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EFFECTS OF PROLACTIN AND PROGESTERONE ON PREPUTIAL GLAND GROWTH AND PROGESTERONE METABOLISM IN HYPOPHYSECTOMIZED, OVARIECTOMIZED IMMATURE RATS

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SUMMARY

Using a 2 × 2 factorial experimental design, treatments of hypophysectomized, ovariectomized, immature rats with prolactin (100 μg twice daily) and progesterone (2 mg/day) resulted in a significant synergism between the two hormones on preputial gland wet weights. Prolactin or progesterone treatment alone, caused gland wet weights to be significantly greater than those of controls. Investigations of the possible modes of action of prolactin on the preputial gland showed that prolactin decreased serum testosterone and/or 17β-hydroxy-5α-androstan-3-one levels but had no effect on serum progesterone levels, indicating that the effect of prolactin is not mediated via an elevation in these serum steroid levels. Prolactin increased the overall rate of metabolism of [^{14}C]-progesterone by preputial gland tissue *in vitro* in proportion to its ability to increase wet weights of the glands, but did not alter the pattern of this metabolism. *In vivo* progesterone treatment caused a significant change in the pattern of [^{14}C]-progesterone metabolites *in vitro*. The main metabolites whose rates of formation were increased by progesterone treatment, were identified as 20α-hydroxy-5α-pregnan-3-one and 5α-pregnan-3α-20α-diol along with its 3β epimer. A less abundant metabolite, whose formation was diminished by progesterone treatment, was identified as 3α-hydroxy-5α-pregnan-20-one. The removal of the adrenal glands did not alter the effects of *in vivo* progesterone treatment on the preputial gland. Therefore, the data indicate that prolactin and progesterone exert separate effects on the preputial gland of the immature rat.

INTRODUCTION

The preputial gland of the rat is a modified sebaceous gland in the dermis of the prepuce and is used as a model for studying sebaceous gland activity. Ebling *et al.*[1] and Ebling[2] reported that progesterone had no significant effect on preputial gland wet weight or sebaceous gland activity in hypophysectomized male and female rats. However, high doses of progesterone did cause preputial gland weights and sebum secretion by sebaceous glands to be greater than controls, when the pituitary glands were intact [1–4].

Lasher *et al.*[5] and Lorincz[6] reported that in the hog anterior pituitary gland there was a sebotrophic hormone which was capable of influencing preputial gland wet weights and augmenting the effect of progesterone on the female preputial gland. Ebling *et al.*[1] reported that neither growth hormone nor adrenocorticotrophic hormone could augment the progesterone effect on the preputial or sebaceous glands and, therefore, did not have similar properties to the sebotrophic hormone described by Lorincz[6].

Since prolactin has been shown to act synergistically with a number of steroids on target organs [7–9], investigations were made of the effects of prolactin alone, and in combination with progesterone on the preputial gland of the female rat. Observations were made to determine whether prolactin could augment the effect of progesterone on the preputial gland and, thereby, display a similarity to the sebotrophic hormone described by Lorincz[6]. The possibility that prolactin treatment influences the metabolism of progesterone by the preputial gland was also examined.

MATERIALS AND METHODS

All Sprague–Dawley female rats were hypophysectomized by Hormone Assay Laboratories, Inc., Chicago, IL, U.S.A. at 22 days of age. They were maintained on 3% glucose solution, water and Purina Rat Chow. All animals were ovariectomized at 26 days of age. In one experiment, animals were adrenalectomized at the same age. The adrenalectomized rats were given 0.9% NaCl solution instead of water. All animals were exposed to 14 h light and 10 h darkness per day.

Experiment I: prolactin–progesterone. The animals were assigned to four groups based on a 2 × 2 factor-

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ial experimental design. Prolactin (NIH-P-S10, 100 μg twice daily) and progesterone (2 mg daily) were injected subcutaneously. Controls received both 0.9% NaCl solution and oil injections. The treatment period ranged from five to seven days.

