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Inhibition of HCG-Induced Ovarian and Uterine Weight Augmentation in the Immature Rat by Analogs of GnRH (39413)

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(Introduction by E. T. Kimura)

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It is firmly established that human chorionic gonadotropin (HCG) increases the sensitivity of the immature rat ovary to exogenous follicle stimulating hormone (FSH) and that the relationship between FSH and ovarian weight as shown by Steelman and Pohley (1) is linear. The reliance of ovarian weight changes following augmentation of FSH by HCG has gained widespread use as a quantitative bioassay for the measurement of FSH in various preparations (1, 2).

In collaboration with Takeda Industries, Ltd., we demonstrated in a number of studies that several analogs of the synthetic decapeptide, *p*-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2 (GnRH), have intensified ovulation-inducing and gonadotropin-releasing activity. For example, replacement of the C-terminal glycine amide of GnRH with various alkylamines resulted in a three- to fivefold increase in ovulation induction and *in vitro* LH and FSH release (3-5). At about the same time, Monahan *et al.* (6) reported that [D-Ala^6]-GnRH was also more active than GnRH. Subsequently, we reported that analogs containing both a C-terminal ethylamide and position 6 D-amino acid modification possessed intense biological activity (7, 8). One of these analogs, [D-Leu^6, des-Gly$NH_2{}^{10}$, Pro-ethylamide^9]-GnRH (I), was at least 30 to 100 times more active than GnRH in causing gonadotropin release or ovulation in either the mature female rat, ewe, or estrous rabbit (9).

On the basis of these observations we designed experiments to determine if I released sufficient FSH *in vivo* to allow for augmentation by HCG in the Steelman-Pohley rat. To our surprise, large doses of I inhibited the usual ovariogenic and uterotropic effects of HCG. Thus, this report examines in detail the inhibitory effect of I on HCG augmentation and describes preliminary findings with other analogs of GnRH.

Methods. The synthesis, purification, characterization, and biological properties of the peptides studied in these experiments were reported previously (3, 7, 8). Sprague-Dawley female rats were obtained at 21 days of age (Sprague-Dawley Co., Madison, Wis.) and maintained in colony cages in quarters with controlled temperature (26 ± 1°) and lighting (lights on 5 AM to 7 PM). Tap water and ground laboratory chow were available *ad libitum*. Hypophysectomized rats (Altech Laboratory, Madison, Wis.) were given access to 10% glucose-saline in place of tap water. Injections were initiated at 22 days of age according to the procedures of Steelman and Pohley (1). Each rat was injected sc at 8 AM and 4 PM for 3 consecutive days, unless indicated otherwise. The rats were killed with an overdose of ether at 25 days of age and the ovaries and uteri removed, cleaned, and weighed to the nearest 0.2 mg on a Roller-Smith balance. One group of animals was injected for various periods of time and checked twice daily for vaginal opening. All solutions were made with buffered saline (10) and stored at 4°. In most cases, HCG[1] and the peptide or the gonadotropin were administered as a single injection. The data were analyzed by analysis of variance and differences between treatments compared using orthoganol contrasts or Duncan's Multiple Range Test (11).

Results. HCG increased both ovarian and uterine weight of the immature rat and augmented the effect of FSH on ovarian weight (Table I: Expt. 1). The relatively large dose of 5 μg of I administered during the 3-day test period did not affect ovarian or uterine

[1] Antuitrin "S," Parke-Davis, Detroit, Michigan.

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TABLE I. Effect of [D-Leu^6, des-GlyNH$_2^{10}$, Pro-Ethylamide^9]-GnRH (I), GnRH, LH, and FSH on Mean Ovarian and Uterine Weight in HCG-Augmented Immature Rats.

