

Effect of Progesterone Alone and in Combination with either Corticosterone, 17 β -Estradiol or Insulin on Prolactin-stimulated Fatty Acid Synthesis in Mammary Explants from Pseudopregnant Rabbits

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Abstract

Progesterone up to 15.9 μ mol/l progressively suppressed prolactin-stimulated fatty acid synthesis in cultured mammary explants from 11-day pseudopregnant rabbits, but did not influence the proportion of fatty acids of medium chain length (C_{8:0}–C_{12:0}). Greater sensitivity to progesterone inhibition was observed at the low end of the ranges of corticosterone (29 nmol/l) and insulin (4.2 nmol/l) concentrations in the culture medium, which suggests an interaction between the hormones. A range of from 0.0001 to 10 μ mol/l of 17 β -estradiol concentrations had no effect on prolactin-stimulated fatty acid synthesis in the presence or absence of progesterone.

Introduction

It is generally accepted that progesterone is both mammogenic (Cowie 1971) and antilactogenic (Denamur 1971; Kuhn 1971) in most mammals. In rabbits, lactogenesis occurs during the last third of pregnancy when the epithelial cells of the well-developed alveolar structures are fully differentiated (Denamur 1971). Lactose is detected in appreciable amounts on day 24 of pregnancy. Injection of prolactin or cortisol during pregnancy in rabbits stimulates lactogenesis, assessed by milk or lactose production, whereas injection of progesterone (10 mg/day) prevents the initiation of these changes (Meites *et al.* 1963; Assairi *et al.* 1974). In rabbit mammary explants, lactose synthesis is inhibited in a dose-dependent manner at a range of 0.51 nmol/l to 5.1 μ mol/l (0.16 μ g/l–1.6 mg/l) of progesterone (Delouis 1975). Fatty acid synthesis is stimulated in a biphasic manner, initially from day 21 of pregnancy and secondly during the perinatal period (Strong and Dils 1972). These changes coincide with a gradual decline in plasma progesterone concentration, an abrupt decrease in the ratio of progesterone and estrogen in blood (Denamur 1971; Challis *et al.* 1973), an increase in the concentration of free glucocorticoids (Denamur 1971) and an increase in plasma prolactin (McNeilly and Friesen 1978).

It has been shown that maximum stimulation of fatty acid synthesis in mammary explants from 11-day pseudopregnant rabbits or mid-pregnant rabbits is achieved by a combination of insulin, corticosterone and prolactin (Forsyth *et al.* 1972; Strong *et al.* 1972). These hormones also induce the synthesis of fatty acids of medium chain length (C_{8:0}–C_{12:0}) which constitute about 68 mole % of the fatty acids of rabbit milk triacylglycerol (Jones and Parker 1981). However, it is not known if progesterone has an inhibitory effect on fatty acid synthesis either *in vivo* or *in vitro*. This paper examines the effect of progesterone on fatty acid synthesis in relation to corticosterone, 17 β -estradiol and insulin concentration in culture.

Materials and Methods

Animals

Virgin rabbits about 9 months old and maintained under natural cycles of light and darkness were made pseudopregnant by a single intravenous injection of human chorionic gonadotropin (500–600 i.u.). The day of injection was taken as day 0 of pseudopregnancy.

Chemicals

Medium 199 was from Commonwealth Serum Laboratories, Parkville, Vic. Bovine insulin (24·9 i.u./mg), corticosterone, 17β-estradiol, fatty acids and BF₃–methanol reagent were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Ovine prolactin (NIH-P-S-14; 25 i.u./mg) was a gift from Endocrine Study Section, National Institutes of Health, Bethesda, Md., U.S.A. Progesterone was from Roussel, U.S.P., France, and Sigma Chemical Company, St. Louis, Missouri, U.S.A. Sodium [1-¹⁴C]acetate, specific radioactivity 1·8–2·09 GBq (49–57 mCi/mmol), was obtained from Amersham Australia Pty Ltd, Sydney.

Preparation and Culture of Mammary Explants

Explants of mammary alveoli were prepared by the method of Forsyth and Myres (1971). Groups of 11 explants were cultured at 37°C in medium 199 containing 0·6 mM sodium acetate, 15 mM NaHCO₃–HEPES buffer (pH 7·4), polymyxin B sulfate and neomycin sulfate antibiotics (1–2 U/ml of medium) in an atmosphere of air. Sterile polythene vials were used for dilution of peptide hormone solution from stock, in order to minimize possible loss of hormones due to adherence to glass. Viability of the mammary epithelial cells and the alveolar integrity were confirmed by histological examination after staining with haematoxylin and eosin.