Experiment II: progesterone-adrenalectomy. The animals were assigned to two groups. Group I received daily subcutaneous injections of sesame oil, while Group II received progesterone (2 mg daily) subcutaneously. The treatment period was five days.

Incubations. The animals were weighed and killed by decapitation. Trunk blood was collected from each animal and the serum was assayed either for progesterone or androgens. The preputial glands were immediately excised, dissected clean of any adhering tissue and weighed individually. The glands were incubated in a flask, previously flushed with a gas containing 95% O_2 and 5% CO_2, in one ml Krebs bicarbonate buffer[10] containing one mg per ml anhydrous dextrose and [^{14}C]-progesterone substrate (Amersham-Searle, 61 mCi per mmol). The flasks were gently agitated for two h in a water bath at 37°. The incubations were halted by placing the flasks in dry ice.

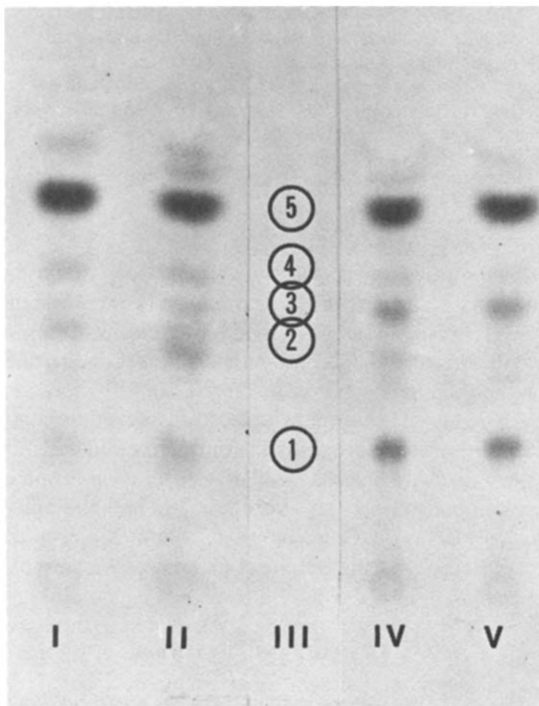

Fig. 1. Radioautogram of a thin layer chromatogram developed in methylene chloride–diethyl ether (5:2 v/v) to separate metabolites of progesterone-4-[^{14}C] produced by preputial gland incubations *in vitro*. Metabolites in channels I and II are from glands of controls and prolactin-treated rats, respectively. Metabolites in channels IV and V are from glands of progesterone-treated and progesterone + prolactin-treated rats, respectively. Chromatographic mobilities of 5α-pregnan-3α-20α-diol and its 3β-epimer (region 1), 20α-hydroxy-5α-pregnan-3-one (region 3), 3α-hydroxy-5α-pregnan-20-one (region 4), progesterone-4-[^{14}C] substrate (region 5) are indicated in channel III

Isolation of steroids. The preputial glands were homogenized in a glass homogenizer containing chloroform–methanol (2:1 v/v). The homogenates were extracted according to the method described by Folch *et al.*[11]. The extracts were evaporated to dryness under a stream of nitrogen. A 500 μl vol. of benzene was added to each tube and a 50 μl aliquot was taken for quantification of radioactivity in a Nuclear Chicago Isocap 300 liquid scintillation spectrometer. The remaining 450 μl was subjected to thin layer chromatography (t.l.c.) on 0.25 mm silica gel (Merck Silica Gel G.F. 254) plates, developed first in *n*-hexane–diethyl ether (95:5 v/v) at 4° and the sterol fractions were removed by scraping off the appropriate region of the chromatogram and then, in the same dimension, the plates were developed in methylene chloride–diethyl ether (5:2 v/v) to separate the labelled progesterone substrate from its radioactive metabolites.