Group[a]	Dose of peptide (μg/rat)	Ovarian weight (mg ± SE)	Uterine weight (mg ± SE)
Experiment 1			
Buffer	–	13.8 ± 0.6	28.8 ± 2.0
HCG	–	41.4 ± 0.3	116 ± 4.4
HCG + FSH (90 μg)[b]	–	120 ± 21	129 ± 7.4
I	5	10.6 ± 0.2	26.8 ± 2.0
HCG + I	0.008	42.6 ± 2.3	109 ± 8.0
	0.04	41.6 ± 3.5	100 ± 4.5
	0.2	27.5 ± 0.6	91.8 ± 8.7
	1	17.2 ± 1.6	41.6 ± 5.6
	5	12.5 ± 1.3	31.8 ± 2.0
HCG + GnRH	500	20.2 ± 1.6	96.4 ± 8.5
	2500	15.8 ± 0.9	38.6 ± 2.8
Experiment 2			
HCG	–	37.3 ± 2.3	110 ± 4.4
HCG + I	5	11.4 ± 1.0	28.0 ± 2.2
HCG + LH (12.5 μg)	–	34.2 ± 1.0	114 ± 4.9
HCG + FSH (90 μg)	–	82.2 ± 13	118 ± 2.3
HCG + LH + I	5	10.9 ± 0.9	26.7 ± 1.2
HCG + FSH + I	5	10.6 ± 0.9	29.4 ± 3.2
Experiment 3			
HCG	–	34.1 ± 2.6	
I	5	10.2 ± 0.9	
HCG + I (−2 hr)[c]	5	10.8 ± 0.4	
(−4 hr)	5	11.8 ± 0.4	
(+2 hr)	5	11.9 ± 1.4	
(+4 hr)	5	10.8 ± 0.7	
HCG (AM only)	–	37.2 ± 2.2	
HCG (AM) + I (PM)	5	22.8 ± 2.0	
I (AM) + HCG (PM)	5	16.8 ± 1.0	
HCG (Days 2 and 3)	–	20.6 ± 1.1	
I (Day 1) + HCG (Days 2 and 3)	5	20.7 ± 1.3	

[a] Five rats per group. Each rat received 50 IU of HCG. All animals were injected twice a day (22–24 days of age) unless indicated otherwise.
[b] Numbers in parenthesis indicate total dose per rat.
[c] Time interval before (+) or after (−) HCG injection.

weight when rats did not receive HCG along with the peptide. However, dose levels of 0.2 to 5 μg of I effected a dose response inhibition ($P < 0.01$) of the increases in ovarian and uterine weight induced by HCG. At the 5-μg dose of I, ovarian and uterine weights were not significantly different from buffer controls. GnRH, injected at much higher levels, was also inhibitory.

The inhibitory effect of I also occurred when rats received either exogenous LH in addition to HCG, or when FSH was augmented by HCG (Expt. 2). In Expt. 3, when I was injected either 2 or 4 hr before or after HCG, ovarian augmentation was inhibited to the same extent as when both hormones were administered together. Injecting I at 8 AM and HCG at 4 PM resulted in a greater inhibition of ovarian augmentation ($P < 0.05$) than when the sequence was reversed. Moreover, when the total dose of I was given on Day 1 and HCG on Days 2 and 3 of the treatment period, inhibition of ovarian weight augmentation did not occur.

Increasing the 3-day augmenting dose of HCG from 50 to 500 IU did not overcome the inhibition by I of ovarian weight increase (Table II). Moreover, graded levels of exogenous LH (10, 20, and 40 μg) did not affect ovarian weights of control or HCG-treated rats. Hypophysectomy did not alter the inhibitory effect of I as relative changes in ovarian weight were similar to those in intacts (Table III).

TABLE II. Effect of HCG, LH, and [D-Leu^6, des-Gly $NH_2{}^{10}$, Pro-Ethylamide^9]-GnRH (I) on Ovarian Weight (Mean ± SE) in Immature Rats.[a]

		Dose of HCG (IU/rat)		
		0	50	500
I (μg/rat)	0	11.7 ± 1.3	38.4 ± 4.1	34.6 ± 2.9
	0.32		19.9 ± 0.9	21.5 ± 1.9
	0.80		16.2 ± 1.7	17.4 ± 1.5
	2.0		14.8 ± 1.4	13.6 ± 0.8
	5.0	10.3 ± 0.8	11.9 ± 0.7	11.0 ± 0.6
LH[b] (μg/rat)	0	13.8 ± 0.8	37.3 ± 2.2	
	10	13.4 ± 0.3	41.2 ± 2.2	
	20	13.6 ± 0.4	42.0 ± 3.9	
	40	14.8 ± 0.8	36.1 ± 4.7	

[a] Injected twice daily: 22-24 days of age.
[b] Potency of 1.6 × NIH-LH-S1.

TABLE III. Mean Ovarian Weights in Intact and Hypophysectomized Immature Rats after Gonadotropin and [D-Leu^6, des-Gly $NH_2{}^{10}$, Pro-Ethylamide^9]-GnRH (I) Treatment.

