Inhibition by Progesterone of the Lactogenic Effect of Prolactin in the Pseudopregnant Rabbit

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Milk secretion can be induced *in vivo* in the pseudopregnant rabbit by the injection of prolactin. When progesterone was injected simultaneously with prolactin, secretion was suppressed. The steroid depressed the increased lactose synthetase (EC 2.4.1.22) activity and the lactose synthesis which can be induced by prolactin in mammary tissue from pseudopregnant rabbits. However, the galactosyl transferase activity was only slightly decreased. The modification induced by prolactin in the polyribosome profile and the increase in the ratio of membrane-bound to free polyribosomes was also completely abolished by progesterone. Measurement of the nucleic acid content of the mammary gland showed that the administration of progesterone suppressed the increase in RNA content which is induced by prolactin. These results suggest that progesterone inhibits the lactogenic action of prolactin without affecting its mammogenic role.

Considerable changes occur in rabbit mammary epithelial cells during the initiation of milk secretion. These changes include cellular hyperplasia, an increase in the total RNA content of cells (Denamur, 1965) and the appearance of intracellular membranes (Bousquet *et al.*, 1969; Tobon *et al.*, 1972). There is also an increased amount both of polyribosomes and of the proportion of polyribosomes bound to endoplasmic reticulum, which is associated with modification of their sedimentation profiles and of their synthetic activity (Gaye & Denamur, 1969). The injection of prolactin induces milk secretion in pseudopregnant rabbits as well as in adrenalectomized, ovariectomized, hypophysectomized pseudopregnant rabbits (Denamur, 1971; Cowie & Tindal, 1971). When mammary explants from pseudopregnant rabbits are cultured with prolactin and insulin with or without a corticoid, secretory activity appears after few days in culture (Forsyth, 1971; Delouis & Denamur, 1972). Lactogenesis occurs at day 19 of pregnancy in the rabbit (Denamur, 1963; Denamur & Delouis, 1972; Strong & Dils, 1972; Mellenberger & Bauman, 1974) and has been correlated with the progressive decrease in the concentration of progesterone in the blood (Denamur, 1971). On the other hand, the concentration of plasma prolactin at this time of pregnancy is unknown. Moreover, the injection of progesterone at day 14 of pregnancy lowers the rate of synthesis of lactose after day 19 of pregnancy (Denamur & Delouis, 1972). The inhibitory effect of progesterone on the induction of milk secretion by prolactin in

mammary-gland explants from pseudopregnant rabbits has been reported (Delouis, 1974). A similar action of progesterone has been previously described at the end of pregnancy in the rat (Kuhn, 1969; Davis *et al.*, 1972), and with mammary explants from pregnant mice maintained in organ cultures (Turkington & Hill, 1969). The inhibitory effect of progesterone on lactogenesis could result from modification by the hormone of prolactin secretion or from a direct action of the steroid on mammary cells or both.

In the present study, we describe the inhibitory effect of progesterone on lactose synthesis and on the other changes which are observed in the mammary cells of pseudopregnant rabbits which have been submitted to prolactin treatment.

Materials and Methods

Animals and hormonal treatments

Rabbits of the strain 'Petit Russe' were used. Pseudopregnancy was induced by mating six-month-old female rabbits with vasectomized males.

Rabbits (group I) received intramuscular injections of ovine prolactin (Laboratoire Byla, Paris, France; batch no. 02-67, activity 20 i.u./mg), 12.5 i.u. twice daily from day 15 of pseudopregnancy onwards. A second group of rabbits (group II) received the same prolactin treatment as well as twice-daily intramuscular injections (5 mg) of progesterone (Improvac, Laboratoire Roussel, Paris, France) from day 14 of pseudopregnancy onwards.

* Deceased April 1973.

