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# Ovulation and Gonadotropin-Releasing Activity of [D-LEU$^6$, DES-GLY $NH_2{}^{10}$, PRO-ETHYLAMIDE$^9$] -GNRH (38715)

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In our previous studies on amino acid substitution in pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$ (GnRH) we found that [des-Gly $NH_2{}^{10}$, Pro-ethylamide$^9$]-GnRH was five times more potent than the native releasing hormone (1, 2). Subsequently, it was reported that D-Ala$^6$-GnRH was nearly four times more active than GnRH both *in vivo* and *in vitro* (3), but that analogs having a bulky side-chain on the D-amino acid in position 6, such as D-Val or D-Pro, were less potent than GnRH. These investigators suggested that the D-Ala$^6$ substitution for Gly$^6$ restricts conformational degrees of freedom of GnRH and improves binding at the receptor, thus increasing biological activity.

Early in our evaluation of the structure-activity relationships of the GnRH molecule, we synthesized both DL-Leu$^6$-GnRH and [DL-Leu$^6$, des-Gly $NH_2{}^{10}$, Pro-ethylamide$^9$]-GnRH (Fukuda *et al.*, unpublished) and found that these analogs were 8 and 20 times more potent than GnRH in the ovulation-induction assay, respectively. However, when the pure optical isomers of Leu were incorporated into position 6, it was found that [L-Leu$^6$, des-Gly $NH_2{}^{10}$, Pro-ethylamide$^9$]-GnRH was only 5% as active as GnRH *in vitro*: whereas, D-Leu$^6$-GnRH was 29 times more active than GnRH in causing ovulation (4). We decided, therefore, to investigate the ovulation-inducing, LH-, and FSH-releasing properties of the nonapeptide [D-Leu$^6$, des-Gly $NH_2{}^{10}$, Pro-ethylamide$^9$]-GnRH in the rat, rabbit, and the ewe.

*Materials and Methods.* The procedures of synthesis, deblocking and purification of [D-Leu$^6$, des-Gly $NH_2{}^{10}$, Pro-ethylamide$^9$]-GnRH (II) and its physicochemical properties were described previously (4).

Mature female Sprague–Dawley rats (Sprague-Dawley, Madison, WI) weighing 200–250 g were individually caged in a controlled 14-hr (5:30 AM–7:30 PM) light and 10-hr dark environment. Only rats showing two consecutive 4-day cycles were selected for experimentation on diestrus (iv injection at 14:30) or proestrus (oral gavage at 13:30). Blood was collected from the rats by jugular puncture at various times after treatment, held at 4° for 24 hr, centrifuged, and the serum decanted and stored at −20°. The rats were killed by cervical dislocation 20–24 hr after treatment and the oviducts compressed between two slides and the ova counted under a low-power microscope. Sodium pentobarbital (30 mg/kg) was injected ip to block the spontaneous LH surge in the proestrous rat. Serum LH was determined by radioimmunoassay (5) and expressed as ng NIH-LH-S16/ml serum. In two experiments a composite serum sample from rats within a treatment group was assayed for FSH (6). NIAMD-Rat-FSH-RP-1 was used as the standard reference preparation.

Ovulation was also determined in mature, virgin, unanesthetized New Zealand does (7). LH release in the cycling and anestrous ewe was also studied according to procedures reported previously (8). The test materials were prepared fresh daily and diluted in saline–0.1% bovine serum albumin. The statistical methods of Bliss (9) were used to compute relative potencies, 95% confidence limits, and deviations from parallelism. The significance of differences among treatments was determined using Duncan's multiple-range test (10).

*Results.* The results of two rat experiments are illustrated in Tables I and II. When given sc to the diestrous or orally to the proestrous rat, II was 47 and 59 times, respec-

TABLE I. EFFECT OF GnRH AND [D-LEU$^6$, DES-GLY NH$_2^{10}$, PRO-ETHYLAMIDE$^9$]-GnRH (II) ON OVULATION AND SERUM LH AND FSH IN THE DIESTROUS RAT.

