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IN VITRO EFFECT OF PROLACTIN, PROSTAGLANDIN F2α AND CYCLOHEXIMIDE ON 20α-DIHYDRO-PROGESTERONE SYNTHESIS IN PSEUDO-PREGNANT RAT OVARIES

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SUMMARY

Experiments were designed to study the effects of prostaglandin F2α and prolactin in the modulation of steroidogenic response of the corpus luteum. Pseudo-pregnant rat ovaries, obtained by treatment with PMSG and HCG, were incubated or perifused in the presence of these compounds and the 20α-dihydro-progesterone (20α-DH-P) secretion and 20α-hydroxysteroid-dehydrogenase (20α-OH-SDH) activity estimated. The 20α-DH-P synthesis during pseudo-pregnancy in the rat increased for 21 days after HCG treatment and decreased thereafter. PG F2α increases 20α-DH-P secretion and 20α-OH-SDH activity whereas prolactin inhibits the amount of 20α-DH-P released and the 20α-OH-SDH activity. Cycloheximide blocked the response to PG F2α and which suggests that protein synthesis plays a part in this mechanism. Prostaglandin and prolactin failed to modify the 20α-OH-SDH activity when added directly to the "105,000 *g*" fraction isolated from these ovaries.

INTRODUCTION

Luteolysis is characterized, in the rat, by a decrease in the serum progesterone. It is now accepted that administration of the prostaglandin F2α (PG F2α) inhibits progesterone secretion and induces structural modifications of the corpus luteum [1].

The effect of this prostaglandin have been studied both *in vivo* and *in vitro* in different species. Intrauterine administration of PG F2α to cyclic ewes reduces blood flow to the ovary after 4 h and serum progesterone after 6 h, while 12 h after administration the ultrastructure of the corpus luteum is modified [2]. In the primates, administration in graded doses induces stimulation of progesterone synthesis at low doses and inhibition at high doses [3]. *In vitro*, opposite effects of PG F2α have been observed: increase of progesterone biosynthesis according to Pharris *et al.*[4], Speroff and Ramwell[5] in the rat, and Santos *et al.*[6], Suginami *et al.*[7] in the human corpora lutea. On the other hand, an inhibitory effect is noted by O'Grady *et al.* in the rabbit [8], Demers *et al.* in the rat [9] and Wilks *et al.*[10] in the rat and in the rabbit corpus luteum.

Three steps of the steroidogenesis mechanism may be influenced by PG F2α: (1) The LH-stimulated cAMP accumulation: PG F2α prevents *in vitro* this accumulation [11]. (2) The availability of cholesterol for conversion to progesterone: *in vivo* this prostaglandin causes a loss in synthetase activity [12]. (3) The 20α-hydroxysteroid dehydrogenase (20α-OH-SDH) activity. *In vivo*, PG F2α induces this activity in pregnant rats [13].

In the last two steps prolactin antagonizes the actions of the prostaglandin [12–16].

In the present work we have studied, *in vitro*, the effects of PG F2α and prolactin on the secretion of 20α-dihydro-progesterone (20α-DH-P) and the 20α-OH-SDH activity in pseudo-pregnant rat ovaries. It was considered of interest to test the possibility that a protein synthesis might play a part in this process. Accordingly the effect of cycloheximide, an inhibitor of protein synthesis [17], was investigated.

MATERIALS AND METHODS

Products. PMSG (1860 U/mg) and HCG (2660 U/mg) were supplied by Organon (Oss, Holland). Prostaglandin F2α was a generous gift of Dr Pike (Upjohn Company, Kalamazoo, MI, U.S.A.). Ovin prolactin (20–50 UI/mg) were supplied by Sigma (Paris). Indomethacin was kindly supplied by Merck (Sharp and Dohme, Rahway, NY, U.S.A.). 20α-DH-P antiserum for radioimmunoassay was purchased from Inst. Pasteur (Paris).

Animals. Immature female rats (Sprague–Dawley, Iffa, -Credo and Wistar from our laboratory) were treated with PMSG (50 UI) at 27 days old and with HCG (50 UI) at 30 days old. Seven, 4, 21, or 28 days after HCG treatment, the animals were killed and the ovaries removed and minced for perifusion or incubation.

