Role of the peripubertal pattern of FSH, LH and prolactin secretion in regulating in-vitro steroidogenesis and follicular growth within juvenile rat ovaries

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Summary. Juvenile rat ovaries were placed in perfusion culture and exposed to (1) tonic FSH (200 ng PR-1 equiv./ml), (2) LH pulses (2/h, amplitude = 80 ng RP-1 equiv./ml), (3) tonic FSH and LH pulses, (4) tonic FSH with LH mini-surges, or (5) tonic FSH with LH and prolactin mini-surges. The LH mini-surge consisted of a series of 80 ng/ml pulses (2/h) with LH increasing to 180 ng/ml for 2 h then returning to the 80 ng/ml pulses. The prolactin mini-surge consisted of a series of 15 ng/ml pulses (2/h) with prolactin increasing to 40 ng/ml for 2 h before returning to the 15 ng/ml pulses. The LH mini-surge occurred at 14:00 h daily while a prolactin mini-surge occurred at 14:00 h and 06:00 h daily. Ovaries were perfused for 0 (in-vivo control), 24 or 48 h, incubated for 1 h in hormone-free medium to assess steroid secretion and subsequently prepared for histological analysis. After a 24 h exposure to FSH, oestradiol secretion was increased, while exposure to LH pulses enhanced progesterone secretion. Treatment with FSH, LH pulses or FSH plus LH pulses decreased the number of small antral follicles by 24 h of perfusion compared to control ($P < 0.05$). The LH mini-surge maintained the small and medium-sized antral follicles after 24 h and increased the number of preovulatory-sized follicles over controls by 48 h ($P < 0.05$). Prolactin/LH mini-surges increased the number of preovulatory-sized follicles within 24 h. These follicles were not present after 48 h of perfusion although both oestradiol ($P < 0.05$) and progesterone ($P < 0.05$) secretion were enhanced. These results suggest that LH mini-surges in the presence of FSH may be the physiological signal which induces the development of preovulatory follicles within a juvenile ovary. Prolactin modifies the ovarian responses to LH mini-surges by accelerating follicular development and subsequently enhancing steroid secretion.

Keywords: puberty; LH; FSH; prolactin; follicular growth; steroidogenesis; rat

Introduction

The first ovulation is an event which marks the end of a complex cascade of hormonal changes that leads to puberty (Ojeda *et al.*, 1986). In rats, the transition from the juvenile stage (21 days of age; no fluid in the uteri) to the peripubertal stage (~35 days of age, fluid in the uteri) is critical for the achievement of puberty (Ojeda *et al.*, 1986). The hormonal factors which facilitate this transition are not clearly understood but follicle-stimulating hormone (FSH), luteinizing hormone (LH) and prolactin play major roles. Throughout the juvenile and peripubertal stages, serum FSH values remain at constant levels of ~200 ng/ml (Urbanski & Ojeda, 1985a). Although the frequency of LH pulses remains at 2/h, the amplitude of each LH pulse increases from 40 ng/ml during the juvenile stage to 80 ng/ml in the peripubertal stage (Urbanski & Ojeda, 1985a). When juvenile stage ovaries are exposed to the peripubertal pattern of FSH and LH, medium-sized antral follicles are

stimulated to grow, suggesting that *in vivo* the gonadotrophin pattern stimulates follicular development (Peluso, 1988). The stimulus needed to continue the growth of these medium-sized antral follicles and stimulate ovarian steroidogenesis has not been identified. One possible stimulus is the LH mini-surge which occurs in the early afternoon during the peripubertal stage (Urbanski & Ojeda, 1985a). The LH mini-surge is about one-tenth of the ovulatory surge and has been shown to stimulate oestradiol secretion from peripubertal stage ovaries (Urbanski & Ojeda, 1985b). Prolactin may also play a regulatory role in stimulating both follicular development and ovarian steroidogenesis since it has the ability to influence several steroidogenic pathways (Bartke *et al.*, 1987) and induce precocious puberty (Advis & Ojeda, 1978). Therefore, the present series of experiments were designed to assess the peripubertal patterns of FSH, LH and prolactin on follicle development and steroidogenesis with the juvenile rat ovary.

