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The role of the suckling stimulus in regulating pituitary prolactin mRNA in the rat

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Summary

Prolactin (PRL) gene expression and the synthesis and secretion of PRL were examined in ovarian-intact lactating rats suckling eight pups on 10 days postpartum. Plasma samples were assayed for PRL concentrations, and pituitary glands were analyzed for total PRL content and PRL mRNA levels. We found that suckling-induced hyperprolactinemia was associated with very high levels of plasma PRL and a doubling in pituitary PRL mRNA levels, whereas pituitary PRL content was not changed. Removal of the suckling pups decreased plasma PRL concentrations 15-fold within 24 h. This decrease in PRL secretion was not accompanied by any significant change in pituitary PRL content. Evidently, both synthesis and secretion of PRL were decreased in the pituitary gland within 24 h following cessation of suckling, as pituitary PRL mRNA content had returned to diestrous levels at this time. To determine whether or not ovarian steroids might have contributed to the changes in PRL synthesis and secretion during lactation and after withdrawal of the suckling stimulus, the experiments were repeated in lactating rats ovariectomized (OVX) on day 2 postpartum. The results in these OVX rats were qualitatively similar to those described in ovarian-intact rats.

We concluded from these findings that the stimulus of suckling induces increases in PRL mRNA levels in the pituitary which provides for the increased PRL synthesis accompanying increased PRL secretion. The cessation of suckling led to prompt decreases in PRL synthesis and secretion within 24 h.

Introduction

In the rat, the pattern of plasma prolactin (PRL) during the estrous cycle is similar to that

for plasma luteinizing hormone (LH). Basal PRL and LH are low except on the afternoon of proestrus (Gay et al., 1970; Smith et al., 1975). In the ovariectomized rat, injection of estradiol induces daily afternoon surges of PRL and LH (Neill et al., 1971; Neill, 1972; Carrillo et al., 1987). However, in some physiological conditions, the plasma levels of PRL and of LH are inversely related. For example, during the first half of pregnancy (Bast et al., 1972; Butcher et al., 1972) and pseudopregnancy (Bast et al., 1972; Smith et al., 1975), during lactation (Lu et al., 1976; Fox et al., 1984), and in

response to stress (Neill, 1970; Ferland et al., 1978; Khorran et al., 1985; Petraglia et al., 1986), plasma PRL is increased while plasma LH is at low basal levels. Recently, we have shown that LH secretion is suppressed during lactation and pituitary α-subunit mRNA and LHβ mRNA levels are also decreased (Fox et al., 1984; Lee et al., 1989a). Moreover, when the suckling stimulus is withdrawn by removing the pups, the reappearance of pulsatile LH secretion is associated with the accumulation of pituitary LH subunit mRNA (Lee et al., 1989a, b). The present experiments were designed to determine how plasma prolactin concentrations, pituitary PRL content and pituitary PRL mRNA levels are affected by lactation and by withdrawal of the suckling stimulus.

Materials and methods

Animals

Female Sprague-Dawley rats from Zivic Miller Laboratories were kept under controlled temperature (20–24 °C) and lighting (lights on 06.00–18.00 h) conditions. Food and water were provided ad libitum. Rats were mated during proestrus and pregnant animals were placed in maternity cages 2 days before the expected day of parturition. Lactating rats (day of delivery designated day 0 postpartum) were ovarian-intact or were ovariectomized on day 2 postpartum and, at that time, litters were adjusted to 8 or 0 pups. Experiments were conducted on day 10 postpartum, and in some groups, litters were removed 12–72 h before day 10.

Blood collection procedures and pituitary gland removal

Rats were decapitated and 5–10 ml of blood were collected into heparinized tubes. Plasma was stored at −20 °C until assayed for PRL. Pituitary glands were removed and homogenized in phosphate-buffered saline (PBS) (pH 7.0), then centrifuged at 3000 × g for 30 min. The supernatant was stored at −20 °C until assayed for PRL. To assess whether the extraction method affected PRL content (Haggi and Aoki, 1981; Lawson et al., 1987), pituitaries from some groups of animals were extracted in high pH PBS (pH 10.5). The

results are expressed as μg PRL/100 μg DNA. In our laboratory, pituitary DNA content averages about 100 μg/pituitary. For RNA determinations, pituitary glands were removed under sterile conditions, rapidly frozen on dry ice and stored at −70 °C.