Perifusion. The tissue slices from several animals were pooled before distribution into the incubation flask. This chamber was placed in an apparatus, similar to that described by Hashimoto[18], immersed in an incubator at 37°C with agitation and exposed to a continuous flow of Krebs–Ringer buffer, pH 7.4, con-

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taining glucose (2 mg/ml) and Bovine serum albumin (BSA) (0.5%). This medium was continuously saturated with a mixture of O_2 and CO_2 (95 + 5%) and pumped at a constant flow rate of 4 ml/h through the chamber by a peristaltic pump (multichannel, Gilson). The effluent medium was collected in 4 ml fractions for 5 h. Following this treatment, the tissue was homogenized in the same buffer, centrifuged at 105,000 *g* and the supernatant frozen for steroid analysis at a later date.

Incubation. In these experiments the sliced ovaries were pooled and then put into incubation flasks. After 30 min of preincubation, the medium was changed and incubation carried out for 3 h in a metabolic shaker at 37°C. The same buffer as for the perifusion studies was used. Following incubation, the tissues were homogenized and treated in the same way as for the perifusion studies.

Enzymic activity. The 20α-OH-SDH activity was determined according to Wiest[19] in the "105,000 *g* supernatant" obtained after homogenization of the ovary tissue in a 0.1 M Tris–HCl buffer pH 7.4 containing 0.1 M sucrose. The homogenate (1 g of wet-tissue/3 ml of buffer) was filtered through a layer of glass wool, and centrifuged for 1 h at 105,000 *g* and the pellet discarded.

An aliquot of supernatant (1.5 ml) was preincubated for 30 min at 37°C in the presence of NADPH (0.45 μmol/0.5 ml buffer). The reaction was initiated by adding progesterone (8×10^{-6} M) and stopped by freezing. The 20α-DH-P formed was measured by radiommunoassay.

Radiommunoassay of 20α-DH-P. This steroid was measured after extraction in petroleum ether (recovery 80%), evaporation at a temperature of 40°C and dissolution in 0.1% gelatin 0.1 M phosphate buffer at pH 7.4. This solution (0.1 ml) or 20α-DH-P standard solution, together with 0.1 ml [^{3}H]-20α-DH-P (2000 c.p.m.) and 0.1 ml diluted antiserum (Inst. Pasteur, Paris) were reared in polypropylene test tubes. The mean association constant of antiserum for 20α-DH-P was 3.9×10^9 M^{-1}. The only steroid showing a significant cross reaction was 20β-dihydro-progesterone: 6%. The tubes were vortexed and allowed to equilibrate for 30 min at room temperature followed by at least 15 min at 0°C. Within 2 min, 1 ml of Dextran charcoal solution (2.5 g charcoal, 0.25 g Dextran in 1000 ml buffer) was added to each tube in the assay. Ten minutes later the tubes were centrifuged at 2200 *g* for 5 min. The supernatant liquid was decanted into a scintillation vial and scintillation mixture added for radioactivity determination. Non-specific binding was less than 4%. The volume of medium extracted was chosen so that samples could be analysed at two dose levels between the 25 and 75% inhibition points of the standard calibration curve. The 50% inhibition level on this curve occurred at a dose of 70 pg. Procedural losses were monitored by addition of the [^{3}H]-steroid and calculated on the basis of the subsequent recovery of the added radioactivity.

The results of the assays were expressed as means ± S.E.M. The significance of the responses was tested by Student *t*-test.

RESULTS

20α-Hydroxysteroid-dehydrogenase (20α-OH-SDH) levels and age of pseudopregnancy in the rat

Preliminary experiments undertaken to determinate the ability of pseudopregnant rat ovaries of different ages, to synthetise 20α-DH-P, were carried out.

Ovaries were removed from rats (Sprague–Dawley) at 7, 14, 21 and 28 days after HCG, homogenized, and the 20α-OH-SDH activity determined (see Materials and Methods). This activity increased progressively: 18 ng of 20α-DH-P synthetized/ovary/20 min. seven days after HCG treatment, 90 ng after 14 days, 203 ng after 21 days and diminished after 28 days of HCG treatment: 80 ng/ovary/20 min.