The radioactive material was localized on the t.l.c. by autoradiography for three days as described by Armstrong *et al.*[12]. The [^{14}C]-progesterone metabolites were subdivided into four regions (see Fig. 1), and eluted off the silica gel using four ml chloroform–methanol (2:1 v/v) and two ml acetone.

The eluate of each region was dried under a nitrogen stream, dissolved in 500 μl benzene and 1/10 aliquots were radioassayed in a Nuclear Chicago Isocap 300 liquid scintillation spectrometer. The remaining amount of radioactivity from each region was pooled by treatment groups for further radiochemical identification by t.l.c. in different solvent systems, derivative formation and rechromatography, and recrystallization to constant specific radioactivity following addition of carrier steroids, according to the method described by Axelrod *et al.*[13]. Details of the specific methods applied to each region are described under Results.

The data are expressed in ng metabolites per mg tissue wet weight on the assumption that the S.A. of the substrate progesterone-4-[^{14}C] was not diluted significantly by endogenous steroids in the tissue.

Radioimmunoassay of steroids. Petroleum ether–benzene (5:2 v/v) extracts of serum were assayed for androgens using the method described by Moger and Armstrong[14], and employing antiserum to testosterone-3-bovine serum albumin. Since this antiserum cross-reacts with 17β-hydroxy-5α-androstan-3-one (5α-DHT) to approximately 53% of reaction with testosterone, the results of these assays are expressed as testosterone-DHT.

Petroleum ether extracts of serum were assayed for progesterone according to the method of Abraham *et al.*[15], using an antiserum to progesterone-11-bovine serum albumin described by Orczyk *et al.*[16].

Statistical analyses. Prior to testing for statistical significance of treatment effects, all data were subjected to the Bartlett's test[17] for homogeneity of variance. When heterogeneity of variance was found to exist, the data were transformed to logarithms.

Table 1. Prolactin and progesterone effects on preputial gland wet weights in hypophysectomized, ovariectomized immature rats (mean ± S.E.)

	Gland wet weight (mg)			
Group	Controls	Prolactin (200 μg)	Progesterone (2 mg)	Progesterone + prolactin
Five-day experiment:				
1	5.9 ± 0.3 (5)*	8.7 ± 0.6 (5)	6.4 ± 0.5 (5)	12.0 ± 1.1 (5)
2	5.6 ± 0.6 (7)	7.5 ± 1.1 (7)	5.7 ± 0.5 (7)	9.9 ± 1.1 (7)
3	5.6 ± 0.2 (7)	8.2 ± 0.4 (7)	7.4 ± 0.6 (7)	11.3 ± 0.9 (7)
4	4.2 ± 0.1 (5)	7.0 ± 0.5 (7)	5.8 ± 0.4 (7)	9.1 ± 0.5 (7)
5	4.6 ± 0.3 (7)	6.9 ± 0.3 (6)	4.7 ± 0.4 (5)	8.3 ± 0.5 (6)
Seven-day experiment:				
1	4.8 ± 0.3 (13)	8.2 ± 0.5 (12)	6.1 ± 0.4 (12)	10.6 ± 0.8 (12)

* Number of animals ( ).

Statistical significance of effects of treatments were established by '*t*' tests or analyses of variance where appropriate.

RESULTS

Prolactin–progesterone

Gland wet weight. Prolactin treatment for seven days caused the preputial gland wet weights to be significantly greater ($P < 0.001$) than those of the controls (Table 1). Progesterone treatment failed to alter gland weights significantly, whereas a statistically significant ($P < 0.05$) synergism was observed to result from treatment with the two hormones together. Similar results were obtained in five experiments in which the treatment period was five days. However, the slight progesterone effect on gland weights reached significance ($P < 0.01$) with increased number of experiments. Furthermore, a significant ($P < 0.01$) interaction occurred between progesterone, prolactin and experiment, indicating that the extent of the synergistic response varied with experiment.