| Group | Dose (ng/100 g body wt) | No. ovulating | Mean number ova | Serum LH (ng/ml) 45 min | Serum LH (ng/ml) 90 min | Serum FSH[a] (ng/ml) 45 min | Serum FSH[a] (ng/ml) 90 min |
|---|---|---|---|---|---|---|---|
| Control | — | 0/3 | — | 4.0 ± 1.0 | 4.5 ± 1.5 | 284 | 230 |
| GnRH | 25 | 0/3 | — | 3.3 ± 0.3 | 2.5 ± 0.5 | 316 | 285 |
| | 50 | 0/4 | — | 6.2 ± 1.3 | 4.5 ± 1.3 | 316 | 332 |
| | 100 | 2/7 | 2.0 ± 1.0[b] | 12.0 ± 2.4 | 8.1 ± 1.6 | 359 | 355 |
| | 200 | 4/5 | 5.8 ± 1.8 | 20.0 ± 1.9 | 24.0 ± 3.0 | 327 | 375 |
| | 300 | 3/4 | 5.7 ± 1.2 | 37.0 ± 3.7 | 30.0 ± 8.5 | 427 | 436 |
| | 400 | 3/4 | 4.0 ± 1.7 | 27.0 ± 3.6 | 28.0 ± 4.9 | 382 | 435 |
| | 500 | 2/2 | 3.0 ± 1.0 | 24.0 ± 1.5 | 58.0 ± 18.0 | 363 | 478 |
| II | 1.25 | 0/4 | — | 1.9 ± 0.5 | 3.3 ± 0.3 | 246 | 257 |
| | 2.00 | 0/2 | — | 2.2 ± 1.8 | 6.5 ± 1.5 | 256 | 338 |
| | 2.50 | 0/4 | — | 2.7 ± 0.3 | 9.0 ± 1.7 | 323 | 335 |
| | 3.00 | 3/5 | 1.0 ± 0.0 | 3.8 ± 0.8 | 14.0 ± 3.0 | 291 | 336 |
| | 4.00 | 2/5 | 4.0 ± 3.0 | 6.2 ± 0.6 | 33.0 ± 11.0 | 316 | 375 |
| | 5.00 | 8/9 | 4.8 ± 0.8 | 10.0 ± 1.6 | 50.0 ± 4.1 | 345 | 462 |
| | 10.00 | 5/5 | 8.2 ± 1.8 | 17.0 ± 1.2 | 125.0 ± 16.0 | 339 | 824 |

$ED_{50}$ of ovulation ±95% confidence limits (ng) for GnRH and [D-Leu$^6$, des-Gly NH$_2^{10}$, Pro-ethylamide$^9$]-GnRH was 160 (70–380) and 3.4 (1.5–8.0), respectively.

[a] Based on duplicate analysis of a single composite serum sample for the group.

[b] SEM.

TABLE II. RESPONSE OF THE PROESTROUS RAT TO ORALLY ADMINISTERED GnRH AND [D-LEU$^6$, DES-GLY NH$_2^{10}$, PRO-ETHYLAMIDE$^9$]-GnRH (II).

| Group | Dose (μg/100 g body wt) | No. ovulating | Mean number ova | Serum LH (ng/ml) 60 min | Serum LH (ng/ml) 120 min | Serum FSH[a] (ng/ml) 60 min | Serum FSH[a] (ng/ml) 120 min |
|---|---|---|---|---|---|---|---|
| Control | — | 0/8 | — | 1.6 ± 0.5 | 2.7 ± 1.5 | 162 | 162 |
| GnRH | 25 | 1/4 | 1.0 ± 0.0[b] | 5.0 ± 1.5 | 5.3 ± 3.2 | 172 | 193 |
| | 50 | 1/4 | 14.0 ± 0.0 | 6.1 ± 4.1 | 32.0 ± 31.0 | 196 | 258 |
| | 75 | 3/5 | 7.3 ± 3.2 | 8.6 ± 3.6 | 82.0 ± 70.0 | 220 | 249 |
| | 100 | 3/3 | 10.7 ± 0.3 | 26.0 ± 17.0 | 13.0 ± 3.5 | 232 | 240 |
| II | 0.31 | 0/3 | — | 1.2 ± 0.3 | 1.8 ± 0.2 | 179 | 180 |
| | 0.47 | 0/4 | — | 1.6 ± 0.1 | 2.3 ± 0.2 | 198 | 199 |
| | 0.62 | 4/7 | 11.8 ± 0.9 | 18.0 ± 13.0 | 61.0 ± 44.0 | 231 | 324 |
| | 1.25 | 6/7 | 13.0 ± 0.7 | 3.5 ± 0.8 | 7.6 ± 2.3 | 196 | 228 |
| | 2.50 | 4/5 | 10.2 ± 2.5 | 11.0 ± 4.2 | 97.0 ± 37.0 | 289 | 360 |

$ED_{50}$ of ovulation ±95% confidence limits (μg) for GnRH and [D-Leu$^6$, des-Gly NH$_2^{10}$, Pro-ethylamide$^9$]-GnRH was 54 (28–100) and 0.92 (0.53–1.60), respectively.

[a] Based on duplicate analysis of a single composite serum sample for the group.

