Effect of LH on progesterone and oestradiol production *in vivo* and *in vitro* by preovulatory rat follicles

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Summary. Temporal changes in follicular oestradiol production induced *in vitro* and *in vivo* by LH were studied. In-vitro changes were measured by incubating preovulatory rat follicles for 12 h, changing the medium every 2 h. Follicles isolated at various intervals after an injection of 10 i.u. hCG were incubated for 2 h to measure changes in oestradiol production *in vivo*. In both studies there was an increase in oestradiol production lasting 4 h followed by a sharp decline. Progesterone production was also increased by LH *in vitro* or hCG *in vivo*, but remained high. A second exposure to LH did not raise oestradiol synthesis, but increased progesterone synthesis *in vitro* only. The decline in oestradiol production is most probably due to a decrease in C17–20 lyase activity, because addition of testosterone, but not of 17 α -hydroxyprogesterone, increased oestradiol production.

Incubation of preovulatory follicles in the absence of LH or incubation of follicles derived from animals in which the spontaneous LH surge was blocked by an injection of pentobarbitone sodium also resulted in a decrease of oestradiol and an increase in progesterone production. This oestrogen–progesterone shift was also caused by a decrease in C17–20 lyase activity.

The results demonstrate that the changes in steroid production *in vivo* and *in vitro* are similar and occur in the presence and absence of LH. It is concluded that the decrease in oestradiol production is dependent on the decrease in the activity of enzymes converting progesterone to aromatizable androgens.

Introduction

Preovulatory follicles are characterized by a high production of oestradiol-17 β . In rats with a 5-day ovarian cycle this ability is achieved the day before pro-oestrus and is terminated on the afternoon of pro-oestrus by the preovulatory LH surge. Effects on steroidogenesis *in vivo* by LH have been studied in immature rats treated with PMSG: follicles explanted after the endogenous LH surge secrete lower amounts of androstenedione and oestradiol than follicles explanted before the LH surge (Hillensjö, Bauminger & Ahrén, 1976). This decrease is due to an inhibition of androgen formation (Katz & Armstrong, 1976; Hillensjö, Hamberger & Ahrén, 1977). The effects of LH on steroidogenesis *in vitro* have been studied by incubating preovulatory follicles and taking samples of the medium at regular intervals (Lieberman *et al.*, 1975). Such studies demonstrate that LH exerts a biphasic effect on oestradiol production: the initial response is a stimulation of overall steroidogenesis followed by a rapid decline in androgen and oestrogen synthesis.

However, changes in steroidogenesis have been observed in the absence of the preovulatory LH surge. Follicles isolated 1 day after an ovulation-blocking dose of pentobarbitone given on the afternoon of pro-oestrus produce considerably lower amounts of oestradiol *in vitro* than do follicles isolated on the morning of pro-oestrus (Uilenbroek, Woutersen & van der Schoot, 1980). This

decrease is due to an inhibition of the conversion of progesterone to androstenedione (Uilenbroek, van der Linden & Woutersen, 1984).

To investigate whether the decrease in oestradiol production after pentobarbitone injection resembled the changes in steroidogenesis after LH, temporal changes in steroid production in the absence and presence of LH were compared in the present study. Furthermore, changes in steroidogenesis *in vitro* induced by an ovulatory dose of LH were compared with those *in vivo* induced by hCG in adult cyclic rats.

Materials and Methods

Animals. Adult female rats of Wistar origin were used. The animals were 3–5 months old and were housed under controlled conditions (20–23°C, lights on 05:00–19:00 h). Vaginal smears were taken daily. Only animals showing at least two successive 5-day cycles were used. Animals were killed at pro-oestrus between 09:00 and 10:00 h to obtain preovulatory follicles for *in-vitro* studies. The ovaries were removed and the 10–12 largest follicles were isolated under a stereomicroscope. To study the effects of LH *in vivo*, rats were injected i.p. with pentobarbitone sodium (37 mg/kg body weight) at 13:30 h on the day of pro-oestrus to block the endogenous LH surge. At 14:00 h the animals were injected s.c. with 10 i.u. hCG (Pregnyl: Organon, Oss, The Netherlands) or vehicle (0.2 ml 0.9% (w/v) NaCl (saline)). The rats were unilaterally ovariectomized 2, 4, 6 or 8 h after hCG injection and 2 or 8 h after injection with saline. The largest follicles were isolated and incubated for 2 h to measure the accumulation of oestradiol-17 β and progesterone. The animals were killed the next morning to count the number of eggs in the oviduct.