Four animals were killed on day 17 and another four animals on day 19 of pregnancy (group I), and their mammary glands removed. Sixteen rabbits which had not been submitted to hormonal treatment were killed on days 14, 15, 17 and 19 of pseudopregnancy (four animals at each time-period). An additional sixteen rabbits, which had received progesterone injections (group II) from day 14 of pseudopregnancy onwards, were killed on days 15, 17 and 19 of pseudopregnancy (four animals at each time-period).

Methods

The total RNA and total DNA contents of the mammary gland were measured as described by Gaye & Denamur (1969). Determinations were made with defatted, dried powder prepared from glands collected on one side of the animal, so that the results are expressed as the total nucleic acid content of half of the mammary gland.

Total and free polyribosomes were isolated by the method of Blobel & Potter (1967), their sedimentation profiles and their synthetic activities were determined as described previously (Gaye & Denamur, 1969).

Lactose synthesis was determined by measuring the synthesis of radioactively labelled lactose after incubation of mammary tissue (which was dissected out immediately after bleeding the animals) with [U- 14 C]-glucose (Delouis & Denamur, 1972).

DNA synthesis was determined after incubating the mammary tissue for 2h with [2- 14 C]thymidine (0.5 μ Ci/ml). The radioactivity was measured after the nucleic acids had been isolated by the method of Schmidt & Tannhauser (1945). Radioactive thymidine (specific radioactivity 48mCi/mmol) was purchased from the Laboratoire des Radioéléments Artificiels, C.E.A., Saclay, France.

Lactose synthetase (EC 2.4.1.22) and galactosyl transferase activities were estimated in crude homogenates of mammary gland which were prepared by the method of Fitzgerald *et al.* (1970). The enzyme activities were determined by the UDP-[14 C]galactose assay described by Brodbeck & Ebner (1966).

Electron microscopy

Mammary tissue was fixed in glutaraldehyde (4%, w/v) and OsO₄ (1%, w/v) in sodium phosphate buffer (0.4M), pH 7.25, and embedded in Epon, as previously described (Bousquet *et al.*, 1969).

Results

Determination of the inhibitory effect of progesterone on lactose synthesis induced by prolactin

Activities of lactose synthetase and galactosyl transferase. These enzymes showed very low activities in mammary tissue obtained from pseudopregnant

rabbits that had not been submitted to hormone treatment; the highest activities were observed between days 15 and 17 of pseudopregnancy (Figs. 1 and 2), which corresponds to the time when the content of RNA and DNA in the mammary gland is maximum (Gaye & Denamur, 1969). The injection of progesterone alone from day 14 of pseudopregnancy decreased both enzyme activities at days 15 and 17 of pseudopregnancy (Figs. 1 and 2).

Fig. 1. Inhibition by progesterone of lactose synthetase activity induced by prolactin in pseudopregnant rabbits

Lactose synthetase activities of mammary tissue were measured as described in the Materials and Methods section. Pseudopregnant rabbits were submitted to: ○, no hormonal treatment; ●, progesterone (10mg/day); □, prolactin (25i.u./day); ■, progesterone (10mg/day)+prolactin (25i.u./day). Each point represents the mean of four determinations (four animals). The vertical bars indicate mean values $\pm$ S.E.M.

Fig. 2. Effect of progesterone on the galactosyl transferase activity induced by prolactin in pseudopregnant rabbits

Galactosyl transferase activity of mammary tissue was determined as described in the Materials and Methods section. Pseudopregnant rabbits were submitted to: ○, no hormonal treatment; ●, progesterone (10mg/day); □, prolactin (25i.u./day); ■, progesterone (10mg/day)+prolactin (25i.u./day). Each point represents the mean of four determinations (i.e. four animals). The vertical bars indicate mean values $\pm$ S.E.M.

The administration of prolactin alone led to an increase in lactose synthetase activity 2 days after the start of the injection and to a further increase after 4 days of treatment (Fig. 1). The increase in galactosyl transferase activity was only significant after 4 days of treatment with prolactin alone (Fig. 2).

