In-vitro secretion of progesterone by the corpus luteum of the tammar wallaby, *Macropus eugenii*

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Summary. Luteal tissue collected from tammars 0, 5, 9 and 16 days after removal of pouch young actively produced progesterone *in vitro*. On Days 5, 9 and 16 luteal progesterone concentration was not significantly different from Day 0 (quiescence). However, the net production of progesterone was significantly higher on Day 5 ($P < 0.05$) than at any other stage, and we suggest that the corpus luteum is the main source of the rise in progesterone in the peripheral circulation at Days 5-8 of the delayed or non-delayed cycle.

Addition of ovine prolactin to corpora lutea incubated on Day 5 after removal of pouch young had no effect on luteal progesterone concentration or the production of progesterone. We therefore conclude that the tonic inhibition exerted by prolactin on the corpus luteum does not affect the steroidogenic capacity of the luteal tissue but may inhibit luteal cell growth during quiescence.

Introduction

In female tammars the corpus luteum (CL) formed after post-partum oestrus is arrested and tonically inhibited by the pituitary (Hearn, 1974). During lactation this suppression appears to be mediated by secretion of prolactin from the pituitary (Tyndale-Biscoe & Hawkins, 1977) but reactivation can be induced experimentally by removal of the sucking pouch young (Renfree & Tyndale-Biscoe, 1973), denervation of the active mammary gland (Renfree, 1979), injection of bromocriptine (Tyndale-Biscoe & Hinds, 1981) or hypophysectomy (Hearn 1974; Tyndale-Biscoe & Hawkins, 1977). When reactivation occurs the corpus luteum increases in size from 10 to about 60 mg (Renfree, Green & Young, 1979) and circulating progesterone rises from < 200 to about 600 pg/ml (Lemon, 1972; Hinds & Tyndale-Biscoe, 1982a). However, until about Day 15 of the reactivated cycle, luteal progesterone concentration does not alter significantly (Renfree *et al.*, 1979) and so the change in plasma progesterone must be due to an increasing rate of production by the CL, an increase in its mass or a change in metabolic clearance rate.

The corpus luteum is devoid of receptors for luteinizing hormone (LH) (Stewart & Tyndale-Biscoe, 1982) but abundant specific binding sites for prolactin are present in luteal tissue during quiescence (Sernia & Tyndale-Biscoe, 1979) and during the delayed or non-delayed cycle (Stewart & Tyndale-Biscoe, 1982). The presence of these receptors strongly suggests that the inhibitory effect of prolactin is exerted directly on the CL. However, it is unclear whether this effect is mediated by an inhibition of luteal cell growth or by a suppression of the steroidogenic activity of the CL. Some preliminary studies have shown that, although progesterone is secreted by quiescent CL *in vitro*, neither the addition of high concentrations of prolactin nor LH affected progesterone production (Sernia, Hinds & Tyndale-Biscoe, 1980). The present study was designed to examine in-

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vitro progesterone production by CL collected at various times after reactivation had been induced by removal of pouch young or injection of bromocriptine and to correlate this with the increasing mass of the CL and the changes in peripheral progesterone levels. In addition the effect of prolactin on the steroidogenic function of CL after reactivation was examined.

Materials and Methods

Ovaries were collected from the tammar at laparotomy or autopsy. A blood sample was taken into a heparinized syringe from the caudal vein or the heart for determination of progesterone. The ovaries were examined and the single CL and non-luteal ovarian tissue separated and placed on ice.

Each CL was weighed and a piece frozen immediately to provide a pre-incubation measure of tissue progesterone concentration. The other piece of tissue was incubated in 1 ml of freshly gassed (95% O₂/5% CO₂) Krebs-Ringer-Bicarbonate buffer (pH 7.0, 118 mM-NaCl, 47 mM-KCl, 0.25 mM-CaCl₂, 1.2 mM-KH₂PO₄, 1.2 mM-MgSO₄·7H₂O, 2.5 mM-NaHCO₃) plus 0.2% glucose, for 4 h at 35°C. After incubation the media and tissues were frozen separately at -15°C until assayed for progesterone. Non-luteal tissue was treated in a similar manner.

Ovine prolactin (NIH-P-S12) was obtained from the National Pituitary Agency, Maryland, U.S.A. Bromocriptine (CB154) was donated by Sandoz (Australia) Pty Ltd, Sydney. All other reagents were analytical grade.