Rate of Fatty Acid Synthesis and Analysis of ¹⁴C-labelled Fatty Acid Methyl Esters by Radio-GLC

The method was essentially as described by Falconer *et al.* 1978; Smith and Falconer 1983.

Statistics

Significance of the difference between treatments was determined by Student's *t*-test or analysis of variance combined with regression analysis where appropriate (Fisher and Yates 1963; Snedecor and Cochran 1978). To stabilize the variance, data in Tables 1 and 2 were transformed using square root and logarithmic transformations where appropriate.

Table 1. Effect of progesterone (Pr) and corticosterone (C) on incorporation of [1-¹⁴C]acetate into fatty acids in explants from 11-day pseudopregnant rabbit mammary glands in the presence of insulin (I, 0·84 μmol/l) and prolactin (P, 0·043 μmol/l)

Results expressed as nmol per hour per milligram explant (mean ± s.e.m.; nine replicates from three rabbits)

Time in culture (h)	Hormones present	Concn of corticosterone (μmol/l)	Incorporation of acetate at progesterone concn (μmol/l) of:			
			0	0·159	1·59	15·9
0	No hormone	0	0·07 ± 0·03			
46	IC	2·89	0·22 ± 0·09			
46	ICP + Pr	0·029	1·86 ± 0·31	1·71 ± 0·39	0·96 ± 0·32	0·82 ± 0·27 ^A
46	ICP + Pr	0·29	2·55 ± 0·56	3·21 ± 0·64	2·26 ± 0·55	0·93 ± 0·27 ^B
46	ICP + Pr	2·89	2·70 ± 0·51	2·30 ± 0·43	1·97 ± 0·45	0·63 ± 0·29 ^C

^A $y^{0·5} = 1·21 - 0·26 \times \log(x)(\text{dose}), d.f. = 34, r = -0·42, P < 0·01.$

^B $y^{0·5} = 1·60 - 0·42 \times \log(x)(\text{dose}), d.f. = 34, r = -0·54, P < 0·001.$

^C $y^{0·5} = 1·51 - 0·46 \times \log(x)(\text{dose}), d.f. = 35, r = -0·59, P < 0·001.$

Results

Effect of Progesterone and Corticosterone on Prolactin Stimulation of Fatty Acid Synthesis

As shown in Table 1 on day 11 of pseudopregnancy fatty acid synthesis was at a low basal rate in the mammary tissue. In the presence of insulin and corticosterone in culture

for 2 days fatty acid synthesis increased slightly. When prolactin also was present, a marked stimulation (about 10-fold) occurred. Increasing the concentration of corticosterone progressively from 0.029 to 2.9 $\mu\text{mol/l}$ (0.01 to 1.0 mg/l) did not significantly enhance the stimulation by prolactin. Addition of progesterone to cultures containing 29 nmol/l to 2.9 $\mu\text{mol/l}$ of corticosterone showed a progressive linear ($P < 0.01$) inhibition of the stimulation by prolactin of fatty acid synthesis (Table 1). Histological examination showed that these explants were as viable and maintained alveolar integrity as well as those cultured in the absence of progesterone, with other lactogenic hormones being present. However, at higher corticosterone concentrations only the highest concentration of progesterone showed an individually significant inhibition of fatty acid synthesis, compared with incubation without progesterone or with lower concentrations ($P < 0.01$).

Table 2. Effect of insulin (I) on incorporation of [$1\text{-}^{14}\text{C}$]acetate into fatty acids in explants from 11-day pseudopregnant rabbit mammary glands in the presence of corticosterone (C, 0.29 $\mu\text{mol/l}$), prolactin (P, 0.0043 $\mu\text{mol/l}$) and progesterone (Pr, 0–15.9 $\mu\text{mol/l}$)

Results expressed as nmol per hour per milligram explant (mean \pm s.e.m.; $n = 9$ observations)

Time in culture (h)	Hormones present	Insulin concn ($\mu\text{mol/l}$)	Incorporation of acetate at progesterone concn ($\mu\text{mol/l}$) of:			
			0	0.159	1.59	15.9
46	CP	0	0.28 \pm 0.06	0.20 \pm 0.07	0.26 \pm 0.06	0.17 \pm 0.07
46	ICP + Pr	0.0042	2.27 \pm 0.05	2.08 \pm 0.63	1.26 \pm 0.36	0.47 \pm 0.10 ^A
46	ICP + Pr	0.042	3.22 \pm 0.88	2.55 \pm 0.57	1.87 \pm 0.61	0.61 \pm 0.06 ^B
46	ICP + Pr	0.42	2.37 \pm 0.41	2.72 \pm 0.51	2.24 \pm 0.56	0.94 \pm 0.19 ^C