Effects of PG F2α and cycloheximide on the 20α-DH-P secretion and 20α-OH-SDH activity

The effects of PG F2α was studied by incubations of pseudo-pregnant rat (Sprague–Dawley) ovary slices (see Materials and Methods). To avoid any action of endogenous prostaglandin, indomethacin at 10^{-5} M (an inhibitor of the prostaglandin synthesis [20]) was introduced in the preincubation and incubation medium. When cycloheximide (9×10^{-5} M) was employed, the antibiotic was present in the preincubation and the incubation medium. At the end of the incubation period, 20α-DH-P is evaluated in the tissue (105,000 *g* supernatant) and in the incubation medium. The 20α-OH-SDH activity was assayed in the 105,000 *g* supernatant.

The possible effect of cycloheximide in 20α-OH-SDH activity was investigated. Two sets of 8 ovaries were incubated, one set serving as control and the second receiving the antibiotic. The results were:

First experiment,
control 160 ng of 20α-DH-P synthetized/ovary/20 min and control + cycloheximide 200 ng/ovary/20 min.

Second experiment,
control 230 ng/ovary/20 min and control + cycloheximide 180 ng/ovary/20 min.

No significant differences of 20α-OH-SDH activity were found in control and treated groups.

The effect of prostaglandin treatment is shown in Table 1. PG F2α (10^{-6} M) increases the release of 20α-DH-P in the incubation medium. An increase in the amount of steroid and in the 20α-OH-SDH activity was observed throughout the experiment in the ovary. Cycloheximide inhibits these effects: *i.e.* these increases were only obtained when the protein synthesis was possible.

Effects of prolactin on the 20α-DH-P secretion and 20α-OH-SDH activity

The long-term experiments (5 h) were necessary to demonstrate the effect of prolactin on 20α-DH-P

Table 1. *In vitro* effects of PG F2α and cycloheximide on the 20α-dehydro-progesterone (20α-DH-P) secretion and the (20α-OH-SDH) activity in pseudopregnant rat ovaries

Experiment[1]	No.	Treatment	Mean ± S.E.M.[2] 20α-DH-P released[3]	20α-DH-P in tissue[4]	20α-OH-SDH activity[5]
I	1	Control	1133 ± 0.1	204 ± 36	172 ± 28
	2	PG F2α (10^{-6} M)	1560 ± 0.03*	341 ± 48*	252 ± 28 (ns)
	3	PG F2α (10^{-6} M) + cycloheximide (9×10^{-5} M)	466 ± 0.03***	74 ± 8*	200 ± 22*
II	1	Control	600 ± 0.03	197 ± 16	83 ± 6
	2	PG F2α (10^{-6} M)	866 ± 0.03**	315 ± 11***	317 ± 8***
	3	PG F2α (10^{-6} M) + cycloheximide (9×10^{-5} M)	266 ± 0.03**	177 ± 11***	83 ± 6***
III	1	Control	700 ± 0.03	133 ± 27	38 ± 2
	2	PG F2α (10^{-6} M)	966 ± 0.03***	300 ± 9***	110 ± 8***
	3	PG F2α (10^{-6} M) + cycloheximide (9×10^{-5} M)	430 ± 0.03***	138 ± 12***	59 ± 12**

1. Three sets of 8 ovaries (experiment I and II) and of 10 ovaries (experiment III) were incubated for 3 h in 10 ml of Krebs–Ringer buffer. Indomethacin was present in the incubation medium at 10^{-5} M.
2. The significance of the responses was tested by the Student *t*-test. In each experiment compared sets were 2/1 and 3/2. Not significant (ns); significant (*$P = 0.05$, highly significant, **$P = 0.01$, and ***$P = 0.001$).
3. Expressed in ng of 20α-DH-P released/ovary/hour.
4. In terms of ng of steroid/ovary.
5. Activity is expressed in terms of ng of 20α-DH-P synthetized/ovary/20 min.

secretion. Sliced ovaries of pseudo-pregnant rats (Wistar) were perifused. 20α-DH-P was measured in the excretion medium collected each hour during the 5 h experiment as well as in the tissue (105,000 *g* supernatant). 20α-OH-SDH was also estimated in this supernatant (see Materials and Methods).

Table 2 shows the results obtained in three experiments where prolactin was used at 10^{-10} M. The excretion of 20α-DH-P is significantly diminished as compared to the control. In the tissue, the amount of steroid was slightly lower than that of the control, but this difference was not statistically significant. The 20α-OH-SDH activity were also decreased under these conditions.