[b] SEM.

tively, more effective than GnRH in causing ovulation. Although the analog increased the number of ova shed at the higher dose levels in the diestrous rat ($P < 0.05$), neither peptide affected the number of ova recovered from proestrous rats. In general, serum LH and FSH levels were related to the dose of the peptide. Serum LH was usually less at 90 than at 45 min in diestrous rats which received GnRH sc, but in rats which were given II the reverse was evident. Thus, the rate of serum LH increase was slower and

the peak concentration occurred at a later time in those rats which received the analog. The greater LH-releasing activity of the analog was also apparent in the orally treated proestrous rat, although serum LH levels were not consistently related to the dose of either peptide. These results may reflect variations in gastrointestinal absorption of both peptides when administered orally. Serum FSH levels increased less dramatically than LH in both experiments and were usually greater at 90 than at 45 min.

A detailed study of the time-course of LH release after a single sc injection of GnRH or II at the respective $ED_{50}$ for ovulation is shown in Fig. 1. The analog released significantly less LH than GnRH by 30 min ($P < 0.01$), but greater amounts of LH were present in the serum at 90 ($P < 0.05$), 120, 150, and 180 min ($P < 0.01$) in rats which received II. The integrated areas under the LH curves indicated a 1.9-fold greater release by II, thus the calculated over-all potency of the analog was nearly 90 times that of GnRH.

In the rabbit, the analog was 31 times more potent than GnRH in causing ovulation (Table III). However, the number of corpora lutea and recovered ova was not related to the dose level of either peptide.

The greater LH-releasing potency of II was also evident in both the mid-luteal and

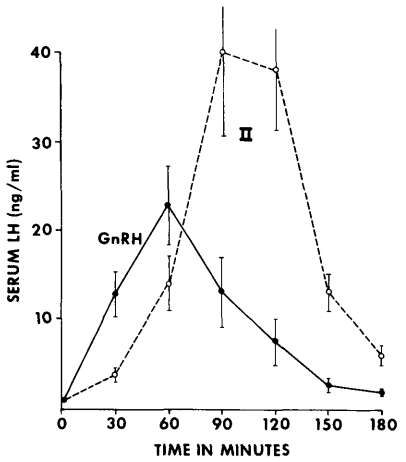


FIG. 1. Serum LH concentrations in the diestrous rat prior to and after sc injection of either GnRH or [D-Leu$^6$, des-Gly $NH_2{}^{10}$, Pro-ethylamide$^9$]-GnRH (II) (160 and 3.4 ng/100 g body wt., respectively). All rats were bled at 0 time with groups of five or six bled at either 30 and 120, 60 and 150, or 90 and 180 min after injection. Ovulation rates and mean number of ova were 8 of 17, 4.0 ± 1.3, and 7 of 15, 4.9 ± 0.8 for GnRH and analog-treated rats, respectively. Vertical bars indicate the SEM.

TABLE III. INTRAVENOUS INJECTION OF GnRH AND [D-LEU$^6$, DES-GLY $NH_2{}^{10}$, PRO-ETHYLAMIDE$^9$]-GnRH [II] ON OVULATION IN THE ESTROUS RABBIT.

| Group | Dose (ng/kg body wt) | No. ovulating | Mean number corpora lutea | $ED_{50}$ of ovulation (ng/kg body wt) |
|---|---|---|---|---|
| Control | — | 0/4 | — | — |
| GnRH | 125 | 1/6 | 6.0 ± 0.0[a] | 195 |
| | 250 | 5/6 | 8.8 ± 1.0 | (125–309)[b] |
| | 500 | 5/6 | 8.8 ± 0.4 | |
| II | 3.12 | 0/7 | — | 6.25 |
| | 6.25 | 4/8 | 8.2 ± 0.6 | (3.5–11) |
| | 12.50 | 6/7 | 6.7 ± 0.7 | |
| | 25.00 | 3/3 | 9.3 ± 0.3 | |

[a] SEM.
[b] 95% confidence limits.

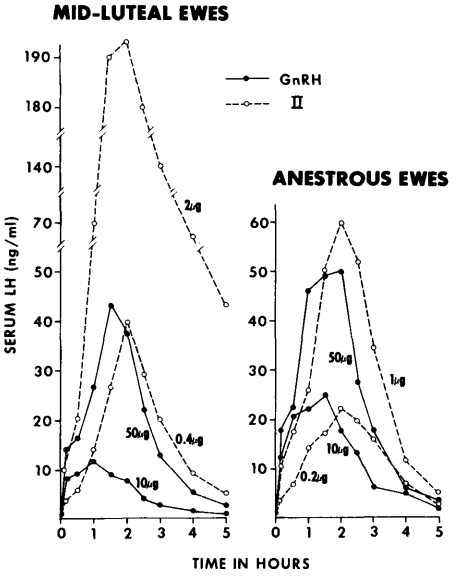


FIG. 2. Comparison of the LH response in the mid-luteal phase (three/dose level) and the early anestrous ewe (two/dose level) after a single im injection of GnRH or [D-Leu$^6$, des-Gly $NH_2{}^{10}$, Pro-ethylamide$^9$]-GnRH (II). Jugular blood was collected at 0, 15, 30, 60, 90, 120, 150, 180, 240, and 300 min after injection.