Materials and Methods

Preparation of culture medium. Ovine LH (NIAMDD-oLH-25), FSH (NIAMDD-oFSH-17) and prolactin (NIAMDD-oPRL-17) were used. LH contamination of the FSH preparation was $0.04 \times \text{LH-S-1}$. In a dose equivalent to 200 ng FSH-RP-1 there are 27 ng LH-RP-1 activity. LH and FSH concentration in the final solutions were expressed in RP-1 units. The amount of LH within the medium refers to the amount of LH activity due to the sheep LH preparation and does not include the 27 ng LH-RP-1 units of activity of the ovine FSH preparation. Prolactin was expressed as ng/ml.

Hormones were added to Medium 199 (Sigma Chemical Co., St Louis, MO, USA) which was supplemented with 2.2 g NaHCO_3/l , 2 g BSA/l, 50 mg streptomycin sulphate/l, 62.9 mg K-penicillin-G/l, 2.38 g Hepes/l and insulin (200 i.u./l; Lente Iletin I, Eli Lilly, Indianapolis, IN, USA). The pH of the medium was adjusted to 7.4.

The culture system. The perfusion culture system used in these experiments has been described in detail (Peluso, 1988). Briefly, changes in the concentrations of LH and prolactin within the culture chambers were achieved by perfusing the culture chambers with media containing different amounts of hormone. In this system the media are changed automatically by programming a microprocessor controller. The mathematics used to calculate the hormonal concentrations which perfused the culture chambers have been previously published (Peluso *et al.*, 1984a).

Animals. Immature female Wistar rats were obtained from Charles River Laboratory (Wilmington, MA, USA) and housed under controlled conditions of temperature, humidity, and photoperiod (12 h light/12 h dark; lights on at 07:00 h). The rats were used when they were 30–34 days of age. On the day of the experiment, the animals were decapitated between 09:30 and 10:00 h. Only animals without fluid in their uteri and ovarian weights of ≤ 12 mg were used, ensuring that the animals were in the juvenile stage. Both ovaries were removed, trimmed of fat and placed in oxygenated medium supplemented with 200 i.u. insulin/l and 400 i.u. heparin/l. After 5 min, the ovaries were weighed and then two ovaries, each from different animals, were placed in a culture chamber. The chambers were placed in a water bath. The medium flow rate through each chamber was 6 ml/h. The medium was continuously gassed with 5% CO_2 in oxygen throughout the culture.

Experimental design. Ovaries were perfused with medium supplemented with (a) tonic FSH (200 ng PR-1 equiv./ml), (b) LH pulses (2/h, amplitude = 80 ng RP-1 equiv./ml), (c) tonic FSH and LH pulses, (d) tonic FSH with daily LH mini-surges, or (e) tonic FSH with LH and prolactin mini-surges. The LH mini-surge consisted of a series of 80 ng/ml pulses (2/h) with LH increasing to 180 ng/ml for 2 h then returning to the 80 ng/ml pulses. The prolactin mini-surge consisted of a series of 15 ng/ml pulses (2/h) with prolactin increasing to 40 ng/ml for 2 h before returning to the 15 ng/ml pulses. The LH mini-surge occurred at 14:00 h while a prolactin mini-surge occurred at 14:00 h and 06:00 h daily (Fig. 1). On each day that an experiment was conducted, ovaries were randomly assigned to the in-vivo control group or one of the treatments. After perfusion for 24 to 48 h, the ovaries were placed into a 13×75 culture tube with 2 ml hormone-free medium and incubated in a shaking water bath for 1 h in a 5% CO_2 /oxygen atmosphere. After this incubation, the ovaries were fixed in formalin and prepared for histological analysis. The media were collected and stored at -20°C until assayed for oestradiol and progesterone.

Steroid assays. The assay for oestradiol-17 β was a direct double-antibody radioimmunoassay (Pantex, Santa Monica, CA, USA). The antiserum to oestradiol cross-reacts 100% with oestradiol-17 β and $<0.001\%$ with oestrone and several other steroids. The assay sensitivity was 5 pg/ml. The intra- and inter-assay coefficients of variation were $<1.0\%$ and 7%, respectively. The progesterone assay was a solid-phase radioimmunoassay (Diagnostic Products, Los Angeles, CA, USA). The antiserum to progesterone cross-reacts 100% with progesterone, 4.3% with 20 α -dihydroprogesterone and $<0.01\%$ with several other steroids. The assay sensitivity was 0.5 ng/ml with intra- and interassay coefficients of variation of 12.3% and 18.9%, respectively.