Direct effects of PG F2α and prolactin on 20α-OH-SDH activity of the "105,000 g supernatant"

When the "105,000 *g* supernatant" was incubated with PG F2α (10^{-6} M to 10^{-12} M) or prolactin (10^{-8} M to 10^{-12} M) no changes in 20α-OH-SDH activity was observed and direct addition had no affect on the enzyme activity.

DISCUSSION

These results demonstrate that *in vitro* PG F2α stimulates the synthesis of 20α-DH-P and induces 20α-OH-SDH activity (only significant in two out of three experiments) in pseudo-pregnant rat ovaries

Table 2. *In vitro* effect of Prolactin on the 20α-dihydro-progesterone (20α-DH-P) secretion and 20α-hydroxysteroid-dehydrogenase (20α-OH-SDH) activity in pseudo-pregnant rat ovaries

Experiment[1]	Treatment	Mean ± S.E.M.[2] 20α-DH-P released[3]	20α-DH-P in tissue[4]	20α-OH-SDH activity[5]
I	Control	26 ± 2.8	20 ± 1.9	2.9 ± 0.5
	LTH (10^{-10} M)	15 ± 0.9***	13 ± 1.3*	1.6 ± 0.1*
II	Control	102 ± 16	17 ± 2.7	9.7 ± 0.7
	LTH (10^{-10} M)	79 ± 11*	23 ± 1 (ns)	8.5 ± 1.2 (ns)
III	Control	63 ± 17	18 ± 3.7	2.6 ± 0.1
	LTH (10^{-10} M)	31 ± 9*	12 ± 2.9 (ns)	1.9 ± 0.1***

1. Two sets of 7 ovaries (experiment I) and of 8 ovaries (experiment II and III) were perifused using constant flow rate of 4 ml/h. One set served as control and the second was treated with prolactin.
2. In each experiment significance of the responses to prolactin was tested vs control. Significance of the responses is given in Table 1.
3. Expressed as ng of steroid released/ovary/hour (determined in the total pooled effluent).
4. In terms of ng of steroid/ovary.
5. Activity is expressed in terms of ng of 20α-DH-P synthetized/ovary/20 min.

slices 21 days after HCG treatment. Under the same conditions prolactin shows an opposite effect. At least in Wistar rats, the 20α-OH-SDH participates in the biochemical mechanism by which PG F2α exerts a lytic effect.

The catabolism of progresterone to 20α-DH-P during pseudo-pregnancy in the rat is increased for 21 days after HCG treatment and then decreases. These results are in agreement with those of Bast and Melampy[21] in pseudopregnant rats and those of Strauss *et al.*[22] in the rabbit, *in vivo*. Ovaries of Sprague–Dawley rats exhibit a greater 20α-OH-SDH activity for unknown reasons.

Prostaglandin and prolactin failed to modify the 20α-OH-SDH activity when added directly to the "105,000 *g* supernatant", nor did HCG under similar conditions [22].

In vivo studies [23] have also shown that PG F2α reduces progesterone and increase 20α-DH-P content in pseudopregnant rat ovaries. The mechanism by which PG F2α exerts this effect is not known. In the present work a stimulation of 20α-OH-SDH activity and of synthesis and release of 20α-DH-P is observed *in vitro*, and under these conditions the vascular theory [23] is excluded. The presence of prostaglandin receptors is reported by Rao *et al.*[24] in pseudo-pregnant rat ovaries and the binding of this prostaglandin to specific receptors may be the first step in the mechanism of action of these compounds. The results obtained with cycloheximide prove that protein synthesis is necessary in this mechanism of action.

On the other hand, LH, which increases progesterone production, also induces endogenous prostaglandin synthesis [25]. It has been shown elsewhere that these compounds inhibit the LH-stimulated progesterone synthesis [26]. This last result may be explained by an increase in catabolism of progesterone to 20α-DH-P.

Nevertheless, this prostaglandin has many effects: loss in content of LH receptors [27], inhibition of cyclic AMP accumulation [11] and loss of cholesterol ester synthetase activity [12].

Prolactin inhibits the amount of 20α-DH-P released and present in the tissue and the 20α-OH-SDH activity in the 105,000 *g* supernatant. Binding sites for LTH also exist in the luteal cells [28].

In conclusion, it appears that prostaglandin F2α and prolactin must participate in the modulation of the steroidogenic response of the corpus luteum through the 20α-DH-P synthesis. The effects of cycloheximide suggest that the synthesis of a protein is involved in the PG F2α regulation [29].

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