Equine Luteinizing Hormone Possesses Follicle-Stimulating Hormone Activity in Hypophysectomized Female Rats

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ABSTRACT

The ability of equine luteinizing hormone (eLH) to promote follicular growth and maturation in hypophysectomized rats has been assessed. A single injection of equine LH has been shown to promote the growth of a large number of antral and preovulatory follicles. In addition, equine LH markedly increased serum estrogen levels and uterine weight. Furthermore, equine LH, like equine chorionic gonadotropin (eCG; PMSG) was able to significantly enhance the incorporation of [^{3}H] thymidine into ovarian DNA, an activity shown to be specific to hormones having follicle-stimulating hormone (FSH) activity. Equine LH treated with an FSH antibody immunoaffinity column to remove any possible contamination still exhibited the above activity, demonstrating that the FSH activity is intrinsic to the eLH molecule. Equine LH has also been shown to be capable of inducing LH receptors in granulosa cells of ovaries of hypophysectomized rats, an activity specific to FSH-like hormones. From the doses required of eLH and the degree of response observed, it is concluded, however, that eLH in the hypophysectomized rat is less active than eCG as an FSH.

INTRODUCTION

The pituitary gonadotropins, LH and FSH, share a common and closely related chemistry. Despite their similar chemical nature, gonadotropin of most mammalian species (ovine, bovine, murine, human, porcine) show little, if any, cross-hormonal activity, i.e., intrinsic FSH activity in LH and vice versa. This is also largely true of human chorionic gonadotropin (hCG) which is biologically and chemically closely related to human LH, although recently it has been demonstrated that the hCG has a low, but intrinsic, FSH-like activity (Siris et al., 1978). Equine chorionic gonadotropin however, is a notable exception because it has long been known to possess both LH and FSH activity comparable to that displayed by pituitary gonadotropins. Both of these activities have

been shown to be intrinsic to the eCG molecule (Papkoff, 1974, 1980; Papkoff et al., 1978).

In the course of studies comparing the properties of equine LH with eCG, we concluded that eLH, like eCG, also exhibited significant FSH-like activity (Licht et al., 1979). These studies showed that eLH had FSH activity when assayed in the intact rat using the rat ovarian hCG augmentation test, as well as in various in vitro FSH radioreceptor assays employing tissue from the pig ovary, horse testis and in the case of the rat, seminiferous tubule preparations obtained from 18-day-old male rats. Equine LH, unlike eFSH, or FSH from other species (Farmer and Papkoff, 1978), however, does not stimulate synthesis of cyclic AMP in the rat seminiferous tubule preparation; rather, it inhibits the FSH response in a dose-dependent manner (Aggarwal et al., 1980).

We report here 2 additional biochemical parameters which have been used to specifically assess the FSH activity of eLH. These are: the stimulation of in vitro [^{3}H] thymidine incorporation into ovarian DNA (Sheela Rani and Moudgal, 1978), and the induction of LH receptors in the granulosa cells of hypophysectomized rats (Zeleznick et al., 1974).

MATERIALS AND METHODS

Animals

Sprague-Dawley female rats were hypophysectomized at 21 days of age and used 1 week later in these studies.

Hormones

With the exception of hCG (CR123), which was obtained as a gift from Dr. R. Canfield through NIAMDD, Bethesda, MD, all the other gonadotropins used were prepared in this laboratory by the procedures detailed earlier (Papkoff et al., 1965, 1967; Schams and Papkoff, 1972; Licht et al., 1979). Hormones were dissolved in 0.9% NaCl and appropriate doses were administered s.c. to rats according to the experimental protocol outlined below.

[³H]thymidine incorporation into ovarian DNA. The method used was essentially that of Sheela Rani and Moudgal (1977, 1978). Four rats were used per experimental group and killed with Nembutal 18 h after treatment at 0900. The ovaries and ovarian fragments suspended in 1 ml of M199 medium containing 1 μCi of [³H] thymidine (New England Nuclear thymidine, [methyl—³H]-80 Ci/mmol). Incubation was carried out in stoppered 10 ml flasks in a Dubnoff shaking incubator at 37°C for 4 h. The reaction was stopped by the addition of 50 μg cold thymidine per flask and, after washing the tissue twice with 2 ml portions of ice cold distilled water, it was homogenized in 1 ml of cold 10% perchloric acid followed by centrifugation and washing of the pellet with 2 ml of ice cold 5% perchloric acid. The macromolecular pellet obtained was hydrolyzed in 1 ml of 10% perchloric acid at 90°C fo 20 min. Aliquots from the supernatant were removed from radioactive counting as well as DNA estimation. DNA was estimated according to the method of Short et al., 1968.