Experiment I

The following tests were undertaken to validate in-vitro procedures for use with the tammar CL.

(1) Aliquants of medium (100 µl) were collected at hourly intervals from duplicate incubations of pieces of one quiescent (Day 0) CL to determine whether the release of progesterone into the medium was at a constant rate over the 4-h incubation period. To establish whether the presence of glucose in the incubation medium was essential, one quiescent CL was bisected and each piece incubated separately in medium lacking glucose. For each experiment the progesterone content of the medium, before and after incubation, was determined to demonstrate that only luteal tissue was synthesizing progesterone.

(2) To check that progesterone production by corpora lutea *in vitro* represented active production and secretion rather than leakage of the steroid from damaged cells in the tissue, CL were obtained from 5 females 16 days after removal of pouch young and incubated as described earlier. By Day 16 each CL weighed more than 40 mg and so could be divided into 3 or 4 pieces. One or 2 pieces were frozen immediately to measure pre-incubation luteal tissue progesterone concentration while the other 2 pieces were incubated. This allowed two independent duplicate determinations to be made of pre- and post-incubation tissue progesterone concentration and of progesterone in the incubation medium.

Experiment II

This experiment was designed to measure the rate of secretion of progesterone by CL at various stages during the first half of a reactivated cycle. The earlier experiments (Sernia *et al.*, 1980; and Exp. I) indicated that the rate of secretion by and progesterone concentrations of luteal tissue were similar on Days 0 and 16. Therefore, CL were collected on Days 0, 5, 9 and 16 after removal of pouch young and rate of progesterone production and tissue progesterone concentrations were determined as above.

Experiment III

Sernia *et al.* (1980) found that addition of high concentrations of prolactin (2 µg/ml) to quiescent or Day 1 CL *in vitro* did not affect the production of progesterone. For this experiment the aim was to determine whether the progesterone secretion rate could be altered by lower, physiological, concentrations of prolactin (Hinds & Tyndale-Biscoe, 1982b) if the CL were already reactivated and actively producing progesterone. In addition non-luteal ovarian tissue was treated in the same way to resolve whether it contributes to plasma progesterone levels at this time. Corpora lutea and non-luteal ovarian tissue collected 5 days after removal of pouch young and simultaneous injection of bromocriptine (2 mg/kg body weight) were incubated with prolactin (50 µg/ml) and progesterone secretion was determined.

Assay of progesterone

Plasma, tissue and incubation medium concentrations of progesterone were determined by radioimmunoassay as described by Sernia *et al.* (1980) using antiserum (Lot S230) supplied by Dr R. I. Cox (CSIRO, Sydney). Antibodies were raised in sheep to progesterone-11-hemisuccinate-BSA. Cross-reactivity relative to progesterone was <0.3% for 17 α -hydroxyprogesterone, 20 α - or 20 β -hydroxy-4 pregnene-3-one, 3 β ,17-dihydroxy-5-pregnene-20-one, 5 β -pregnane-3 α ,20 β -diol, 5 β -pregnane-3 α ,20 α -diol, cortisol and corticosterone. Higher cross-reactivity was present with 11-deoxycorticosterone (1.0%), pregnenolone (1.6%), 5 β -pregnane-3,20-dione (3.5%) and 11 β -hydroxyprogesterone (6.7%). Assay sensitivity, defined as the least measurable amount of progesterone was 25 pg/ml. Intra- and inter-assay coefficients of variation of 9.0 and 13.0% respectively were determined from repeated measures of a single pool of plasma within the same assay ($n = 10$) and in separate assays ($n = 29$). Before assay tissues were homogenized in 1 ml glass-distilled water using a ground-glass homogenizer. The homogenates and the incubation media were extracted three times with 10 ml freshly redistilled hexane, dried under air, redissolved in 1 ml absolute ethanol and stored at -15°C . The ethanol solution was diluted 1:50 and two different duplicate aliquants of between 50 and 200 µl were taken for assay. Therefore, each concentration is the mean of 2 results for assays of different volumes.

Calculations

The net production rate was expressed as ng steroid/mg tissue incubated/4 h. The expression was derived by subtracting the initial concentration of steroid/mg tissue at time 0 from the sum of the steroid concentrations in the incubated tissue and its incubation medium. All tissue weights were expressed as mg wet weight tissue. Results were analysed by the Student's t test or analysis of variance followed by Duncan's multiple range test.