RNA extraction and measurement of PRL mRNA

Pituitaries were quickly thawed and homogenized in 220 μl buffer (10 mM Tris-HCl, 0.5% Nonidet P-40, and 1 mM EDTA, pH 7.4). The homogenate was centrifuged at 13 000 × g for 5 min in the cold room. The nuclear pellets were sonicated to assay DNA content (Labarca et al., 1980). The RNA in the supernatant was extracted with a mixture of phenol/chloroform/isoamyl alcohol (100 : 100 : 1). Total cytoplasmic RNA was quantitated by absorbance at 260 nm (Papavasiliou et al., 1986). Specific PRL mRNA levels were measured by an RNA dot-blot hybridization as described previously (Papavasiliou et al., 1986), using a rat PRL cDNA probe provided by Dr. Richard A. Maurer (Maurer, 1983). In this study, all samples were measured in the same dot-blot assay. We performed a number of experiments to optimize the dot-blot assay for PRL mRNA. To determine the amount of PRL cDNA probe that was necessary to completely saturate the binding sites per filter, identical quantities of RNA from an adult female rat pool were spotted onto several nitrocellulose filters (1200 ng of RNA spotter per filter) and each filter was hybridized with different concentrations of labeled single-stranded PRL cDNA probe (25–200 ng/6 ml hybridization buffer). Following 16 h of hybridization, filters were washed and dots cut out and counted for 1 min. The results in Fig. 1 show that under the conditions used, the hybridization of PRL mRNA to cDNA was complete using 100 ng or more probe/filter. We have selected 150 ng cDNA/1200 ng RNA spotted as a constant, to ensure saturation in our assay system. Fig. 2 shows that when a saturating amount of PRL probe was used, increasing the quantity of RNA per dot resulted in a linear increase in PRL mRNA per dot. We spot 25 or 50 ng RNA per dot in triplicate for each sample in our optimized assay system.

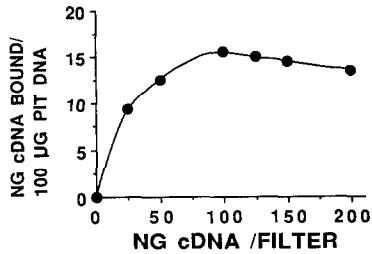During the characterization of the PRL mRNA dot-blot assay, we compared dot blots to Northern

Fig. 1. Determination of saturating concentrations of PRL cDNA probe for a constant amount (1200 ng) of total cytoplasmic RNA per nitrocellulose filter. Each point represents triplicate measurements. (Note: in our assay system we spot 1200 ng of RNA per filter (25 or 50 ng RNA per blot) and hybridize to 150 ng of PRL cDNA, to insure saturation.)

blot analysis using mRNA samples from several rat models (intact males, OVX females, cycling females). In general, PRL mRNA levels determined by dot-blot analysis showed parallel changes to samples examined by Northern blot. Also, the PRL cDNA probe selectively hybridized to a single mRNA band. Results are expressed as ng cDNA bound/100 μg pituitary DNA to equalize variations in pituitary size and cell number between samples. The equation used to calculate the results is as follows:

ng probe bound/100 μg pituitary DNA

$$= \frac{A \times R \times 1.22 \times 100}{SA \times e^{-kt} \times C \times D} \times 10^3$$

where A is the counts per min specifically bound, R is the pituitary RNA content (μg/180 μl cytosol), 1.22 is a volume correction factor for the
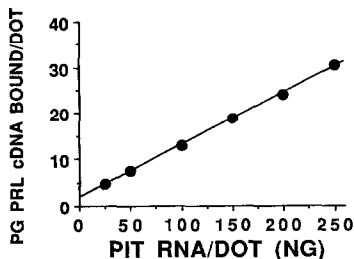

Fig. 2. The linearity of the PRL cDNA binding to increasing amounts of total cytoplasmic RNA per dot (from a random cycling female rat pituitary pool), when a saturating amount of cDNA was applied to a nitrocellulose filter and hybridized (each point is the result of triplicate determinations).