The simultaneous injection of progesterone prevented the increase in lactose synthetase activity induced by prolactin which was seen after 2 days of treatment and produced a 70% decrease after 4 days of treatment with prolactin (Fig. 1). The galactosyl transferase activity was not affected by the simultaneous injection of progesterone in the presence of prolactin after 2 days of treatment, but was 30% lower 4 days after the start of the prolactin treatment (Fig. 2).

Table 1. Effect of progesterone on the incorporation of [14 C]glucose into lactose during lactogenesis induced by prolactin in pseudopregnant rabbit mammary gland

Mammary explants were incubated for 15 min in medium 199 (Flobio, Paris, France), supplemented with insulin (Endopancrine, Paris, France; 120m-i.u./mg) and [U- 14 C]glucose (C.E.A., Saclay, France; specific radioactivity 150mCi/mmol; 25 μ Ci/ml). The explants were then transferred to medium 199 supplemented with insulin (5 μ g/ml) and glucose (16.7mM) and incubated for a further 3 h. The total lactose synthesized (i.e. in the medium and in the tissue) was determined as previously described (Delouis & Denamur, 1972). The values are means $\pm$ S.E.M. with the number of animals in parentheses.

Hormonal treatment	[14 C]Lactose synthesized (c.p.m./mg wet wt. of tissue)
Day 14 pseudopregnancy, no hormone treatment	12 $\pm$ 3 (4)
Day 19 pseudopregnancy, no hormone treatment	157 $\pm$ 47 (4)
Day 14 pseudopregnancy, injected with prolactin (25i.u./day) for 48 h	1082 $\pm$ 500 (4)
Day 14 pseudopregnancy, injected with prolactin (25i.u./day)+progesterone (10mg/day) for 48 h	53 $\pm$ 11 (4)

Lactose synthesis. The incorporation of [U- 14 C]glucose into lactose was increased in explants of mammary tissue from rabbits which had been injected for 2 days with 25i.u. of prolactin per day. However, the results were not as reproducible as those usually obtained by using 100i.u. of the hormone per day (Delouis & Denamur, 1972), although the incorporations of radioactive glucose into lactose were linear during these incubation periods. [14 C]Lactose synthesis was almost completely inhibited in tissue obtained from animals which had been injected with progesterone (Table 1).

Limitation by progesterone of the increased nucleic acid content induced by prolactin

The total DNA and the total RNA content of the mammary gland were increased by injections of prolactin alone (2 $\times$ 12.5i.u./day). The maximum DNA response had already occurred after 2 days of treatment, but the content of RNA was still increasing after 4 days of treatment. The RNA content was considerably decreased by the simultaneous injection of progesterone and prolactin, but still remained higher than in untreated animals. The DNA content of the mammary gland was the same when animals were injected with prolactin or with prolactin plus progesterone for 2 days. It was slightly lower when pseudopregnant rabbits were injected with prolactin

Fig. 3. Effect of progesterone on the total RNA and DNA contents of mammary glands of pseudopregnant rabbits during lactogenesis induced by prolactin

Nucleic acid content of mammary tissue was measured as described in the Materials and Methods section. Pseudopregnant rabbits were submitted to: ○, no hormonal treatment; ▲, progesterone (10 mg/day); □, prolactin (25 i.u./day); ■, prolactin (25 i.u./day)+progesterone (10 mg/day). Each point represents the mean of four determinations (i.e. four animals). The vertical bars indicate mean values $\pm$ S.E.M.

and progesterone than when they received prolactin injections alone for 4 days (Fig. 3).

The incorporation of [14 C]thymidine into mammary-gland DNA was measured in pseudopregnant rabbits and in those injected with prolactin alone, with progesterone alone or with both hormones (Fig. 4). The incorporation of [14 C]thymidine into DNA was similar when prolactin was injected alone or simultaneously with progesterone. This result, when compared with slight differences in the total DNA content of mammary glands obtained from animals which had received the same hormone treatments (Fig. 3) suggests that the effect of progesterone on the mammogenic action of prolactin is low. On the other hand, progesterone alone was able to maintain a high degree of DNA synthesis (Fig. 4). This corresponds to the ability of progesterone alone when injected into pseudopregnant rabbits to maintain the total DNA content of the mammary gland (R. Denamur, unpublished work).