Immunoaffinity chromatography. A monkey antiserum to ovine FSH (Rao and Moudgal, 1979; Sheela Rani and Moudgal, 1977) was coupled to Affigel-10 (Bio-Rad Labs., Richmond, CA) using standardized procedures as described by the manufacturer. Three hundred μl of the antiserum was coupled to 3 ml of Affigel-10 after initial washing of the gel with 9 ml of ice-cold deionized water followed by 9 ml of 0.1 M sodium bicarbonate buffer, pH 8.25. The coupling reaction was carried out in the bicarbonate buffer (2 ml per 3 ml gel) for 18 h at 4°C using a reciprocal shaker to keep the gel well mixed. At the end of this period the gel was centrifuged at 4°C, resuspended in 2 ml of bicarbonate buffer containing 20 μl of 1 M leucine methylester solution and put back on the reciprocal shaker for an additional 2 h. The last step was essential to block any free amino groups in the gel. The gel was extensively washed with bicarbonate buffer followed by 0.1 M, pH 7.4 phosphate buffered saline (PBS) until the optical density of the supernatnat at 280 nm dropped below 0.05. The gel was packed into a column in a 3 ml disposable syringe and a solution of eLH in PBS (1 mg/ml) was applied to the gel and allowed to stand at 4°C for several hours prior to elution with PBS. The unabsorbed protein which appears in the first 3 tubes (1 ml/tube) was pooled, dialyzed free of salt and lyophilized; 80—90% of the applied material could be recovered. The antibody gel can be recycled following treatment sequentially with 30 ml each of PBS containing 3 M KI and PBS alone (Muralidhar et al., 1974).

LH receptor induction studies. Female rats hypophysectomized at 21 days of age were given 2 mg of diethylstilbesterol/ rat on Days 27, 28 and 29. On the last day they received, in addition, a s.c. injection of 0.2 ml of saline, eLH, (40 μg), eCG (4 μg), or eFSH (20 μg). Animals were sacrificed 44 h later, the ovaries removed and granulosa cells expressed into medium M199 in petri dishes. The cells of ovaries from each group were pooled, washed twice with the medium, suspended in a known volume of M199 and counted. Aliquots of cell suspension in triplicate were incubated with ¹²⁵I-hCG for 1 h at 37°C to determine the binding capacity of the cells. Nonspecific binding was determined by including in 1 set of tubes an excess of cold hCG. Iodination of hCG was essentially carried out according to the procedure of Catt and Dufau (1975) and the binding studies according to the procedure of Sheela Rani et al., (1980).

Another set of aliquots of the cell suspension were incubated in the presence or absence of 20 ng of ovine LH (oLH) for 2 h at 37°C and the progesterone produced monitored by appropriate radioimmunoassay.

Other procedures. The serum samples of gonadotropin-treated rats were analyzed for estradiol by a standardized radioimmunoassay procedure as described earlier by Mukku and Moudgal (1975) using an antiserum provided by Dr. G. Erickson, University of California, San Diego. Ovarian histology was kindly done, according to standard procedures, by Dr. P. Goldsmith, Reproductive Endocrinology Center, University of California, San Francisco.

Standard statistical methods were employed for analysis of data. Student's *t* test was used for comparison of different groups. Results were considered to be "statistically significant" when the calculated P value was 0.05 or less.

RESULTS

In the initial study the effect of a single injection of equine gonadotropins on the ovarian and uterine weights of hypophysectomized 27-day-old female rats (5—7 in each group) was assessed. Both eLH and eCG were able to markedly increase the uterine weight (Fig. 1). Equine LH increased ovarian weight by only 50% and did not provide a strict dose-dependent effect when compared with the effect of eCG. The increment in uterine weight brought about by equine or ovine FSH, though marginal (45—50% compared to 200% in the case of eLH or eCG), was statistically significant; these hormones had no effect on ovarian weight. Ovine LH, even at a high concentration, did not increase either the ovarian or uterine weight.