Analysis of follicular growth. The formalin-fixed ovaries were dehydrated, embedded in paraffin wax, sectioned at 10 μm and stained with haematoxylin and eosin. The number of cells in each follicle was determined as previously

Fig. 1. Changes in LH and prolactin concentrations within the culture chamber during the afternoon of each day of culture. Ovaries exposed to the LH mini-surge treatment received tonic FSH (200 ng/ml), LH pulses of 80 ng/ml (2/h) and the LH mini-surge between 14:00 and 16:00 h daily. Ovaries treated with the prolactin/LH mini-surge, also received a series of prolactin pulses (15 ng/ml; 2/h) and a prolactin mini-surge at 14:00 and 06:00 h daily.

described (Peluso *et al.*, 1984b) with the following modifications. Every 4th section was examined and only non-atretic follicles that contained the oocytes with a visible nucleus and/or nucleolus were counted. An image analyser (Image Technology, Deer Park, NY, USA) was used to estimate the total number of granulosa cells for each follicle. The perimeter was estimated by tracing the basement membrane, thereby inscribing the granulosa cell layers. The perimeters of the antral cavity (if present) and the ovum were also calculated. The volume of each follicular component was mathematically determined and the volume of the antral cavity and ovum was subtracted from the volume of the entire follicle to determine the volume of the granulosa cell layers. The number of granulosa cells in the follicle was determined by dividing the volume of the granulosa cell layer by the average volume of a granulosa cell ($100 \mu\text{m}^3$).

The number of observed follicles was multiplied by 4 to correct for the fact that not every section of the ovary was examined and then normalized for differences in ovarian weight.

Statistical analysis. The data generated in these experiments were analysed by analysis of variance followed by Duncan's new multiple range test with only P values ≤ 0.05 considered to be significant.

Table 1. The effect of gonadotrophins on follicular distributions within juvenile rat ovaries after 24 h of perfusion

Follicle size class†	Treatments					
	In-vivo control	FSH	LH	FSH + LH	LH mini-surge	Prolactin/LH mini-surge
> 320	1.7 ± 0.5	0.3 ± 0.3	0.9 ± 0.6	0.4 ± 0.4	2.0 ± 0.8	$3.7 \pm 0.7^*$
160–320	5.7 ± 1.3	1.3 ± 0.6	1.9 ± 0.5	2.3 ± 1.6	4.1 ± 1.9	8.2 ± 1.2
80–159	7.7 ± 1.9	2.5 ± 1.2	2.3 ± 0.5	2.6 ± 0.7	4.1 ± 1.7	2.2 ± 0.5
40–79	22.5 ± 3.3	17.3 ± 4.2	9.8 ± 3.3	24.3 ± 5.9	15.3 ± 3.1	$3.0 \pm 1.9^*$
20–39	30.0 ± 4.6	28.8 ± 8.3	19.8 ± 4.6	26.7 ± 5.3	22.5 ± 4.8	$6.3 \pm 1.6^{**}$
10–19	53.1 ± 8.1	$4.8 \pm 2.1^*$	$7.5 \pm 2.9^*$	$4.8 \pm 1.1^*$	$22.1 \pm 5.6^{**}$	$32.2 \pm 9.4^{**}$
5–9	83.5 ± 14.6	37.3 ± 7.3	27.5 ± 1.6	50.8 ± 11.5	51.0 ± 7.4	76.7 ± 20.5
< 5	19.4 ± 4.4	10.5 ± 0.9	7.0 ± 1.7	11.6 ± 2.5	16.8 ± 5.4	17.5 ± 2.2
No. of observations	15	4	4	10	11	6

*Value significantly different from control ($P < 0.05$).

**Value significantly different from control and other in-vitro groups ($P < 0.05$).

†No. of cells/follicle $\times 1000$.

Fig. 2. The effects of a 24-h exposure to various gonadotrophin treatments on the amount of oestradiol-17β and progesterone secreted during a 1-h incubation. Values are means ± s.e.m. of 4–15 ovaries per group. * $P < 0.05$ compared to the in-vivo control group.