Results

Experiment I

(1) The progesterone concentrations in aliquants of medium collected at hourly intervals show that the release of progesterone into the medium occurred at a constant and linear rate over the 4-h incubation period. However, if glucose was absent from the medium progesterone release was reduced at 1 h and total release by 4 h was one eighth that of CL incubated in medium containing glucose (Text-fig. 1). The concentration of progesterone in medium alone, before and after incubation, was <25 pg/ml in all experiments.

(2) Corpora lutea collected at Day 16 actively produced progesterone *in vitro*. The concentration of progesterone in pre-incubation tissue was not significantly different from that observed after incubation (paired-sample t test, $0.2 > P > 0.1$) indicating that progesterone concentrations in the medium represented a net gain of steroid (Table 1). The rate of production was 19.9 ± 3.0 ng/mg/4 h.

Text-fig. 1. Secretion of progesterone into the medium by pieces of quiescent CL in the presence or absence of glucose in the medium.

Table 1. Progesterone production by pieces of CL incubated 0, 5, 9 and 16 days after removal of pouch young (RPY)

Exp.	Days after RPY	Wt of CL (mg)	Tissue content (ng progesterone/mg tissue)		Progesterone (ng/mg/4 h)		Plasma progesterone (pg/ml)
			Pre-incubation	Post-incubation	Secretion into medium	Net production	
I	16	53.2 ± 9.1 (5)	35.2 ± 2.2	30.3 ± 2.3	18.9 ± 2.8	19.9 ± 3.0	—
II	0	7.0 ± 0.2 (4)	26.9 ± 8.6	19.1 ± 2.3	31.6 ± 4.2	23.8 ± 2.7	228.3 ± 25.4
	5	11.6 ± 3.8 (5)	32.1 ± 4.9	36.0 ± 5.5	55.0 ± 6.7*	58.8 ± 8.3†	440.5 ± 107.2
	9	20.7 ± 2.6 (4)	19.4 ± 4.1	15.9 ± 1.6	13.6 ± 1.8	9.2 ± 2.5	290.6 ± 21.7
	16	49.7 ± 3.1 (4)	28.6 ± 1.6	27.9 ± 3.0	20.3 ± 2.7	19.6 ± 6.2	412.8 ± 28.9

Values are mean ± s.e.m. for the no. of CL shown in parentheses.

Values significantly different from Day 0 CL: * $P < 0.01$, † $P < 0.05$.

Experiment II

The increase in the mass of the CL from Day 0 to 16 (Table 1) agrees with the data of Renfree *et al.* (1979). As in Exp. I, pre-incubation and post-incubation tissue progesterone concentrations at each stage were not significantly different, while the progesterone concentration in the medium showed a net increase (Table 1).

The pre-incubation tissue progesterone concentrations of CL collected from females on Days 5, 9 and 16 were not significantly different from that for the quiescent CL (Day 0) ($P > 0.4$) (Table 1). However, the rate of secretion of progesterone into the medium was higher ($P < 0.01$) on Day 5 than on Days 0, 9 and 16 (Table 1). This difference was also seen ($P < 0.05$) when the net production of progesterone was derived (Table 1). The high net production *in vitro* on Day 5 was reflected in higher plasma progesterone values on this day (Table 1), although the differences between Day 5 and Days 0 and 9 are not statistically significant because of the large variation around the mean on Day 5.

Experiment III

The progesterone content and production rate of this set of CL collected on Day 5 after simultaneously removing the pouch young and injecting bromocriptine were similar to those collected on the same day in Exp. II (Table 1). The addition of ovine prolactin to the incubation medium had no effect on the tissue progesterone content or the rate of secretion of progesterone into the medium (Table 2). The progesterone content and secretion rate of non-luteal ovarian tissue from the same group of animals were negligible (Table 2).

Table 2. The effect of addition of ovine prolactin (NIH-P-S12, 50 ng/ml) on luteal tissue progesterone content and progesterone secretion rate of (a) corpora lutea collected 5 days after removal of pouch young and (b) non-luteal ovarian tissue

