parallelism between the dose response line for the standard (69/104) and a female plasma pool when assayed over an eight fold dilution range was assessed from the difference in slope values between the respective preparation. The difference was not significant ($P > 0.05$) between the slope values for the logit log-dose response line of the 69/104 preparation (2.37 with 95% confidence limits at 2.09 and 2.68) and those of the plasma pool (2.50 (2.32 : 2.68) ; $n = 5$). The within-assay and between-assay variations, assessed in a similar manner as in the case of bioassays, were 5.3% (400 comparisons) and 4.4% (11 assays), respectively.

In the calculation of results, a lognormal distribution of individual observations (Gaddum 1945) was assumed, i.e., all calculations were performed using logarithmically transformed values to give geometric means and confidence limits. An analysis of variance and a calculation of appropriate contrasts were also carried out on the logarithmically transformed values.

**Classification of control and treatment cycles.** – It is general practice in combining LH data obtained throughout the menstrual cycle to use the day of the midcycle LH surge and, in addition, the day of the onset of menstruation as reference points around which the LH activity throughout the remainder of the cycle can be arranged. In the present study, no difficulties were encountered with the classification of the LH data from the control cycles; however, it was not possible to use this scheme with all the treatment cycles, since the LH peak in these cases was either suppressed or abolished. Furthermore, since the bleeding patterns on average are irregular during minipill treatment, the correct designation of the day of the onset of menstruation would be liable to error. As a consequence, the following design was used to characterize the treatment and control cycles. For the control cycles, the LH profiles were characterized by the day of the LH surge (designated as day LH) and by the day of the onset of menstruation (designated as day M). The normal menstrual cycle was thus described in terms of days LH -8 to LH + 8 and M -4 to M + 4. For the treatment cycles with an LH peak, as arbitrarily defined as an elevation in biological LH activity of at least 100% over the average LH levels during the preceding and succeeding 16 to 20 days of the cycle, the activities were grouped around the LH peak only (days LH - 8 to LH + 8 Group I). For the treatment cycles without an LH surge (Group II), the LH activities were arranged according to the days of collection (1 to 21) from the first day of preceding bleeding. In these latter cycles, it is recognized that the first day of collection may not necessarily correspond to that of the initiation of progesterone withdrawal bleeding (menstruation) of the previous cycle.

An alternative classification was also examined, which was based on evidence of luteal activity in the same cycles as assessed from the profile of plasma progesterone (Landgren et al. submitted). Following this classification, three groups could be formed according to whether the progesterone pattern was normal (>5 ng/day for 5 days), suppressed (<2 ng/ml) or partially suppressed (>2 < 5 ng/ml).

**RESULTS**

Of the 29 second month treatment cycles with NET, two groups could be formed, based on the presence (10 cycles, Group I), or absence (19 cycles, Group II) of a significant LH surge. The plasma levels of biological and immunological LH activities and their ratios throughout the treatment and...
the corresponding control cycles for these two groups are presented in Figs. 1 and 2. In Group I, while the biological and immunological activities were significantly reduced throughout the treatment cycles ($P < 0.001$), the corresponding B/I ratios remained unchanged ($P > 0.05$). In Group II, the plasma levels of both biological and immunological activities were lower ($P < 0.01$ and $P < 0.05$, respectively) in the treatment cycles in comparison to those found in the follicular phase of the control cycles (days LH-2 to LH-8), although higher ($P < 0.05$ and $P < 0.001$, respectively) than those of the luteal phase (days LH + 2 to LH + 8) of the control cycles. The B/I ratios were nevertheless significantly higher in both phases of the control cycle ($P < 0.001$) than throughout the treatment cycles.

A significant decrease in B/I ratios was observed in the midcycle region (days LH-1, LH, LH+1) of the control cycles of both groups ($P < 0.001$). The relationship between the two activities in plasma before and after NET.