Table 2. The effect of gonadotrophins on follicular distributions within juvenile rat ovaries after 48 h of perfusion

Follicle size class†	Treatments		
	In-vivo control	LH mini-surge	Prolactin/LH mini-surge
> 320	1.7 ± 0.5	7.2 ± 2.4*	2.2 ± 0.4
160–320	5.7 ± 1.3	8.8 ± 2.0	7.6 ± 2.7
80–159	7.7 ± 1.9	1.5 ± 0.7	4.0 ± 0.9
40–79	22.5 ± 3.3	9.7 ± 3.2*	0.8 ± 0.8*
20–39	30.0 ± 4.6	21.5 ± 4.6	4.8 ± 2.6*
10–19	53.1 ± 8.1	43.3 ± 8.2	14.8 ± 5.3*
5–9	83.5 ± 14.6	63.0 ± 12.8	61.4 ± 7.2
< 5	19.4 ± 4.4	27.7 ± 5.8	27.6 ± 6.7
No. of observations	15	6	5

*Value significantly different from control and LH mini-surge groups ($P < 0.05$).

†No. of cells/follicle × 1000.

Results

Most of the follicles within a juvenile ovary (in-vivo controls) were preantral with $\leq 19 \times 10^3$ granulosa cells. Small antral follicles, with between 20 and 80×10^3 cells, and medium-sized antral follicles ($\sim 350 \mu\text{m}$ in diameter; $80\text{--}160 \times 10^3$ granulosa cells) were also numerous. Occasionally, large antral ($160\text{--}320 \times 10^3$ cells) or preovulatory-sized ($\geq 320 \times 10^3$ cells) follicles were also observed (Table 1). The juvenile ovary secreted oestradiol and progesterone at relatively low rates (Fig. 2).

Compared to in-vivo controls, the number of preantral follicles was reduced after 24 h of perfusion regardless of the gonadotrophin treatment ($P < 0.05$). However, more preantral follicles were present in the LH mini-surge and the prolactin/LH mini-surge treatments than in any of the other in treatments ($P < 0.05$). In addition, the number of preovulatory-sized follicles present after exposure to the prolactin/LH mini-surge was slightly but significantly increased over in-vivo

Fig. 3. The effects of a 48-h exposure to various gonadotrophin treatments on the amount of oestradiol-17β and progesterone secreted during a 1-h incubation. Values are means $\pm$ s.e.m. of 4–15 ovaries per group. * $P < 0.05$ compared to the in-vivo control group.

control values ($P < 0.05$) (Table 1). After 24 h, the juvenile ovaries perfused with FSH secreted more oestradiol ($P < 0.05$), while ovaries exposed to LH pulses secreted more progesterone compared to in-vivo control ($P < 0.05$). The oestradiol and progesterone secretion rates for all the other gonadotrophin treatments were comparable to the in-vivo controls ($P > 0.05$) (Fig. 2).

By 48 h, the number of preantral follicles present within the juvenile ovary exposed to the LH mini-surge, was similar to the in-vivo control ($P > 0.05$) and greater than the number present at 24 h ($P < 0.05$). Further, the number of preovulatory-sized follicles was increased over control values ($P < 0.05$) (Table 2). In spite of the increased follicular development, oestradiol and progesterone secretion was not enhanced over in-vivo controls ($P > 0.05$) (Fig. 3). The prolactin/LH mini-surge group at 48 h showed a decrease in the number of preantral and small antral and preovulatory-sized follicles ($P < 0.05$), although oestradiol ($P < 0.05$) and progesterone ($P < 0.05$) secretion were elevated (Table 2; Fig. 3).

Discussion

The present data demonstrate that FSH, LH and prolactin interact in a complex manner to regulate both follicle growth and steroidogenesis. Over a 24-h period, tonic FSH enhances oestradiol secretion; while LH pulses promote progesterone secretion. Although these observations confirm the well-described effect of each gonadotrophin on ovarian steroidogenesis (Dorrington, 1978), combined exposure to FSH and LH pulses does not enhance the secretion of either steroid. The cellular mechanism by which simultaneous exposure to LH pulses and tonic FSH neutralizes the steroidogenic action of each gonadotrophin is not yet known. A similar neutralizing interaction has been demonstrated previously with tonic FSH antagonizing the growth-promoting action of tonic LH (Peluso *et al.*, 1984b). However, in the presence of FSH, the growth-promoting action of LH can be restored if LH is given in a pulsatile manner (Peluso *et al.*, 1984b). Ovarian responses to FSH and LH therefore depend on both the amount of each gonadotrophin and the temporal pattern of gonadotrophin exposure.