Follicle incubations. Individual follicles were incubated in 500 μ l Medium 199 (GIBCO, Grand Island, NY, U.S.A.) pH 7.4 containing 25 mM-Hepes (Sigma, St Louis, MO, U.S.A.). The incubations were carried out at 37°C under an atmosphere of 95% O₂ and 5% CO₂ in a shaking waterbath.

To study the effects of LH *in vitro*, follicles isolated on the morning of pro-oestrus were incubated for 12 h. Every 2 h the medium was aspirated and replaced by fresh medium. LH (NIH-LH-S19: with LH activity of $1.01 \times$ NIH-LH-S1 and FSH activity of $<0.05 \times$ NIH-FSH-S1) was added to the medium in a final concentration of 40 ng/ml as indicated. The amount of LH (40 ng/ml) is equivalent to the amount circulating during the preovulatory LH surge at pro-oestrus.

At the end of incubation the medium was stored at –20°C until assayed for oestradiol-17 β and progesterone.

After incubation for 8 h or 8 h after hCG or saline injection, aromatase and C17–20 lyase activity in the follicles were measured. The follicles were incubated in the presence of testosterone (50 ng/ml) or 17 α -hydroxyprogesterone (50 ng/ml) and the accumulation of oestradiol in the medium was measured.

Steroid assays. Concentrations of oestradiol-17 β and progesterone in the incubation medium were measured by radioimmunoassay without prior extraction as described previously (Uilenbroek *et al.*, 1980). The oestradiol antibody was raised against oestradiol-17 β -6-(O-carboxymethyl)oxime-BSA complex. The properties of this antibody with regard to specificity have been described by Exley, Johnson & Dean (1971). The sensitivity of the assay was 10 pg/tube and the intra- and inter-assay variation was 7.2 and 8.3% respectively. Addition of testosterone or 17 α -hydroxyprogesterone to the culture medium (50 ng/ml) did not interfere with the oestradiol assay. The progesterone antibody was raised against 11 α -hydroxyprogesterone-hemisuccinate-BSA complex. Data on the specificity of the antibody have been described by de Jong, Baird & van der Molen (1974). The sensitivity of the progesterone assay was 25 pg/tube and the intra- and interassay variations were 13.7 and 16% respectively.

Statistical analysis. The results have been expressed as means $\pm$ s.e.m. Differences between groups were measured by Student's *t* test. A difference was considered to be statistically significant if $P < 0.05$.

Results

Effect of LH in vitro

Incubation of preovulatory follicles in LH-free medium resulted in a significant decrease of oestradiol production from 1.12 ± 0.07 ng/2 h/follicle at 0–2 h to 0.38 ± 0.05 ng/2 h/follicle at 10–12 h ($P < 0.001$) (Text-fig. 1a). In contrast, basal progesterone production increased from 0.15 ± 0.05 ng/2 h/follicle during the first incubation (0–2 h) to 0.46 ± 0.11 ng/2 h/follicle during the final incubation (10–12 h) ($P < 0.02$).

Incubation in the presence of LH (40 ng/ml) during the entire incubation period raised oestradiol and progesterone production (Text-fig. 1b). The production of oestradiol decreased after 4 h to levels not different from those in untreated rats. Maximal progesterone production was reached between 4 and 6 h and remained high thereafter. LH given during the first incubation period (0–2 h) resulted in a pattern of oestradiol production not different from that after stimulation with LH during the whole incubation period (0–12 h) (Text-fig. 2a). Exposure of follicles to LH at the end of 10 h of incubation resulted in a small but significant ($P < 0.01$) increase of oestradiol production (Text-fig. 2b). When the follicles had been exposed to LH from 0 to 2 h, no increase in oestradiol was found after LH given at 10–12 h (Text-fig. 2c).

Progesterone production after LH from 0 to 2 h reached a maximum between 2 and 4 h and decreased thereafter (Text-fig. 2a). LH given at the end of the incubation (10–12 h) resulted in a high progesterone production, even if follicles were exposed to LH at 0–2 h.

Addition of testosterone (50 ng/ml) to the culture medium after 8 h of incubation increased oestradiol production significantly between 10 and 12 h ($P < 0.002$) (Text-fig. 3). However, addition of 17α -hydroxyprogesterone (50 ng/ml) had no effect. Oestradiol production at 8–10 and

Text-fig. 1. Time-course of the accumulation of oestradiol and progesterone in the medium by individual preovulatory follicles of rats. The follicles were incubated in the absence (a) or presence (b) of LH (40 ng/ml). Each bar represents the mean $\pm$ s.e.m. of 8–10 determinations.