Serum steroid levels. Measurements of serum steroid levels showed that there was no significant difference in progesterone levels between controls and prolactin-treated rats. Testosterone-dihydrotestosterone levels were significantly ($P < 0.001$) decreased by prolactin treatment paralleling a slight, but statistically significant ($P < 0.01$) effect on the part of prolactin to decrease adrenal weights (Table 2).

[^{14}C]-Progesterone metabolism. In presenting the data, [^{14}C]-progesterone metabolism refers to the conversion of [^{14}C]-progesterone into metabolites of the four major chromatographically separable regions, denoted as regions one to four in Fig. 1.

Progesterone treatment *in vivo* significantly ($P < 0.001$) increased [^{14}C]-progesterone metabolism by the preputial gland *in vitro*. However, the progesterone effect varied significantly ($P < 0.001$) with experiment (Table 3). In addition to affecting the extent of [^{14}C]-progesterone metabolism, *in vivo* progesterone treatment caused a change in the pattern of [^{14}C]-progesterone metabolites *in vitro*. The metabolites were subdivided into four major regions based on areas of radioactivity on the t.l.c. (Fig. 1).

Region 1. Rechromatography of the pooled samples of radioactive material of region 1 in methylene chloride–diethyl ether (5:2 v/v) followed by chromatography in ether–acetone (98:2 v/v) revealed two dense areas of radioactivity. The two radioactive areas were designated as regions 1(a) and 1(b). The compound in region 1(a) had a similar chromatographic mobility to 5α-pregnan-3α-20α-diol both as an acetate derivative and as a free compound. Following oxidation of the [^{14}C]-metabolite in region 1(a)

Table 2. Prolactin effects on serum steroid levels and adrenal weights in hypophysectomized, ovariectomized immature rats (mean ± S.E.)

Group	Adrenal gland weights (mg)	Serum progesterone (ng/ml)	Serum testerone-DHT (ng/ml)
Control	5.8 ± 0.1 (13)*	0.70 ± 0.32 (5)	0.57 ± 0.04 (7)
Prolactin (200 μg)	5.2 ± 0.2 (13)	0.93 ± 0.39 (5)	0.33 ± 0.03 (10)

* Number of animals ( ).

Table 3. Prolactin and progesterone effects on formation of [^{14}C]-metabolites from [^{14}C]-progesterone by preputial glands of hypophysectomized, ovariectomized immature rats (mean ± S.E.)

Group	Number of animals	[^{14}C]-Metabolites (ng/mg tissue) in: Region 1	Region 2	Region 3	Region 4
Experiment 1*					
Control	7	0.47 ± 0.09	0.65 ± 0.16	1.34 ± 0.24	0.77 ± 0.18
Prolactin (200 μg)	7	0.50 ± 0.10	0.66 ± 0.14	1.09 ± 0.14	0.60 ± 0.14
Progesterone (2 mg)	7	1.83 ± 0.21	0.49 ± 0.11	3.10 ± 0.36	0.18 ± 0.10
Prolactin + progesterone	7	1.23 ± 0.11	0.25 ± 0.02	2.23 ± 0.10	0.07 ± 0.02
Experiment 2**					
Control	5	0.53 ± 0.03	0.29 ± 0.09	0.89 ± 0.09	0.67 ± 0.14
Prolactin (200 μg)	5	0.49 ± 0.07	0.29 ± 0.06	0.94 ± 0.06	0.43 ± 0.04
Progesterone (2 mg)	5	1.37 ± 0.06	0.19 ± 0.03	1.58 ± 0.08	0.33 ± 0.04
Prolactin + progesterone	6	1.13 ± 0.03	0.13 ± 0.02	1.35 ± 0.09	0.14 ± 0.03

* [^{14}C]-Progesterone concentration in the incubation, 3.87×10^{-7} M.
** [^{14}C]-Progesterone concentration in the incubation, 2.35×10^{-7} M.

with Jones reagent[18], the product was recrystallized to constant S.A. with 5α-pregnan-3-20-dione (Table 4). The region 1(b) [^{14}C]-metabolite was recrystallized to constant S.A. with 5α-pregnan-3β-20α-diol (Table 4). It is therefore concluded that region 1(b) is predominantly 5α-pregnan-3β-20α-diol, and that region 1(a) is its 3α-epimer.