Analysis of variance of data in line 3

Source of variation	d.f.	Sum of squares	Mean squares	Significance level
Between treatments				
Regression (linear)	1	3.519	3.519	$P < 0.05$
Residual	2	7.679	3.840	$P < 0.05$
Total	3	11.198	3.733	
Between rabbits	2	9.921	4.961	$P < 0.05$
Error	6	3.237	0.540	

^A $\text{Log}(y) = 0.43 - 0.77 \times \text{log}(x)$, d.f. = 34, $r = -0.61$, $P < 0.001$.

^B $\text{Log}(y) = 0.75 - 0.74 \times \text{log}(x)$, d.f. = 34, $r = -0.63$, $P < 0.001$.

^C $\text{Log}(y) = 0.80 - 0.59 \times \text{log}(x)$, d.f. = 34, $r = -0.57$, $P < 0.001$.

Effect of Progesterone and Insulin on Prolactin Stimulation of Fatty Acid Synthesis

As Table 2 shows, the presence of insulin was absolutely essential for the stimulatory effect of prolactin on fatty acid synthesis. Raising the concentration of insulin from 0.0042 $\mu\text{mol/l}$ (0.025 mg/l) to either 0.042 $\mu\text{mol/l}$ (0.25 mg/l) or 0.42 $\mu\text{mol/l}$ (2.5 mg/l) had no significant effect on the action of prolactin. Similarly to the data in Table 1 progesterone showed consistent ($P < 0.001$) progressive inhibition of prolactin-stimulated fatty acid synthesis provided that insulin was also present. Only in the absence of insulin was no significant effect of progesterone observed. Analysis of variance of line 3 of Table 2 is presented in that table.

Effect of Progesterone and 17β -Estradiol on Prolactin Stimulation of Fatty Acid Synthesis

Varying the amount of 17β -estradiol exponentially from 0.0001 to 10 $\mu\text{mol/l}$ (0.027 to 2700 $\mu\text{g/l}$) either alone or in the presence of 1.59 $\mu\text{mol/l}$ of progesterone had no significant effect on fatty acid synthesis stimulated by prolactin (0.043 $\mu\text{mol/l}$), in the

presence of insulin ($0.84 \mu\text{mol/l}$) and corticosterone ($2.9 \mu\text{mol/l}$) [result of analysis of nine observations derived from three rabbits for each treatment].

Effect of Progesterone on the Proportion of Fatty Acids of Medium Chain Length
($C_{8:0}$ – $C_{12:0}$)

On day 11, when the rate of fatty acid synthesis was at a low basal level, the proportion of fatty acids of medium chain length synthesized was 47 ± 3.4 (moles % of acetate incorporation). Stimulation of fatty acid synthesis by prolactin was not accompanied by a significant change in the proportion of fatty acids of medium chain length. The presence

Table 3. Effect of progesterone on medium-chain fatty acid synthesis by explants from 11-day pseudopregnant rabbit mammary gland after culture for 44 h

Mammary explants from 11-day pseudopregnant rabbits were cultured in medium 199 with insulin (I, $0.84 \mu\text{mol/l}$), prolactin (P, $0.043 \mu\text{mol/l}$), corticosterone (C, $2.9 \mu\text{mol/l}$) and progesterone (Pr, $15.9 \mu\text{mol/l}$) for 44 h and then groups of 10–11 explants were incubated at 37°C in medium 199 containing 0.6 mM sodium $[1\text{-}^{14}\text{C}]$ acetate (370 kBq) and 5 mM glucose for 2 h. Mean \pm s.e.m. of data from three rabbits are given

Time in culture (h)	Hormones in culture	Fatty acid synthesis ^A	Moles % of $[1\text{-}^{14}\text{C}]$ acetate incorporated into fatty acid:						
			$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}^+$ $C_{16:1}$	$C_{18:0}^+$ $C_{18:1}^+$ $C_{18:2}^+$	$\Sigma C_{8:0}$ – $C_{12:0}$ ^B
0	None	0.6 ± 0.3	16.9 ± 2.1	11.6 ± 1.3	18.8 ± 2.4	16.0 ± 1.4	13.4 ± 0.9	23.3 ± 2.4	47.3 ± 3.4
46	ICP	4.4 ± 1.4	18.5 ± 3.5	13.7 ± 3.6	22.8 ± 3.4	16.5 ± 1.9	13.5 ± 4.1	15.0 ± 2.9	55.0 ± 6.9
46	ICP + Pr	0.8 ± 0.3^C	18.2 ± 2.8	8.5 ± 2.8	18.5 ± 4.0	13.0 ± 1.4	11.7 ± 2.3	30.2 ± 8.0	45.1 ± 7.8

^A Units: nmoles of $[1\text{-}^{14}\text{C}]$ acetate incorporated per hour per milligram tissue.