These results show that progesterone inhibits the increase in the RNA/DNA ratio brought about by prolactin alone (Fig. 3). This effect seems to be due mainly to an inhibition of the prolactin-induced increase in the total RNA content of the mammary cells.

Influence of progesterone on polyribosome modification induced by prolactin

Ratios of polyribosomes bound to endoplasmic reticulum/free polyribosomes. In rabbit mammary gland at day 14 of pseudopregnancy about 20–25% of the total polyribosomes were bound to membranes. After 96 h of prolactin injections, this proportion had increased to 70%. This effect of prolactin was completely suppressed by the simultaneous injections of progesterone, since the proportion of bound polyribosomes was never higher than 15–20% of total polyribosomes when the two hormones were injected together.

The results of examinations of the mammary cells by electron microscopy are in full agreement with the biochemical data. Most of the polyribosomes appeared free in the cytoplasm and the endoplasmic reticulum was only slightly developed in cells of mammary tissue from rabbits which had been injected simultaneously with prolactin and progesterone (Plate 1).

Sedimentation profile of polyribosomes. After 96 h of prolactin treatment, the sedimentation profile of the total polyribosomes were similar to those usually observed in rabbit mammary gland during lactation. They were characterized by the presence of a peak

EXPLANATION OF PLATE I

Electron micrographs of mammary-gland cells from pseudopregnant rabbits

(a) Day 14 of pseudopregnancy; the cytoplasm contains mainly free ribosomes (arrows) (ER, endoplasmic reticulum) (magnification $\times 10000$). (b) The pseudopregnant rabbit had been treated with prolactin as described in the Materials and Methods section for 48 h (25 i.u./day). The cytoplasm contains a well-developed endoplasmic reticulum (ER) with numerous ribosomes and a Golgi apparatus (G) filled with protein granules (magnification $\times 10000$). (c) The pseudopregnant rabbit had simultaneously been injected with prolactin (25 i.u./day) and progesterone (10 mg/day). The cytoplasm contains only poorly developed endoplasmic reticulum (ER). Ribosomes in small clusters are free in the cytoplasm (arrows) (magnification $\times 10000$).

Fig. 4. Effect of progesterone on DNA synthesis induced by prolactin in mammary glands of pseudopregnant rabbits

The incorporation of [^{14}C]thymidine into the DNA of mammary tissue was measured as described in the Materials and Methods section. Pseudopregnant rabbits were killed at the time indicated on the abscissa. Vertical bars indicate mean values $\pm$ S.E.M. Each point represents the mean of four determinations (i.e. for four animals).

□, Controls; ▨, progesterone (10mg/day) from day 14 of pseudopregnancy; ▩, prolactin (25i.u./day) from day 15 of pseudopregnancy; ■, progesterone (10mg/day) from day 14 of pseudopregnancy + prolactin (25i.u./day) from day 15 of pseudopregnancy.

corresponding to 7–9 ribosomes (Fig. 5b) which was not observed in pseudopregnant rabbit mammary glands (Fig. 5a). This modification was almost totally

suppressed by simultaneous injections with prolactin and progesterone (Fig. 5c). The differences in the sedimentation profile of the total polyribosomes (Fig. 5) may correspond to differences in the sedimentation profile of bound polyribosomes since the latter represent about 70% of total polyribosomes after 96h of prolactin treatment. The sedimentation profile of free polyribosomes which had been isolated from mammary glands of rabbits injected for 96h with prolactin alone (Fig. 6b) show a large peak of monomers associated with polymers which were more aggregated than those observed when they were isolated from mammary glands of pseudopregnant rabbits (Fig. 6a). After simultaneous injections of prolactin and progesterone, the sedimentation profiles (Fig. 6c) were almost identical with those observed in lactating-rabbit mammary gland or in mammary gland of pseudopregnant rabbits which had been injected with prolactin alone for 4 days (Fig. 6b).