difference between the volume of homogenization (220 μl) and the volume of phenol-extracted cytosol (180 μl), SA is the specific activity of the probe (ranged between 2–5 $\times 10^8$ cpm/μg PRL cDNA), e^{-kt} is the correction factor for radioactive decay, where t is the time difference in days between the data of nick translation and counting of the nitrocellulose spots and k is the ^{32}P decay constant (0.0485), C is RNA (μg per spot), D is the pituitary DNA content (μg), and 10^3 is the mass conversion constant from micrograms to nanograms (Papavasiliou et al., 1986).

Prolactin radioimmunoassay and data analysis

Plasma PRL concentrations (5–20 μl duplicates from each sample) and pituitary PRL content (1:1000 dilution, 5–10 μl duplicates) were assessed by the radioimmunoassay (RIA) described previously (Smith, 1978a). NIAMD RP-3 standard was used, and the sensitivity of the assay was 0.01 ng/tube. The data were analyzed by one-way analysis of variance and Newman-Keuls test ($P <$ 0.05).

Results

Plasma prolactin levels, and pituitary prolactin content in ovarian-intact rats during lactation and after withdrawal of the suckling stimulus

Fig. 3 shows that the suckling stimulus from eight pups increased plasma PRL concentrations by 15-fold when compared to control, diestrous rats (99.2 $\pm$ 6.3 vs. 6.3 $\pm$ 1.8 ng/ml). At 24 h after pup removal, plasma PRL concentrations had dropped sharply and were not significantly different from diestrous, control values. Pituitary PRL content was similar in lactating rats and in diestrous rats (22.1 $\pm$ 4.3 vs. 26.8 $\pm$ 4.2 μg/100 μg DNA). After pup removal, pituitary PRL content did not change significantly compared to lactating rats. Table 1 shows the effect of extraction at a high pH on pituitary PRL content. Animals whose pups had been removed for 48 h were included to more carefully determine the effect of withdrawal of the suckling stimulus. Increasing the pH of the homogenization buffer from 7.0 to 10.5 resulted in about a 10-fold increase in PRL content (see Fig. 3 for comparison). However, qualitatively, the results were very similar to those shown in Fig. 3.
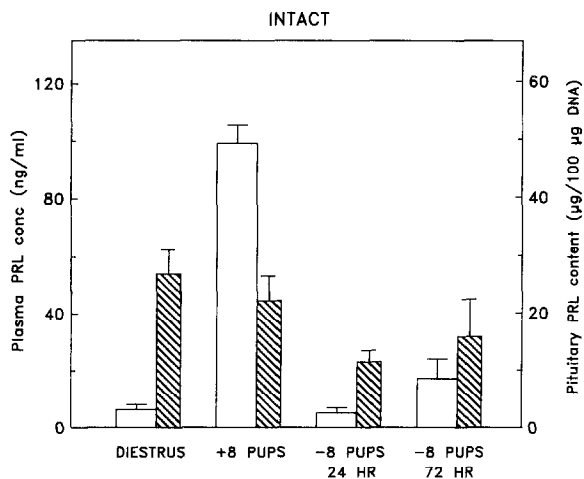
Fig. 3. The effect of lactation and of withdrawing the suckling stimulus on plasma PRL levels (open columns) ($n = 6$–13 animals) and pituitary PRL content (hatched columns) ($n =$ 3–6 animals) in ovarian-intact rats on day 10 postpartum. Plasma PRL was significantly higher ($P < 0.05$) in the +8 pup group than in any other group.
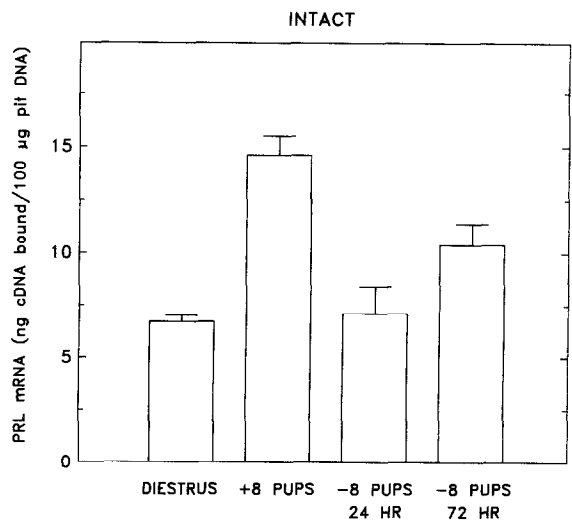