The ability of the individual gonadotropins to increase serum estradiol was tested using the
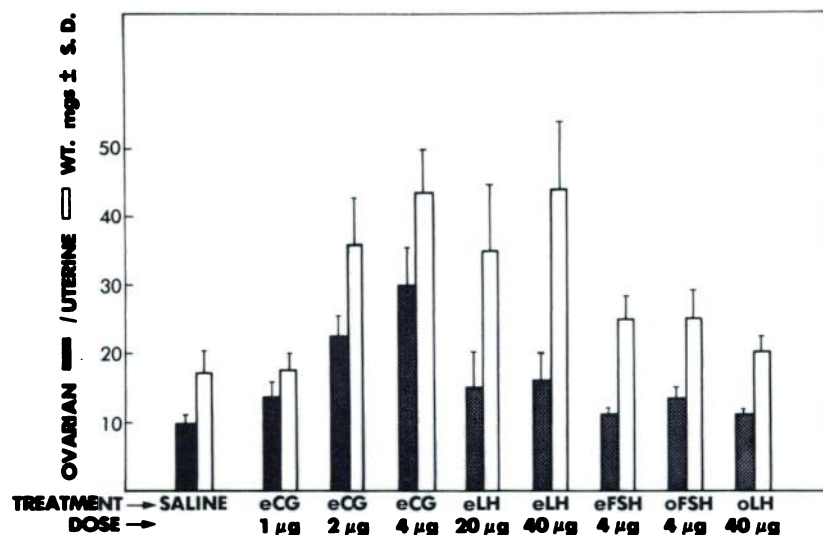
FIG. 1. Ability of a single injection of different doses of eCG and eLH to increase the ovarian and uterine weights of hypophysectomized immature rats. Sprague-Dawley rats were hypophysectomized at 21 days of age and were injected (s.c.) on Day 27 with different doses of hormones (eCG and eLH). Four groups of controls were included: saline, eFSH, oFSH, and oLH, respectively. All animals were sacrificed 48 h after injection, the ovaries and uterus carefully dissected from adhering tissue and weighed on a torsion balance. Number (n) of each group was 5–7.

same experimental protocol as above. Equine CG in this system also was much more effective than eLH in increasing serum estradiol levels (Fig. 2). The other gonadotropins tested had virtually no effect on this parameter. Since production of estrogen in the ovary is dependent upon the interplay of both FSH and LH activities, this experiment provides, once again, an assessment of the total gonadotropic potency of the hormones under study.

Confirmatory evidence for the total gonadotropic activity of eLH was obtained by histological examination of the ovary. Thus, the eLH and eCG treated rats showed the presence of a large number of antral and preovulatory follicles (Fig. 3C and D). No such antral follicles were present in the saline and eFSH treated groups (Fig. 3A and B); an increase in granulosa cell layers, however, is evident in the eFSH treated group of rats.

A better assessment of the FSH activity of eCG and eLH was obtained by determining their ability to stimulate the in vitro incorporation of [³H] thymidine into ovarian DNA. This test was based on an earlier observation of Sheela Rani and Moudgal (1978) that in intact immature rats maximal stimulation of this event can be obtained 18 h post-injection

of eCG or FSH. The results of the preliminary experiment showed clearly that in vitro [³H] thymidine incoporation into ovarian DNA can be used as a specific biochemical response to FSH since oLH, even at a dose of 40 μg, did not have any activity (Table 1). Equine LH, like other FSHs, showed a clear stimulatory activity. The fact that there was no increase in the radioactivity of the acid soluble fraction between the experimental and control groups (data not shown) showed that this stimulatory activity was not just due to an increase in the uptake of labeled thymidine.