Progesterone treatment *in vivo* caused a highly significant ($P < 0.001$) increase in formation of region 1(a) and 1(b) metabolites *in vitro* in both experiments (Table 3). Prolactin also caused a significant ($P < 0.001$) increase in region 1 metabolites which was nullified by expressing the data per mg tissue wet weight (Table 3). The combination of prolactin and progesterone resulted in an additive response. It should be noted that the effect of progesterone on formation of regions 1 metabolites varied significantly ($P < 0.001$) with experiment in a similar fashion to that of [^{14}C]-progesterone metabolism.

Region 2. The labelled compounds in region 2 have not been identified. Upon further separation of the metabolites, it was found that at least four [^{14}C]-compounds were present—all possessing low amounts of radioactivity. There was no significant difference in amounts of radioactivity in this region when comparisons were made between all four treatment groups. However, a significant interexperimental variation ($P < 0.001$) in incorporation of label into region 2 existed (Table 3).

Region 3. Reduction of the region 3 metabolite by $NaBH_4$ followed by chromatography in ether–acetone (98:2 v/v) revealed two areas of radioactivity with similar chromatographic mobilities to 5α-pregnan-3α-20α-diol and its 3β epimer. The radioactive metabolites in the 3β epimer region was recrystallized to constant S.A. with 5α-pregnan-3β-20α-diol (Table 4), indicating that the major metabolite in region 3 was 20α-hydroxy-5α-pregnan-3-one.

Analysis of variance of ^{14}C incorporation into region 3 per mg tissue weight showed that progesterone caused a significant ($P < 0.001$) increase in labelled metabolites (Table 3). A significant ($P < 0.001$) interaction existed between progesterone and experiment, indicating that the progesterone effect varied with experiment. Prolactin treatment had no effect.

Table 4. Identification of metabolites of [^{14}C]-progesterone by recrystallization

Carrier steroid	Recrystallized from	Specific activity (d.p.m./mg) 2*	3	4	5
5α-Pregnan-3-20-dione [Region 1(a) oxidized using Jones reagent]	Methylene chloride and *n*-hexane		895	868	889
5α-Pregnan-3β-20α-diol [Region 1(b)]	Methylene chloride–methanol (95:5 v/v) and *n*-heptane	676	679	659	
5α-Pregnan-3β-20α-diol [Region 3 reduced using $NaBH_4$]	Methylene chloride–methanol (95:5 v/v) and *n*-heptane	1242	1228	1234	
3α-Hydroxy-5α-pregnan-20-one [Region 4]	Acetone and *n*-hexane	543	524	519	

* Number of recrystallizations.

Table 5. Effects of progesterone (2 mg) on preputial gland weights and [^{14}C]-progesterone metabolism in hypophysectomized, ovariectomized, adrenalectomized, immature rats (mean ± S.E.)

Group	Gland weights (mg)	^{14}C-Progesterone metabolites (ng/mg tissue) in: Region 1	Region 2	Region 3	Region 4
Control (4)*	4.6 ± 0.3	1.00 ± 0.11	0.36 ± 0.05	2.37 ± 0.26	1.97 ± 0.18
Progesterone (7)	7.1 ± 0.4	1.38 ± 0.12	0.35 ± 0.06	3.17 ± 0.17	0.33 ± 0.08

Note: [^{14}C]-Progesterone concentration in the incubation, 3.37×10^{-7} M.
* Number of animals ( ).