**Fig. 1.**

Group I: The plasma levels of biological (○) and immunological (●) LH activity before and during the second month of daily oral administration of 300 µg of norethisterone. This group consists of treatment cycles and the corresponding control cycles in ten women in which a midcycle LH surge was present. Both activities were assayed in terms of the 69/104 preparation. LH = day of LH peak, M = day of the onset of menstruation. Geometric mean values with 95% confidence limits are presented. The decrease in B/I ratios at midcycle in the control cycles is significant ($P < 0.001$).
For further details see Fig. 1.

Group II: The plasma levels of biological (○) and immunological (●) LH activity before and during the second month of daily oral administration of 300 µg of norethisterone to 19 women. No LH surge was present in the treatment cycles. C.D. = collection day.

Treatment was examined also in other ways. The first was based on the comparison of the geometric mean B/I ratios. As indicated in Table 1, no significant differences were found between the treatment cycles of Groups I and II and the corresponding control cycles.

The second approach consisted of the regression analysis of the two activities for each cycle, with the biological activity as the independent variable. Because of the large differences in LH values in comparison with the remainder of the cycle, the LH levels associated with the midcycle LH surge (days LH-1, LH, LH + 1) were excluded from the analysis, since their inclusion had a disproportionate influence on the slope and intercept values of the regression line. From the regression analysis of each cycle, the slope and the regression line and its intercept on the X or Y axis were calculated. A comparison of these regression coefficients for the treatment and control cycles showed no significant differences in Group I, while a significant difference ($P < 0.01$) was observed in the intercept value of the regression line between the treatment and control values in Group II.
The differences observed between the intercept values of the regression lines for the Group II treatment and control cycles suggest that a large difference in the B/I ratios could also be found in plasma samples with low levels of biological activity in Group II cycles, which would not be observed in Group I cycles. To test this possibility, the mean B/I ratio of 5 samples with the lowest biological activity was calculated for each cycle in both Group I and Group II treatment and control cycles. As seen in Table 1, a significant difference in B/I ratios ($P < 0.05$) was observed between treatment and control periods in Group II, which was not observed in Group I. This difference was due to significantly higher plasma levels of immunological LH activity ($P < 0.01$) in the treatment periods of Group II compared to the corresponding control cycles.

No differences were observed ($P > 0.05$) in the regression coefficients and B/I ratios found in the sixth month of treatment in comparison with either the corresponding second month treatment cycles or the control cycles from the same subjects.

When the biological and immunological LH activities and their ratios were regrouped according to the level of luteal activity in the same cycles, no

Table 1.
The geometric mean biological : immunological ratio of hLH in plasma samples (n = 5) with the lowest biological activity found in each treatment and control cycle when grouped according to the presence (Group I) or absence (Group II) of a midcycle LH surge in the 2nd month of norethisterone administration. For comparison, the B/I ratios obtained throughout the whole cycle or treatment period are also indicated.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>No. of cycles</th>
<th>B/I ratios</th>
<th>Bioassay (mIU/ml)</th>
<th>Radio-immunoassay (mIU/ml)</th>
<th>B/I ratios&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (Group I + II)</td>
<td>29</td>
<td>7.7 (6.9: 8.6)</td>
<td>11 (10 :12)</td>
<td>1.4 (1.3:1.6)</td>
<td>7.7 (7.2:8.1)</td>
</tr>
<tr>
<td>T (Group I + II)</td>
<td>29</td>
<td>6.6 (5.6: 7.6)</td>
<td>9.8 ( 8.9:11)</td>
<td>1.6 (1.4:1.8)</td>
<td>7.2 (6.5:7.6)</td>
</tr>
<tr>
<td>C (Group I)</td>
<td>10</td>
<td>8.2 (6.3:11 )</td>
<td>13 (11 :16)</td>
<td>1.6 (1.2:2.0)</td>
<td>8.2 (7.0:9.4)</td>
</tr>
<tr>
<td>T (Group I)</td>
<td>10</td>
<td>8.2 (6.3:11 )</td>
<td>9.9 ( 8.5:11)</td>
<td>1.4 (1.0:1.8)</td>
<td>7.9 (6.9:9.1)</td>
</tr>
<tr>
<td>C (Group II)</td>
<td>19</td>
<td>7.4 (6.5: 8.4)</td>
<td>10 ( 9.1:11)</td>
<td>1.4 (1.2:1.5)</td>
<td>7.4 (6.9:8.0)</td>
</tr>
<tr>
<td>T (Group II)</td>
<td>19</td>
<td>5.8 (4.9: 7.0)</td>
<td>9.8 ( 8.5:11)</td>
<td>1.7 (1.5:1.9)</td>
<td>6.8 (6.1:7.5)</td>
</tr>
</tbody>
</table>