Text-fig. 2. Time-course of the accumulation of oestradiol and progesterone in the medium by individual preovulatory follicles of rats. The follicles were incubated in the absence and presence of LH (40 ng/ml). Each bar represents the mean $\pm$ s.e.m. of 8–10 determinations.

10–12 h by follicles that had been exposed to LH (0–2 h) was significantly lower than that by follicles incubated without LH ($P < 0.001$). Also in the latter follicles oestradiol production was increased by addition of testosterone ($P < 0.001$), but not by addition of 17α -hydroxyprogesterone.

Effect of hCG in vivo

Follicles isolated 2 h after an injection of 10 i.u. hCG produced significantly higher concentrations of oestradiol ($P < 0.001$) during incubation for 2 h than did follicles isolated from rats that had not been treated with hCG or follicles isolated at the morning of pro-oestrus (Table 1). In contrast, oestradiol production by follicles isolated 4, 6 or 8 h after hCG injection decreased significantly. Oestradiol production by follicles isolated 8 h after injection with saline was also lower than that by follicles isolated after 2 h ($P < 0.05$), but higher than in hCG-treated rats. Addition of LH to the incubation medium increased oestradiol production in the saline-treated rats after 2 h ($P < 0.001$) and 8 h ($P < 0.01$), but was ineffective in the hCG-treated rats. However, in the saline-treated rats LH-stimulated oestradiol production was lower at 8 h than at 2 h.

Progesterone production by follicles isolated 2 h after hCG injection was 10-fold higher than in saline-treated rats (Table 1). Progesterone production by follicles isolated 6 and 8 h after hCG was lower than after 2 and 4 h. Addition of LH to the incubation medium increased progesterone

Text-fig. 3. Accumulation of oestradiol in the medium by individual preovulatory follicles of rats. Follicles were incubated from 0 to 2 h in the absence (a) or presence (b) of LH (40 ng/ml) and incubated from 8 to 12 h in control medium (C) or medium containing testosterone (T) or 17α-hydroxyprogesterone (17α-OHP) (50 ng/ml). Each bar represents the mean $\pm$ s.e.m. of 8–10 determinations.

Table 1. Accumulation of oestradiol and progesterone in the medium by individual preovulatory follicles isolated at various intervals after an injection of 10 i.u. hCG or saline and incubated for 2 h in the presence or absence of LH (40 ng/ml)

	Follicles isolated after	LH	Steroid (ng/2 h/follicle)	
			Saline	10 i.u. hCG
Oestradiol-17β	2 h	—	1.16 $\pm$ 0.21	4.90 $\pm$ 0.86†
		+	5.25 $\pm$ 0.95*	3.92 $\pm$ 0.72
	4 h	—	—	0.73 $\pm$ 0.10
		+	—	0.87 $\pm$ 0.20
	6 h	—	—	0.11 $\pm$ 0.01
		+	—	0.12 $\pm$ 0.01
	8 h	—	0.62 $\pm$ 0.09†	0.10 $\pm$ 0.01‡
		+	2.07 $\pm$ 0.45*	0.10 $\pm$ 0.01
Progesterone	2 h	—	0.65 $\pm$ 0.16	63.9 $\pm$ 4.5‡
		+	12.4 $\pm$ 4.7*	65.5 $\pm$ 8.1
	4 h	—	—	57.8 $\pm$ 9.3
		+	—	68.3 $\pm$ 15.0
	6 h	—	—	33.4 $\pm$ 5.2
		+	—	33.8 $\pm$ 3.9
	8 h	—	1.05 $\pm$ 0.28	35.1 $\pm$ 5.4‡
		+	6.3 $\pm$ 2.2*	35.2 $\pm$ 6.7

Values are mean $\pm$ s.e.m. of 7–10 follicles.

* $P < 0.05$ compared with follicles incubated in LH-free medium.

† $P < 0.05$ compared with follicles isolated after 2 h.

‡ $P < 0.05$ compared with follicles isolated from saline-treated rats.

Text-fig. 4. Accumulation of oestradiol in the medium by individual preovulatory follicles of rats. Follicles were isolated 8 h after an injection of 10 i.u. hCG (b) or saline (a) and incubated for 4 h in control medium (C) or medium containing testosterone (T) or 17 α -hydroxyprogesterone (17 α -OHP) (50 ng/ml). Each bar represents the mean $\pm$ s.e.m. of 8–10 determinations.

production in the saline-treated rats, but was unable to increase progesterone in the hCG-treated rats.