Region 4. The major metabolite of region 4 was identified by recrystallization to constant S.A. as 3α-hydroxy-5α-pregnan-20-one (Table 4). No significant interexperimental variation occurred in incorporation of label into this region. Examination of hormonal effects on formation of region 4 metabolites demonstrated a significant ($P < 0.001$) decrease due to progesterone treatment *in vivo* (Table 3). Prolactin treatment had no effect.

Progesterone–Adrenalectomy

Gland wet weight. Progesterone treatment caused preputial gland weights to be significantly ($P < 0.01$) greater than those of the controls (Table 5).

[^{14}C]-Progesterone metabolism. Progesterone treatment significantly ($P < 0.01$) increased [^{14}C]-progesterone metabolism *in vitro* and changed the pattern of metabolites in a manner similar to that seen in hypophysectomized, ovariectomized animals (Tables 3 and 5).

DISCUSSION

Results from five and seven day treatments with 200 μg prolactin demonstrated that prolactin had a highly significant effect on preputial gland wet weights in hypophysectomized, ovariectomized immature rats. Our data are supported by the findings of a number of workers [19–21], and lead us to propose that prolactin may be involved in the development of this modified sebaceous gland.

Our study also demonstrated that the combined treatments with prolactin and progesterone for five days resulted in a significant synergism with respect to increasing preputial gland wet weights in most experiments.

Because the rats that were given prolactin were not adrenalectomized, it is possible that prolactin may have exerted its effect by increasing the production of adrenal steroids or by enhancing the effects of adrenal steroids at the site of the preputial gland. However, measurements of serum steroid levels indicated that prolactin had no significant effect on serum progesterone levels, and serum testosterone-dihydrotestosterone levels were actually significantly decreased by prolactin treatment. It cannot be concluded whether this latter effect of prolactin was caused by a decrease in adrenal androgen production or by increases in androgen clearance, e.g. greater uptake by target tissues or increased metabolism. Since there was an effect by prolactin to decrease adrenal gland weights, this suggests that prolactin may decrease adrenal steroid output. Because the levels of steroids in hypophysectomized, ovariectomized rats are low, it is difficult to explain the effect of prolactin on the preputial gland in terms of a "permissive" action—allowing steroids to exert a greater stimulatory effect. Therefore, our data suggest that prolactin can act independently of steroids on influencing the weight of the preputial gland. We have also shown an action of prolactin on preputial glands, independent of steroids, when squalene was used as a measure of glandular activity (Wielgosz, G. and Armstrong, D. T., unpublished observations).

Progesterone treatment *in vivo* influenced the pattern of [^{14}C]-progesterone metabolism *in vitro*. This change in pattern of [^{14}C]-metabolites involved an increased formation of 5α-reduced and 20α-reduced [^{14}C]-metabolites (regions 1 and 3), suggesting that the effect of *in vivo* treatment with high levels of progesterone results in an increase in activities of the 5α-reductase and 20α-hydroxysteroid dehydrogenase (20α-SDH) in the preputial gland. Such substrate-induced increases in enzyme activities could provide an effective means of controlling local levels of progesterone as has been suggested for other organs [22–25].

When the animals were adrenalectomized as well as hypophysectomized and ovariectomized, progesterone treatment *in vivo* had significant effects on both preputial gland wet weights and [^{14}C]-progesterone metabolism, indicating that progesterone does not act via the adrenal glands, ovaries or pituitary gland.

In summary, prolactin and progesterone acted synergistically on preputial gland wet weight, indicating that prolactin possesses properties of a sebotrophic hormone. *In vivo* progesterone treatment slightly increased preputial gland wet weights and changed the pattern of [^{14}C]-progesterone metabolites *in vitro*. It is suggested that this change may be due to an increase in 5α-reductase and 20α-SDH activities, and that the rise in 20α-SDH activity could be a means of controlling local levels of progesterone in the preputial gland.

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