NS: not significant.
* $P < 0.05$.
** $P < 0.01$.

<sup>a</sup> Estimated throughout the whole cycle.
significant differences were observed between treatment and the corresponding control cycles, neither with respect to the geometric mean (daily) B/I ratio for each cycle nor with regard to the regression coefficients.

**DISCUSSION**

There is a high degree of similarity between the profiles of biologically and immunologically active hLH in plasma of women after treatment with NET. The differences that are observed between the treatment and control periods, although statistically significant, would seem to be rather limited. These differences, which were noted in the group in which no discernible LH peak was observed during the treatment periods (Group II), were attributed to the higher levels of immunologically active, biologically inactive material compared to those in the corresponding control cycles. This conclusion was based on the differences between the treatment and control cycles in the intercept values of the regression line obtained from the regression analysis of the two activities. In support, significant differences in the B/I ratios were obtained for samples chosen from throughout the treatment and control cycles with the lowest biological activity. These differences might be attributed to the higher plasma levels of biologically-inactive immunologically-active material (e.g. LH subunits) in the treatment cycles than in the corresponding control cycles. Additional studies involving the fractionation of the biological and immunological activities of plasma into their individual components will be needed to resolve the nature of this material.

The elevated B/I ratios (i.e. greater than unity) observed in this study can, to a large extent, be accounted for by the reagents employed in the RIA. Studies conducted concurrently with the present investigation have shown that such elevated B/I ratios are due to the purity of the LH preparation used as standard (*Robertson & Diczfalusy 1977*) as well as to the presence of iodinated subunits in the iodinated LH preparation used as tracer in the RIA, and to the specificity of the antiserum employed (*Suginami et al. 1978*).

In the present study, which was conducted in 1976–1977, the preferred standard of this laboratory (the 1st IRP for LH, for immunoassy, code No. 68/40) was not employed, since this preparation did not give parallel response lines with plasma in the RIA system used, while, conversely, a less suitable standard (the 1st IRP for LH/FSH for bioassay, code No. 69/104) exhibited parallelism. As a consequence and as a direct result of the elevated levels of biologically inactive, immunologically active material in the 69/104 preparation, the immunological values determined in plasma with this preparation as standard were underestimated in comparison with the corresponding levels of biological LH activity, resulting in grossly elevated B/I ratios. In a previous study (*Suginami et al. 1978*), a B/I ratio of the 68/40 standard in terms of the
69/104 preparation with the same RIA reagents as those employed in the present study was estimated as 8.3 with 95% confidence limits at 7.1 and 10.0. This ratio can be used to re-express, in terms of the 68/40 preparation, the B/I ratios obtained in the present study. The calculated ratios (0.87–0.93) (cf. Table 1) are similar to those recently reported for plasma LH (Bártfai et al. 1979; Robertson et al., submitted) with the use of considerably improved reagents in the RIA. Thus, the elevated ratios obtained in the present study can be attributed to a very large extent to the choice of the LH reference standard preparation employed in the RIA. It is to be stressed that this aspect does not influence the conclusions drawn in the present investigation, since this study is essentially a comparison between treatment and control cycles from the same subjects.

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