In a second study, an attempt was made to establish whether this effect was dose related. In order to increase the sensitivity of the system to low doses of the hormone, the hypophysectomized rats received, along with different doses of gonadotropins, 2 mg of diethylstibestrol per rat. Preliminary studies had shown that this did indeed increase the sensitivity of the system, diethylstibestrol in itself not having any stimulatory activity in the hypophysectomized rats. Even though the results showed that no apparent dose-dependent response could be established with the doses tried (Table 2), it appeared that as little as 1 μg of eLH or FSH could elicit a significant re-
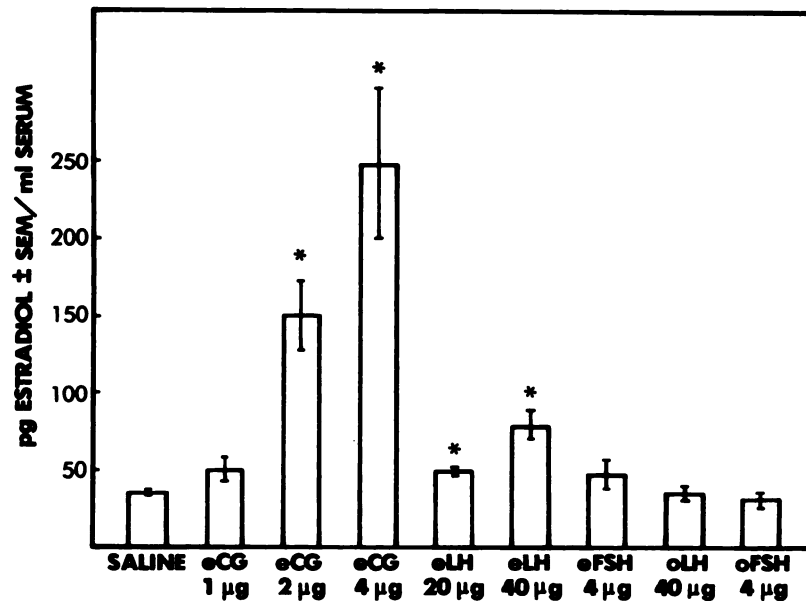
- FIG. 2. Ability of a single injection of different doses of eCG and eLH to increase serum estradiol levels in hypophysectomized immature female rats. Experimental protocol was essentially the same as in Fig. 1. Estradiol in ether-extracted samples of serum was determined by radioimmunoassay (see text). Number (n) of each group was 5—7. An *asterisk* (*) above the vertical bars indicates that these values are significantly different from the saline controls.

sponse. The slight reduction in response obtained using 5 μg of eLH or FSH was initially puzzling. However, the difference between the 2.5 μg and 5.0 μg dose group was not found to be statistically significant. The response obtained with 1 μg eFSH, however, was significantly greater than that obtained with 1 μg eLH (P<0.01). It is interesting that when the 2 chorionic gonadotropins (eCG and hCG) were compared for their relative ability to stimulate [³H] thymidine incorporation into DNA, hCG surprisingly showed minimal but significant activity. This essentially confirms the observation of Siris et el., (1978) that hCG has intrinsic FSH activity.

In order to be sure that the FSH activity associated with eLH was not due to FSH contamination, the eLH preparation was passed through an oFSH antibody Affigel column. This antibody was observed to cross-react with eFSH to the extent of 30% by radioimmunoassay. The FSH antibody affinity chromatography purified eLH was found to still be capable of significantly stimulating [³H] thy-midine incorporation into ovarian DNA (Table 3).

The FSH activity of eLH was further assessed by determining its ability to induce LH receptors in ovaries of hypophysectomized rats. The pilot study conducted to test the system clearly showed that eCG (4 μg) or eFSH (20 μg) was able to induce LH receptors (7- to 10-fold increase over control). Equine LH (20 μg) or oLH (40 μg) failed to induce such activity. Upon increasing the dose of 40 μg, eLH, however, was able to induce LH receptors (Table 4). That this increase in binding relates __ to induction of functional receptors is shown by the ability of oLH to stimulate progesterone production (Table 4).