Fig. 4. Pituitary PRL mRNA content in ovarian-intact lactating rats on day 10 postpartum ($n = 6$–8 animals). PRL mRNA levels were significantly increased by the suckling stimulus compared to the other groups and had returned to diestrous levels 24 h after pup removal.

There was no significant change in pituitary PRL content after pup removal.

Prolactin mRNA concentrations in ovarian-intact lactating rats and after pup removal

Pituitary PRL mRNA levels in diestrous rats ($n = 8$) averaged 6.7 ± 0.3 ng cDNA/100 μg pituitary DNA (Fig. 4). During lactation, the mRNA levels increased to 14.6 ± 0.9 ng cDNA/100 μg pituitary DNA. By 24 h after pup removal, pituitary PRL mRNA levels had returned to diestrous, control levels.

Plasma prolactin levels and pituitary prolactin content in ovariectomized lactating rats and after withdrawal of the suckling stimulus

Lactation increased plasma PRL concentrations to 119 ± 12 ng/ml (Fig. 5), levels similar to

TABLE 1

EFFECT OF HIGH pH HOMOGENIZATION BUFFER (PBS, pH 10.5) ON PITUITARY PRL CONTENT

Numbers are mean ± SEM.

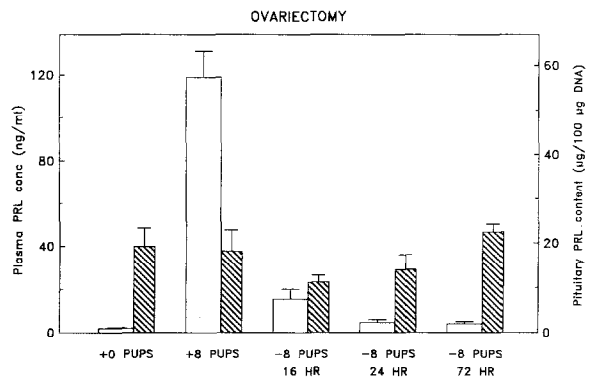| Group	Pituitary PRL (μg PRL/100 μg DNA)
Diestrus	216 ± 18 [a]
Intact + 8 pups	159 ± 18
Intact − 8 pups, 24 h	152 ± 18
Intact − 8 pups, 48 h	150 ± 9

[a] $P < 0.05$ compared to other groups.

Fig. 5. The effect of lactation and of withdrawing the suckling stimulus on plasma PRL levels (open columns) and pituitary PRL content (hatched columns) in OVX lactating rats on day 10 postpartum ($n = 4$–7 animals). Ovariectomy was performed on day 2 postpartum. Suckling greatly increased plasma PRL concentrations when compared to all other groups. Plasma PRL had returned to nonsuckled control levels by 16 h after pup removal. Pituitary PRL content did not differ among these groups.
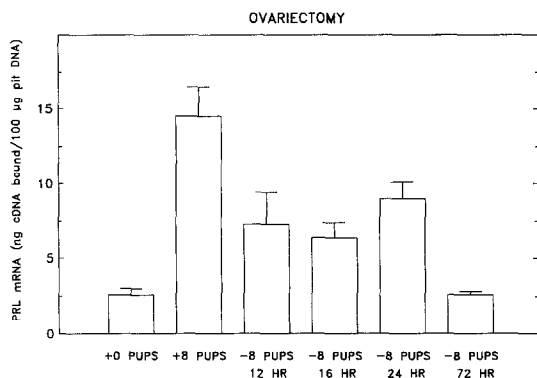
OVARIECTOMY