^B $\Sigma C_{8:0}$ – $C_{12:0}$ values are not significantly different from each other (Student's *t*-test).

^C Significantly different from ICP value, $P < 0.01$.

of progesterone at $15.9 \mu\text{mol/l}$ inhibited fatty acid synthesis stimulated by prolactin ($P < 0.01$) but had no significant effect on the proportion of fatty acids of medium chain length synthesized by explants (Table 3).

Discussion

The results presented earlier show a progressive inhibition of milk-fat synthesis by progesterone up to $15.9 \mu\text{mol/l}$, in mammary explants in media containing insulin (4.2 to 420 nmol/l) and corticosterone (29 to 2900 nmol/l). However, the highest concentration of progesterone used is well above the maximum physiological concentration in the rabbit ($0.06 \mu\text{mol/l}$, Challis *et al.* 1973). Pharmacological dosage of rats with progesterone (5 mg/day) resulted in plasma concentrations above $1.5 \mu\text{mol/l}$ (Elkarib *et al.* 1983) which are closer to the concentrations which showed inhibitory effects *in vitro*.

At the lowest concentration of corticosterone used in the culture medium (29 nmol/l) an increased sensitivity to progesterone inhibition was observed (Table 1). Prolactin addition resulted in an eight-fold stimulation of milk-fat synthesis at this low corticosterone concentration, which was significantly reduced by $1.59 \mu\text{mol/l}$ of progesterone. The normal concentration of total plasma corticosterone in pregnant rabbits was 72 nmol/l (Gala and Westphal 1967). Similarly, at the lowest concentrations of insulin used (4.2 nmol/l) an increased sensitivity to progesterone was also observed (Table 2). This insulin concentration was just above the physiological range (0.34 – 0.85 nmol/l in the rat around parturition, Kuhn 1977) and resulted in a maximal response to prolactin in the explants cultured in the absence of progesterone. Both of these insulin and corticosterone concentrations are appreciably lower than those used by Carrington *et al.* (1983) in rabbit lobular cultures from mammary gland. However, their preparation of mammary lobules appeared to have a lowered overall sensitivity to hormones. Teyssot and Houdebine (1981) investigated the

effect of progesterone *in vivo* (10 mg/day for 5 days) on stimulation by prolactin of β -casein gene transcription in nuclei from mammary tissue from pseudopregnant rabbits. They observed a marked inhibition due to progesterone, which was greater at the lower prolactin dosage. Nuclei from lactating rabbits treated with progesterone showed little inhibition. Culture of mammary gland explants for 24 h with prolactin (4.3 nmol/l) and progesterone (3.2 or 15.9 μ mol/l), however, showed no significant suppression of casein synthesis. Teyssot and Houdebine suggest that progesterone receptors may have decreased during culture, hence lowering sensitivity to progesterone but it is also possible that the culture was not continued long enough to observe inhibitory effects.

One possible cause for the inhibition of fatty acid synthesis by progesterone is through competition between the pharmacological concentrations of progesterone and the low physiological concentration of corticosterone. In lactating rat mammary tissue progestagens have been shown to bind to glucocorticoid receptors (Quirk *et al.* 1983), which may thereby be prevented from functioning. Examination of the effects of estrogen concentration on fatty acid synthesis of cultured explants showed no effects over a range of 0.0001 to 10 μ mol/l whether progesterone was present or absent. Thus there is no support for the idea that estrogen may induce or maintain progesterone receptors in mammary tissue in culture thereby enabling progesterone to exert an inhibitory effect (Haslam and Shyamala 1979; Quirk *et al.* 1983).

Examination of the fatty acid composition of milk fat synthesized in culture showed no significant alterations in chain length of the fatty acids with any treatment, despite marked changes in rates of synthesis (Table 3). The high initial proportion of fatty acids of medium chain length may be a consequence of the high dose of chorionic gonadotropin used to induce pseudopregnancy, which may cause physiological precocity.

We conclude that within the wide range of progesterone concentrations used, progesterone *in vitro* showed a progressive inhibitory effect on prolactin-stimulated fatty synthesis in mammary gland explants. However, at near-physiological concentrations of corticosterone and insulin, only pharmacological and not physiological concentrations of progesterone were significantly inhibitory.

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