After 24 or 48 h of perfusion, neither FSH nor LH pulses alone or in combination stimulate follicular development. In fact, these treatments result in a reduction in the numbers of preantral follicles. Previous work has demonstrated that tonic FSH plus LH pulses increases the number of medium-sized antral follicles within 3 h of perfusion (Peluso, 1988). In the present study, this treatment fails to maintain this population of follicles after 24 h of perfusion, suggesting that tonic

FSH plus LH pulses is the hormonal signal that initiates the rapid growth of small and medium-sized antral follicles but does not sustain their growth. Since the LH mini-surge treatment maintains the number of small and medium-sized antral follicles and, when extended for 48 h, increases the number of preovulatory follicles, it is proposed that this gonadotrophin pattern promotes the development of preovulatory follicles during the transition from the juvenile to the peripubertal stage of puberty.

While this hormonal sequence controls follicular development, an additional hormonal factor must regulate steroidogenesis within the juvenile ovary. Prolactin may be this factor since prolactin influences ovarian steroidogenesis. Prolactin increases progesterone secretion from cultured rat granulosa cells (Fortune & Vincent, 1986) or isolated hamster follicles (Roy & Greenwald, 1987). At physiological levels (i.e. 20–25 ng/ml) prolactin does not affect oestradiol secretion from cultured rat granulosa cells, although high doses inhibit secretion of this steroid (Fortune *et al.*, 1986). When prolactin concentrations are elevated in intact rats, both basal and hCG-induced secretion of oestradiol and progesterone are increased (Advis *et al.*, 1981). Prolactin could mediate its steroidogenic action by increasing granulosa cell LH receptors (Advis *et al.*, 1981) and/or inhibiting ovarian 20 α -hydroxysteroid dehydrogenase activity (Eckstein & Nimrod, 1979; Jones & Hsueh, 1981). Both of these actions could explain the prolactin-induced increase in steroid secretion.

In our study, the prolactin/LH mini-surge treatment increased the number of preovulatory follicles by 24 h, confirming the mitogenic action of FSH, LH and prolactin (Roy & Greenwald, 1988). However, the prolactin/LH mini-surge only promotes follicular growth during the first 24 h of culture while enhancing both oestradiol and progesterone secretion after the second 24 h culture period. Since neither preovulatory follicles nor corpora lutea were observed at 48 h, it is assumed that the few preovulatory-sized follicles present at 24 h became atretic. Similarly, the prolactin/LH mini-surge treatment reduced the number of small and medium-sized antral follicles, apparently enhancing their atresia and subsequent differentiation into steroidogenic interstitial tissue. The putative prolactin/LH-induced follicular atresia and/or differentiation would increase the amount of steroidogenically-active tissue within the juvenile rat ovary and gradually increase serum oestradiol concentrations. Since the initiation of prolactin mini-surges is not dependent on the ovary (Kimura & Kawakami, 1981), it is proposed that prolactin mini-surges in the presence of FSH and LH induce follicles to develop. These follicles would ultimately differentiate into a steroidogenically-active component within the juvenile ovary and increase serum oestradiol values. As the rat approaches puberty, the pattern of prolactin secretion changes. In the juvenile stage prolactin mini-surges occur in the early morning between 02:00 and 05:00 h and the evening between 14:00 and 17:00 h. At 1–2 days before the first ovulation, the morning prolactin mini-surge disappears (Kimura & Kawakami, 1980); oestradiol concentrations increase and subsequently induce LH mini-surges (Urbanski & Ojeda, 1986). With prolactin levels reduced, in-vivo conditions would be more similar to the LH mini-surge treatment which stimulates the development of preovulatory-size follicles *in vitro* and presumably *in vivo*. While this is an attractive hypothesis, considerably more studies must be conducted to identify the precise pattern of hormonal stimulation which controls ovarian function during the transition for the juvenile to the peripubertal stage of puberty.

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