DISCUSSION

The foregoing study provides additional evidence that eLH, like eCG, possesses both LH and FSH bioactivity. The histological, morphological, and biochemical data in this study supports and justifies such a conclusion. Although eLH appears to be decidedly less active than eCG in its effect on ovarian weight, estrogen production, and [³H] thymidine incorporation into ovarian DNA, it is evident that eLH is able to support follicular growth through
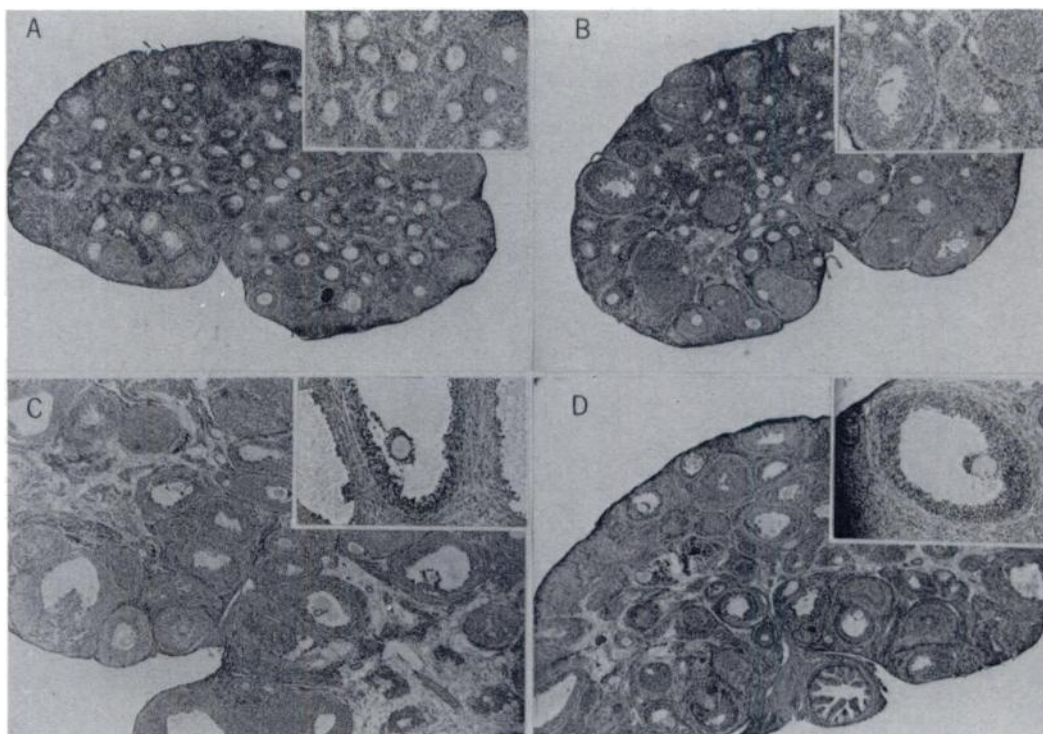
FIG. 3. Histological sections of ovaries of hypophysectomized rats treated with (A) saline; (B) 20 μg eFSH; (C) 4 μg eCG and (D) 40 μg eLH. Rats were autopsied 48 h after a single injection of the hormone and the ovaries processed for histology by standard procedures (H and E stain). Magnification: whole ovarian sections × 30 and *inserts* × 80.

all its stages. Though both eCG and eLH (at a higher dose) bring about an equivalent increase in uterine weight, there appears to be a wide disparity in the amount of estrogen production elicited by the 2 gonadotropins. A possible explanation for this may be that the estrogen produced by eLH stimulation, though less than that produced by eCG, may be enough to

TABLE 1. Effect of administering equine and ovine gonadotropins in vivo to hypophysectomized immature female rats on in vitro [³H] thymidine incorporation into ovarian DNA.

Group	Treatment	[³H] Thymidine incorporated into 100 μg/DNA/4 h mean cpm ± SEM	P (compared with control)
I	Saline	45,578 ± 6,023	...
II	eCG 4 μg	143,575 ± 15,453	<0.001
III	eLH 40 μg	110,339 ± 9,801	<0.001
IV	eFSH 20 μg	98,734 ± 17,037	<0.025
V	oLH 40 μg	53,624 ± 6,761	NS
VI	oFSH 20 μg	113,037 ± 14,070	<0.001

[a]The animals (4 rats in each group) were autopsied 18 h after initiation of treatment. The ovaries were removed, minced and incubated in M-199 in the presence of 1 μc [³H] thymidine for 4 h. The tissues were processed for DNA and the incorporation of [³H] thymidine (see text for details). The hormone treatment did not bring about any change in pool size (acid soluble fraction).

TABLE 2. Effect of a single injection of different doses of gonadotropins on in vitro [³H] thymidine incorporation into ovarian DNA in hypophysectomized female rats.