	Intact		Hypophysectomized	
Group[a]	No. rats	Ovarian weight (mg ± SE)	No. rats	Ovarian weight (mg ± SE)
Control	15	15.0 ± 0.5	18	5.5 ± 0.3
HCG (50 IU)	15	51.6 ± 3.2	11	12.5 ± 1.4
HCG + FSH (180/μg)	5	121.0 ± 11.0	6	37.2 ± 5.4
HCG + I (5 μg)	15	15.2 ± 1.0	14	4.7 ± 0.3

[a] Injected twice daily: 22-24 days of age.

Table IV shows that both HCG and HCG + I injected on Days 22-24 of age significantly ($P < 0.01$) advanced the age at vaginal opening (29 vs 40 days for both controls and I alone, respectively). Twice a day administration of 0.05 or 0.5 μg of I on Days 22-31 of age also resulted in a significant ($P < 0.01$) reduction in age at vaginal opening (36 vs 40 days for controls). However, continuing the twice daily injections until puberty effected a 5-day delay in vaginal opening at 0.5 μg ($P < 0.01$), but not at the 0.05-μg dose.

That inhibition of ovarian augmentation in the HCG model is not limited to one analog of GnRH is shown in Table V. For example, [D-Ala^6, des-Gly$NH_2{}^{10}$, Pro-ethylamide^9]-GnRH (analog IV), another potent agonist of GnRH, was as active as I in inhibiting HCG augmentation. Two less active agonists of GnRH, [des-Gly$NH_2{}^{10}$, Pro-ethylamide^9] (II) and [D-Leu^6] (III), also inhibited HCG augmentation but to a lesser extent than either I or IV. In contrast, the des-His^2 analog of [D-Leu^6]-GnRH (analog V) increased ovarian weight at 1 and 5 μg ($P < 0.05$) as did the des-His^2 analog of I (analog VI) at the 0.2-μg dose level.

Discussion. In addition to an intense gonadotropin agonist activity, [D-Leu^6, des-Gly$NH_2{}^{10}$, Pro-ethylamide^9]-GnRH (I) exerts a pronounced inhibition of ovarian and uterine augmentation of HCG in the immature rat, as demonstrated in the present experiments. This surprising biological effect was dose-related. Moreover, the peptide effectively inhibited ovarian and uterine weight increase when injected either with or at a site separate from HCG, or when administered up to 4 hr before or after HCG. An interval of 8 to 16 hr between injection of I and HCG, regardless of which was given first, resulted in a lesser inhibition of HCG augmentation than at reduced intervals. Moreover, a level of HCG 10 times that normally used in the Steelman-Pohley bioassay model for FSH did not reduce the inhibitory effect of I. The increase in ovarian weight after HCG augmentation of exogenous FSH was also inhibited by the analog.

The mechanism whereby I inhibited ovar-

ian and uterine augmentation of HCG in the present studies remains obscure. Johnson and Mallampati (12) observed that the immature 23-day-old rat released large quantities of both FSH and LH when injected ip with a relatively low level of GnRH (40 ng). However, in our experiments exogenous LH (0.5–64 μg of activity, per rat) did not affect ovarian weight either in the presence or absence of HCG, suggesting that the inhibitory effect of I on HCG augmentation may be extrapituitary, possibly uterine or ovarian. In support of this supposition, we observed that I also inhibited HCG augmentation in the hypophysectomized rat. It is unlikely that sufficient residual gonadotropin is present in the acutely hypophysectomized rat which accounts for a response similar to that observed with the intact animal. Moreover, we recently observed (unpublished) that I effectively inhibited ovarian weight augmentation of HCG in mature rats at 10 weeks after hypophysectomy.

Nevertheless, the inhibition of HCG-induced ovarian and uterine augmentation by I and other analogs of GnRH was, in general, proportional to their intrinsic LH and FSH releasing activities reported previously (3–9). In the immature male rat, 0.5 μg of synthetic GnRH administered once daily for 2 weeks significantly decreased accessory sexual organ weight (13). These workers presented evidence that the response was related to altered steroid biosynthesis. Recently Banik and Givner (14) reported that analog IV (our designation) advanced ovulation in the diestrous rat and prevented

TABLE IV. Influence of [D-Leu^6, des-GlyNH$_2$^{10}, Pro-Ethylamide^9]-GnRH (I) and HCG on Age at Vaginal Opening in the Immature Rat.