Incubation of follicles isolated at 8 h in saline-treated rats produced significantly higher amounts of oestradiol in the presence of testosterone ($P < 0.002$), but not in the presence of 17 α -hydroxyprogesterone (Text-fig. 4). A similar pattern of oestradiol production was observed for follicles isolated 8 h after an injection of 10 i.u. hCG.

Discussion

The present findings show that the changes in oestradiol production by preovulatory follicles induced by LH *in vitro* and by hCG *in vivo* are essentially the same. A single dose of LH (hCG) increased oestradiol production during 4 h *in vitro* and *in vivo*. This was followed by a rapid decline and a second dose of LH was unable to stimulate oestradiol production. A similar loss in oestradiol production *in vivo* 2 days after injection of PMSG in immature female rats has been reported (Hillensjö *et al.*, 1976). The decrease in oestradiol production after a 2-h exposure to LH *in vitro* or after a single injection with hCG *in vivo* is due to a decreased C17–20 lyase activity converting 17 α -hydroxyprogesterone to androstenedione, thus limiting the amount of substrate available for aromatization. This conclusion has been reached because of the findings that addition of testosterone in the medium increased oestradiol production, while addition of 17 α -hydroxyprogesterone was without effect. Furthermore, addition of LH in the culture medium at the end of incubation for 10 h *in vitro* increased progesterone production, demonstrating that the follicles were still responsive. A difference between the effect of hCG *in vivo* with the effect of LH *in vitro* is that progesterone production could not be increased by LH 8 h after a single injection of 10 i.u. hCG. However, a dose of 10 i.u. hCG *in vivo* resulted in a much larger progesterone production than did 40 ng LH *in vitro* (64 and 9 ng respectively). A specific lesion of 17:20 side-chain cleavage after the gonadotrophin surge has been reported for PMSG-treated rats (Hillensjö *et al.*, 1977; Suzuki & Tamaoki, 1979).

The pattern of oestradiol production after exposure to LH was the same regardless of whether the follicles were exposed during the entire incubation period or only for 2 h. In contrast, progesterone production was maintained when LH is continuously present. The 2-h exposure to LH stimulated cholesterol–progesterone conversion, but maximal progesterone production was reached later than maximal oestradiol production. This might be due to the rapid conversion of progesterone by the initial active 17 α -hydroxylase/C17–20 lyase system or that LH acts at a level beyond progesterone, stimulating the conversion of progesterone to androstenedione. This might explain why addition of LH is much more effective in stimulating oestrogen production than is addition of 17 α -hydroxyprogesterone as substrate. Regulation of 17 α -hydroxylase and C17–20 lyase activity by LH has also been proposed by Bogovich & Richards (1982). They observed that the increased oestradiol production in preovulatory follicles was dependent on an increase in thecal 17 α -hydroxylase and C17–20 lyase activity. This correlated with the small sustained increases in serum LH which are indispensable for differentiation of small antral follicles to oestradiol-producing preovulatory follicles (Bogovich, Richards & Reichert, 1981). Therefore, low doses of LH might, in addition to a stimulation of cholesterol side-chain cleavage, stimulate the 17 α -hydroxylase/C17–20 lyase system. With higher doses of LH this is soon followed by an inactivation of this enzyme system resulting in the oestrogen–progesterone shift. It has been suggested that the acute decrease of this enzyme activity is due to a decrease in the concentrations of microsomal cytochrome P-450 (Suzuki & Tamaoki, 1983). This hCG-induced decrease is dependent on RNA and protein synthesis (Lieberman *et al.*, 1975).

It was surprising that oestradiol production decreased even in the absence of a preovulatory LH surge. In earlier studies we found a decrease in follicular oestradiol production the day after injection of pentobarbitone on the day of pro-oestrus (Uilenbroek *et al.*, 1980), which was due primarily to a decline in C17–20 lyase activity (Uilenbroek *et al.*, 1984). The present study demonstrates that even at 22:00 h on the day of pro-oestrus (8 h after injection of saline at 14:00 h) oestradiol production is significantly decreased. A decrease in oestradiol production was also found during incubation in LH-free medium. The mechanism seems similar because addition of LH after incubation for 10 h *in vitro* or 8 h after injection of saline *in vivo* resulted in an increase of oestradiol production. Addition of testosterone, but not 17 α -hydroxyprogesterone, also increased oestradiol production. At present we have no explanation why, in the absence of a preovulatory LH surge *in vivo* or during incubation *in vitro*, C17–20 lyase activity decreases.

In conclusion, the changes in steroid production in the absence and presence of LH are essentially similar *in vitro* and *in vivo*. This justifies the use of this in-vitro technique to study changes of steroidogenesis *in vivo*.

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