Synthetic activities of total and free polyribosomes. In all cases, the synthetic activity of polyribosomes was measured with an enzyme fraction prepared from lactating rabbit mammary gland which was used as a source of translational factors.

The rate of incorporation of [^{14}C]leucine into proteins synthesized by the total polyribosomes (pmol of [^{14}C]leucine incorporated/mg of ribosomal RNA) increased during the prolactin treatment of the pseudopregnant rabbits. It was 3.5 and 4.5 times higher after 48 and 96h respectively than in control pseudopregnant rabbits. The administration of progesterone diminished the increase in the rate of [^{14}C]leucine incorporation caused by prolactin injections. However, the synthetic activities of total polyribosomes were 2.6 and 2.7 times higher after 48 and 96h respectively than those prepared from the mammary gland of control animals (Table 2).

Free polyribosomes prepared from mammary glands of pseudopregnant rabbits which received injections of prolactin or progesterone plus prolactin for 96h were as active as were the total polyribosomes prepared from the same mammary glands. In addition, the synthetic activities of the free polyribosomes were not modified by the simultaneous administration of progesterone and prolactin (Table 2).

Discussion

The lactose synthetase activity of the mammary gland of pseudopregnant rabbits increased after injecting low concentrations of prolactin. This effect occurred earlier (after 48h) and was more marked than was the increase in galactosyl transferase activity (after 48 and 96h). This suggests that the stimulation of lactose synthetase activity which occurs during the first 2 days of prolactin treatment without modification of galactosyl transferase activity corresponds to

Fig. 5. Sedimentation profiles of total mammary-gland polyribosomes in a sucrose gradient

Pseudopregnant rabbits were submitted for 96h to: (a) no hormonal treatment; (b) prolactin (25i.u./day); (c) prolactin (25i.u./day)+progesterone (10mg/day).

Fig. 6. Sedimentation profiles of free mammary-gland polyribosomes in a sucrose gradient

Pseudopregnant rabbits were submitted for 96h to: (a) no hormonal treatment; (b) prolactin (25i.u./day); (c) prolactin (25i.u./day)+progesterone (10mg/day).

the induction or the stimulation of a low initial rate of synthesis of α -lactalbumin. The considerable increase in the two enzyme activities 48 and 96h after prolactin treatment might be the result of a stimulated synthesis of the two components. It might also be due to an augmentation of the number of sites present on the membranes able to accumulate the A protein (galactosyl transferase). This interpretation is compatible with (a) the moderate increase in the amount

of total mammary-gland RNA and the increase in the RNA/DNA ratio which occurred during the first 2 days of prolactin treatment, (b) the prolactin-induced development of endoplasmic reticulum which occurs mainly between 2 and 4 days of prolactin treatment (Bousquet *et al.*, 1969); and (c) the sedimentation profile of free polyribosomes after 2 days of prolactin treatment which suggests that the synthesis of membranes might follow the appearance of

Table 2. *Synthetic activities of polyribosomes after prolactin or prolactin and progesterone administration to 14-day pseudo-pregnant rabbits*