Group[a]	Dose of I per injection (μg/rat)	Days of age injected	Age at vaginal opening (mean ± SE)
Control	–	22–24	40.0 ± 0.4
HCG (50 IU)[b]	–	22–24	29.2 ± 0.4
HCG + I	0.83	22–24	29.4 ± 0.4
I	0.83	22–24	39.6 ± 0.3
	0.05	22–31	36.2 ± 0.4
	0.5	22–31	36.2 ± 0.5
	0.05	22–open	40.2 ± 1.2
	0.5	22–open	45.0 ± 2.0

[a] Five rats per group injected daily at 8 AM and 4 PM.

[b] Total dose per rat.

TABLE V. Effect of Various Analogs of GnRH on HCG-Augmentation of Ovarian Weight (mg ± SE) in the Immature Rat.

Peptide	Total dose of peptide (μg/rat)[a] 0.2	1.0	5.0
I	27.4 ± 1.3	18.2 ± 1.4	11.9 ± 0.3
II	38.4 ± 3.0	30.2 ± 1.8	22.2 ± 1.6
III	32.0 ± 1.3	24.6 ± 2.4	17.6 ± 2.0
IV	25.9 ± 2.4	18.3 ± 1.8	13.2 ± 1.5
V	34.8 ± 2.7	42.0 ± 3.0	47.2 ± 4.2
VI	42.0 ± 3.5	39.7 ± 3.6	37.8 ± 2.3
	100 μg	500 μg	
GnRH	22.4 ± 0.93	16.1 ± 1.1	

[a] Five rats per group injected at 8 AM and 4 PM for 3 consecutive days. Ovarian weight of HCG controls (50 IU/rat) was 33.3 ± 2.0 mg.

Peptide I: [D-Leu^6, des-GlyNH$_2$^{10}, Pro-ethylamide^9]-GnRH; Peptide II: [des-GlyNH$_2$^{10}, Pro-ethylamide^9]-GnRH; Peptide III: [D-Leu^6]-GnRH; Peptide IV: [D-Ala^6, des-GlyNH$_2$^{10}, Pro-ethylamide^9]-GnRH; Peptide V: [des-His^2, D-Leu^6]-GnRH; Peptide VI: [des-His^2, D-Leu^6, des-GlyNH$_2$^{10}, Pro-ethylamide^9]-GnRH.

mating behavior and pregnancy during the ensuing expected estrus period. It was suggested that this antifertility effect was the result of a change in ovarian steroidogenesis as measured by vaginal cytology. In our studies vaginal opening was delayed 5 days when 0.5 μg of I was administered twice a day from 22 days of age, but advanced 4 days if the peptide injections terminated at 32 days of age. In another series of experiments (Johnson *et al.*, in press), chronic administration of I inhibited normal ovarian growth, maturation, and vaginal opening in the immature rat, and prevented normal cycling and caused atrophy of the ovaries and uterus in mature animals. These results together with the work of others suggest an effect of large doses of I on ovarian steroidogenesis, but do not indicate if the mechanism is direct.

In conclusion, we have demonstrated a pronounced negative relationship between the dose of potent agonists of GnRH and the ability of HCG to augment ovarian and uterine weight in the immature rat. It remains to be demonstrated if this effect involves other endocrine organs, such as the pineal or adrenal, or if the analogs act directly on ovarian or uterine tissue.

Summary. Experiments were conducted to study the inhibitory effect of large doses of a few analogs of GnRH on ovarian and

uterine weight in the immature rat. In most experiments the 21-day-old HCG-primed animal was used. Extensive study of [D-Leu^6, des-GlyNH_2^{10}, Pro-ethylamide^9]-GnRH (I) indicated that ovarian weight increase induced by HCG was inhibited in both the intact and hypophysectomized rat. Increasing the augmenting dose of HCG from 50 to 500 IU in the intact animal did not overcome the inhibitory effect of I. Furthermore, twice-a-day injections of I into normal immature rats from 22 to 31 days of age significantly advanced puberty; however, when injections were continued until puberty, vaginal opening was significantly delayed. In general, the inhibitory effect of other analogs of GnRH in the HCG model was proportional to their intrinsic LH and FSH releasing activities. These data constitute direct evidence that large doses of the intensely active analogs of GnRH have inhibitory effects on the ovary, uterus, and reproductive function in the rat.

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