Animal No.	Post-incubation tissue content (ng progesterone/mg tissue)		Progesterone secretion into medium (ng/mg/4 h)	
	Control	+ Prolactin	Control	+ Prolactin
(a) <i>Corpora lutea</i>				
1	46.3	48.4	38.9	40.8
2	42.1	41.5	47.2	52.4
3	34.8	29.8	45.7	46.8
4	42.5	36.2	74.4	57.2
5	19.1	17.8	19.3	20.8
Mean $\pm$ s.e.m. (n = 5)	37.0 $\pm$ 4.8	34.7 $\pm$ 5.2	45.1 $\pm$ 8.8	43.6 $\pm$ 6.4
(b) <i>Non-luteal ovarian tissue</i>				
Mean $\pm$ s.e.m. (n = 18)	0.05 $\pm$ 0.01	0.06 $\pm$ 0.02	0.05 $\pm$ 0.01	0.06 $\pm$ 0.02

Discussion

These results show that the CL of the tammar can actively produce progesterone *in vitro* at a steady rate for 4 h but non-luteal ovarian tissue cannot produce progesterone under these conditions. In these respects the tammar CL resembles those of eutherian mammals such as the hamster (Terranova, Connor & Greenwald, 1978), rat, rabbit and guinea-pig (Terranova, Saidapur & Greenwald, 1980), pig (Hunter, 1981) and man (Hunter & Baker, 1981). Also, like the CL of eutherians the rate of secretion varied at different days of the cycle; the rate of secretion of quiescent CL was similar to that of the newly formed rabbit CL but was much less than that of the newly formed rat CL (Terranova *et al.*, 1980). The high rate of secretion on Day 5 was similar to that of the 6-day pig CL (Hunter, 1981) and early human CL (Hunter & Baker, 1981). Conversely, the rates on Day 9 and 16 were higher than the unstimulated rates of the later stages of CL lifespan of either of these species but were comparable to their rates when incubated with hCG. This is particularly interesting as the CL of the tammar did not respond to LH or prolactin in the medium (Sernia *et al.*, 1980), is devoid of LH receptors (Stewart & Tyndale-Biscoe, 1982) and can function normally after hypophysectomy (unpublished observations).

Since the non-luteal ovarian tissue of the tammar produced negligible amounts of progesterone, as does the rabbit (Terranova *et al.*, 1980), it is highly likely that the CL is the major source of elevated plasma progesterone during the oestrous cycle and pregnancy in this species. There is a marked but transient peak of progesterone on Day 5 or Day 6 after removing the pouch young, which is followed by a return to basal concentrations by Day 9 and then a slow rise to maximum levels by Day 16 (Hinds & Tyndale-Biscoe, 1982a). As previously reported by Renfree *et al.* (1979),

and confirmed in this study, the concentration of progesterone in luteal tissue remains unchanged between quiescence (Day 0) and Day 16, so that the changes observed in plasma progesterone must arise from changes in total progesterone production by the CL or changes in metabolic clearance of progesterone. The significant increase in production rate *in vitro* on Day 5 occurred before the CL had begun to enlarge and so, if the secretion rate *in vivo* is similarly elevated, this alone would be sufficient to account for the peak in plasma progesterone on Day 5 or 6. Because the peak occurs on only 1 day in any individual animal the variance around the mean in the present experiments was high and not statistically significant. After Day 5 the progesterone production rate *in vitro* declined and so could not account for the subsequent major rise in plasma progesterone by Day 16. However, by this day the mass of the CL has increased 5- or 6-fold and this would account for the rise without an increased secretion rate.

Not only is the progesterone production rate unusually high on Day 5 but the luteal cells undergo a transient hyperplasia then (Berger, 1970; Renfree & Tyndale-Biscoe, 1973) before the hypertrophy that is evident by Day 16. Furthermore, it is only during the brief from Day 4 to Day 8 that ablation of the CL severely affects embryo survival (Sharman & Berger, 1969; Tyndale-Biscoe, 1970, 1979). Therefore, if prolactin exerts its effect by inhibiting steroidogenesis this is the most probable period. The results of Exp. III, which was designed to test this, did not support the hypothesis that prolactin inhibits steroidogenesis, nor did prolactin affect production of progesterone by quiescent or Day 1 CL (Sernia *et al.*, 1980). We conclude that prolactin has no significant role after the CL has reactivated. Since the removal of inhibition appears to be an all-or-none effect the role of prolactin must be to prevent the initiation of CL growth by hyperplasia and hypertrophy and so only indirectly prevent the rise in plasma progesterone. This is a novel function for prolactin in ovarian control but is analogous to its morphogenetic role in mammogenesis. This conclusion, however, leaves uncertain the reason for the high concentration of prolactin-binding sites on luteal cells on Day 16 (Stewart & Tyndale-Biscoe, 1982) long after the CL has been released from prolactin constraint.

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