The assay mixture contained in a total volume of 250 μ l: 200 μ M-Tris-HCl buffer, pH 7.6, 5 μ M-MgCl₂, 1 μ M-dithiothreitol, 19 amino acids minus leucine (0.06 μ M each), 2 μ Ci of [¹⁴C]leucine (specific radioactivity 150 μ Ci/mmol) (Laboratoire des Radioéléments Artificiels, C.E.A., Saclay, France), 1 μ M-ATP, 0.4 μ M-GTP, 10 μ M-creatine phosphate, 40 μ g of creatine phosphokinase (EC 2.7.3.2.); 45 μ g of polyribosomes (rRNA), 75 μ g of tRNA and 350 μ g of the enzyme fraction extracted from lactating-rabbit mammary glands [i.e. the 105000_{g_{av}} supernatant which was then precipitated with 70% (w/v) (NH₄)₂SO₄ and the precipitate then dialysed]. Incubations were of 30 min at 37°C. At the end of the incubation 1 ml of 10% (w/v) trichloroacetic acid containing an excess of unlabelled leucine was added to the incubation and the mixture was heated to 90°C for 15 min. Proteins synthesized were collected on glass filters and the radioactivities were measured in a liquid-scintillation spectrometer. The values are means $\pm$ S.E.M. with the number of rabbits in parentheses except for the control (no hormone) where the value is the mean $\pm$ half the difference between the two results.

Hormonal treatment	[¹⁴ C]Leucine incorporated (pmol/mg of RNA)	
	Total polyribosomes	Free polyribosomes
No hormone	100 $\pm$ 25 (2)	—
Prolactin (25 i.u./day) 48 h	350 $\pm$ 30 (4)	—
Prolactin (25 i.u./day)+progesterone (10 mg/day) 48 h	270 $\pm$ 40 (4)	—
Prolactin (25 i.u./day) 96 h	450 $\pm$ 120 (5)	400 $\pm$ 140 (3)
Prolactin (25 i.u./day)+progesterone (10 mg/day) 96 h	270 $\pm$ 50 (5)	300 $\pm$ 140 (3)

polyribosomes which are able to bind to them (Gaye & Denamur, 1969).

The induction of lactose synthetase was completely suppressed by the simultaneous injection of progesterone during the first 2 days of treatment with prolactin and this corresponds to the absence of lactose synthesis during this period. Galactosyl transferase activity was not affected by the simultaneous injection of progesterone during this period. Therefore the decrease in lactose synthesis caused by progesterone is probably the result of an inhibition of α -lactalbumin synthesis. This interpretation agrees with the work of Turkington & Hill (1969). They found, by using mouse mammary gland in organ culture, that the induction of lactose synthetase activity by prolactin plus cortisol was inhibited by a lower concentration of progesterone than was the galactosyl transferase activity. This latter finding was confirmed by experiments using mammary explants from pseudopregnant rabbit in organ cultures (Delouis, 1974). However, the total absence of variation of polyribosome profiles compared with the untreated controls and the small increase in the amount of RNA observed when progesterone was injected with prolactin do not correspond to a selective inhibition of α -lactalbumin synthesis without a correlated inhibition of total milk-protein synthesis.

In addition, the stimulation of the synthesis of immunoprecipitable casein mammary-gland fragments by prolactin injected into pseudopregnant rabbits did not occur when progesterone was injected simultaneously (L. M. Houdebine & P. Gaye, unpublished work). Progesterone decreases the rate of α -lactal-

bumin synthesis at the same rate as the synthesis of total protein and of immunoprecipitable casein in pseudopregnant rabbit mammary-gland organ cultures (C. Delouis, unpublished work).

Low concentrations of prolactin (25 i.u./day) induced the formation of polyribosomes similar to those found in the secretory cell which has a high ratio of bound/free and high amount of large aggregates. Progesterone completely prevented this effect of prolactin. The low ratio of bound/free polyribosomes observed after progesterone and prolactin treatment might be the result of the absence of a developed endoplasmic reticulum in the mammary cell, but sedimentation profiles of free or total polyribosomes after injection of progesterone plus prolactin differed from those obtained with prolactin alone. The complete absence of large aggregates of polyribosomes from mammary cells submitted to progesterone and prolactin treatment might be simply due to the lack of mRNA coding for milk proteins in these polyribosomes.

Finally, the limited effect of progesterone on the DNA as compared with the RNA content of the mammary cell showed that the inhibitory effect of this steroid is restricted to the lactogenic action of prolactin. This suggests that the mammogenic action of prolactin was only slightly affected.

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