anestrous ewe (Fig. 2). A dose of 0.4 μg II effected a similar LH release in the mid-luteal phase ewe to that of 50 μg GnRH; whereas, in the anestrous ewe the analog was approximately 50 times more effective since 0.2 and 1 μg released approximately the same amount of LH as 10 and 50 μg GnRH, respectively. The increase in serum LH after the analog was delayed and peaked at a later time compared with GnRH in both stages of reproduction. However, the disappearance rate of LH from the serum after peak levels was similar for both peptides.

*Discussion.* The results of these experiments indicate that II, which has a bulky side-chain at position 6, possesses surprisingly intense ovulation-inducing and *in vivo* LH-releasing activity. Moreover, the potency of this analog is at least as great as that of the corresponding D-Ala$^6$ analog (11, 12), which does not have a bulky side-chain. Apparently the side-chain of the D-amino acid in position 6 does not contribute a significant spatial effect to the interaction with the binding site of the hormone receptor or to intermolecular interactions.

From our studies in both the rat and the anestrous ewe it was evident that the onset in serum LH increase was delayed after injection of II compared to GnRH. With the analog, peak serum concentrations of LH also occurred later, reflecting the delayed onset in LH release rather than a prolonged release stimulus. However, the decline in serum LH after peak concentrations was similar for both releasing substances. Thus, the pattern of serum LH change was not greatly different after either GnRH or II, except during the early phase of the response, if the dose was adjusted for differences in potency. This suggests that the increased ovulating potency of II can be accounted for almost entirely by the intrinsic LH-releasing activity of the nonapeptide and not be a pattern of LH release different from that of GnRH.

In a recent publication, Vilchez-Martinez *et al.* (13) reported that both the D-Ala$^6$ and D-Leu$^6$ analogs of [des-Gly $NH_2^{10}$, Pro-ethylamide$^9$]-GnRH were potent LH- and FSH-releasing substances in the immature male rat. When compared on an identical dose basis to GnRH, both analogs induced a greater LH release and effected a delay in peak serum concentrations of the hormone. However, the decline in serum LH after peak concentrations was similar for the three peptides. In an earlier report, Coy *et al.* (11) suggested that the D-Ala$^6$ analog prolonged the release of LH in the immature rat when the peptide was infused over a 4-hr period. In contrast, we observed similar LH-release patterns in the cycling rat (Rippel, unpublished) when these two analogs were compared to GnRH on an equal ovulating-activity basis (4). The reasons for these discrepancies are unknown, but probably reflect peculiarities of the assay systems or the different basis used for the dose-level comparisons.

It also appears from the pattern of LH release that the increased activity of II was not the result of a delayed disappearance of the peptide from the systemic circulation. This was apparent in our studies when GnRH and II were compared on an equal ovulating basis. Thus, it seems likely that the D-amino acid residue in position 6 of [des-Gly $NH_2^{10}$, Pro-ethylamide$^9$]-GnRH restricts freedom of the central part of the molecule, enabling a more desirable conformational fit to the receptor site(s) of the target organ, resulting in intense ovulation-inducing and gonadotropin-releasing activity.

*Summary.* The ovulation-inducing and gonadotropin-releasing activities of [D-Leu$^6$, des-Gly $NH_2^{10}$, Pro-ethylamide$^9$]-GnRH (II), were evaluated in rats, rabbits, and sheep. A sc dose of 3.4 ng/100 g body wt of the analog was equal to 160 ng/100 g body wt of GnRH in causing ovulation in the diestrous rat. At these dose levels, the integrated LH release was 1.9 times greater for the analog. Both the time of increase and maximum serum concentrations of LH were delayed after injection of the analog. Oral administration of II and GnRH to the proestrous rat resulted in an $ED_{50}$ for ovulation of 0.92 and 54 μg/100 g body wt, respectively. Serum levels of LH and FSH were highly variable for the various treatment groups when both releasing substances were administered orally. The intense ovulating activity of II was also evident in the estrous rabbit as indicated by

an activity 31 times greater than that of GnRH. Additionally, the analog was at least 50 times more active than GnRH in releasing LH in both the mid-luteal and anestrous ewe. From our experiments with the cycling rat it appears that the intense ovulation-inducing activity of II can be accounted for by the intrinsic LH-releasing activity of the nonapeptide, rather than by a prolonged release stimulus.

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