Treatment (4 rats/group)	Dose (μg/rat)	[³] Thymidine incorporated/100 μg ovarian DNA/4 h mean cpm ± SEM	Net cpm incorp.	Percent increase
Saline	. . .	22,631 ± 3,150	. . .	. . .
eLH	1.0	48,403[b] ± 4,376	25,772	113
	2.5	59,198[b] ± 6,153	36,567	161
	5.0	48,359[b] ± 4,649	25,728	113
eFSH	1.0	69,393[b] ± 3,098	46,762	206
	2.5	100,661[c] ± 17,883	78,030	344
	5.0	66,683[a] ± 11,101	44,052	194
eCG	5.0	106,047[c] ± 6,617	83,416	368
hCG	5.0	37,879[a] ± 2,351	15,248	67

[a,b,c]P values of groups compared to saline control: [a]$P<0.01$; [b]$P<0.005$; [c]$P<0.001$.

TABLE 3. Ability of immunoaffinity purified equine LH to stimulate [³H] thymidine incorporation into ovarian DNA.

Group	Treatment	(n)	[³H] Thymidine incorporated/100 μg ovarian DNA/4 h mean cpm ± SEM
I	Saline control	(3)	23,564 ± 1,771
II	eLH − 2.5 μg	(4)	44,108 ± 6,628[a]
III	eCG − 1.0 μg	(4)	60,525 ± 4,040[b]

[a]Significantly different from Group I, $P<0.025$.

[b]Significantly different grom Group I, $P<0.025$ and Group II, $P<0.05$.

TABLE 4. Ability of equine LH to induce LH receptors in granulosa cells of hypophysectomized rats.

In vivo treatment[a]	¹²⁵I-hCG bound/mg prot/1 h mean cpm ± SD	Progesterone produced[b] ng/4 X 10⁵ cells/2 h mean ± SD	
		−LH	+LH
Saline	2355 ± 424	ND	0.25
eCG 4 μg	66753 ± 7750	. . .	. . .
eLH 40 μg	44574 ± 6450	3.66 ± 0.42	55.00 ± 0.86
eFSH 20 μg	20440 ± 1902	2.04 ± 0.26	53.75 ± 1.70

[a]For experimental details see text. Each group had 4 rats and the granulosa cells recovered from the 8 pairs of ovaries were distributed into 3 tubes.

[b]Cells were incubated in the presence and absence of 25 ng oLH/tube.

satisfy the minimal requirement of this steroid for maximal increase in uterine weight. Alternately, the difference in the effects of the eCG and eLH might be attributed to differences in their circulatory half-lives. Thus, while eCG could be stimulating the continuous production of estradiol, resulting in high levels being monitored at 48 h, the effect of eLH on this parameter could be tapering off much earlier, leading to the measurement of relatively lower titers of estradiol 48 h posttreatment. Supportive evidence for the sustained action of eCG is shown in the data of Sheela Rani and Moudgal (1978) in immature rats.

The ability of eLH to stimulate the rate of [³H]thymidine incorporation into ovarian DNA, as well as induce LH receptors, a response shown to be specific to hormones having FSH activity (Sheela Rani and Moudgal, 1978; Sheela Rani et al., 1980; and the present study), points clearly to eLH exhibiting intrinsic FSH activity in in vivo situations. From the various parameters used in this study to assess both total gonadotrophic and FSH activity of eLH, it appears that the hormone is decidedly less active than eCG as an FSH. Whether this can primarily be ascribed to differences in circulatory half-lives or affinity of binding to LH/FSH receptors of the hormones is presently unclear.

Equine LH has earlier been shown to exhibit cross-reactivity in an eFSH homologous radioimmunoassay system, though in a nonparallel fashion (Farmer and Papkoff, 1979). Since this suggested that the eLH preparation used could be contaminated with eFSH, it was further purified by passing through an oFSH antibody Affigel column. The oFSH antibody was observed to cross-react with eFSH to the extent of 30%. The Affigel-purified eLH no longer cross-reacted with eFSH antibody by radioimmunoassay, showing that the cross-reactant had been removed. This preparation was still able to elicit the specific FSH response, indicating that the FSH activity of eLH is truly intrinsic to the molecule. The question of why the mare produces 3 distinct gonadotropins having differing degrees of FSH activity remains an enigma.

ACKNOWLEDGMENTS

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