Fig. 6. Pituitary PRL mRNA during lactation and after pup removal in OVX rats ($n = 6$–10 animals). Pituitary PRL mRNA levels were increased significantly by suckling. PRL mRNA levels had decreased by 12 h after pup removal but did not return to nonsuckled control levels until 72 h.

those observed in ovarian-intact lactating rats (Fig. 3). Plasma PRL concentrations had decreased markedly by 16 h after pup removal, to levels not significantly different from levels observed in OVX + 0 animals. Pituitary PRL content in the OVX lactating rat (19.2 ± 4.2 μg/100 μg DNA) was similar to the content observed in intact lactating rats (26.8 ± 4.2 μg/100 μg DNA). Pituitary PRL content did not change in the presence of the suckling stimulus (Fig. 5), and similar to the intact rats, did not change significantly after pup removal. The primary differences between intact and OVX animals was that plasma PRL was significantly higher in diestrous controls and at 72 h after pup removal than in comparable OVX groups.

Pituitary prolactin mRNA concentrations in OVX lactating rats and after pup removal

Ovariectomized nonsuckling rats had low pituitary PRL mRNA content than diestrous rats (2.6 ± 0.4 vs. 6.7 ± 0.3 ng cDNA bound/100 μg pituitary DNA, Figs. 5 and 6). The suckling stimulus increased pituitary PRL mRNA in OVX rats to the same levels as in ovarian-intact lactating rats (14.5 ± 2.0 vs. 14.6 ± 0.9 ng cDNA bound/100 μg pituitary DNA) (Figs. 5 and 6). By 12 h after pup removal, PRL mRNA content had decreased when compared to lactating rats suckling eight pups. By 72 h, pituitary PRL mRNA (2.6 ± 0.2 ng cDNA bound/100 μg pituitary DNA) had returned to

the levels observed in nonlactating control rats. These levels were significantly lower than those observed at 72 h after pup removal in intact animals.

Discussion

During lactation, we observed very high plasma PRL levels which correlated with high pituitary PRL mRNA levels. Moreover, plasma PRL and pituitary PRL mRNA were increased to the same degree in both OVX and ovarian-intact lactating rats. We have shown previously that plasma estrogen concentrations are very low during lactation (Smith, 1978b; Lee et al., 1989b). Thus, it appears that estrogen does not play a role in increasing PRL synthesis during lactation. Therefore, the mechanisms mediating PRL synthesis during lactation are different from those during other conditions such as the estrogen-induced PRL surge. Recently, Carrillo et al. (1987) reported that estrogen-induced PRL surges are associated with very high levels of pituitary PRL and PRL mRNA content, suggesting that estrogen increases PRL synthesis. Estrogen is known to have a stimulatory effect on pituitary PRL gene expression and on PRL synthesis in vivo and in vitro (Maurer, 1982; Shull and Gorski, 1984). Our data did show that basal levels of plasma PRL and pituitary mRNA content were significantly higher in intact animals, i.e., diestrous controls and at 72 h after pup removal, than in the OVX animals, which is probably due to much higher levels of plasma estradiol in the intact animals.

In investigating the time course of the decline in PRL secretion and the change in pituitary PRL stores following withdrawal of suckling pups from the lactating rat, our findings show that in both OVX and intact lactating rats, the return to basal levels of plasma PRL and of pituitary PRL mRNA occurred within 12–24 h after pup removal. Surprisingly, pituitary PRL content remained quite constant in the face of very high or low plasma PRL concentrations and PRL mRNA levels. These data differ from those of Torres and Aoki (1985) who reported that PRL content was increased 24 h after withdrawal of suckling. However, our data consistently showed that PRL content tended, if anything, to decrease 12–24 h after pup removal,

whether in intact or OVX rats, and regardless of the pH of the homogenization buffer. Therefore, our results cannot be explained by a failure to adequately extract PRL. It has been reported that within 24 h after pup removal there is a marked increase in pituitary lysosomal enzyme activity and prolactin protease, which would cause lysosomal degradation of PRL (Farquhar, 1977; De Marco et al., 1982). This crinophagy could prevent large increases in PRL content in the face of decreases in synthesis and secretion. Perhaps, if these parameters were studied during a dynamic episode of suckling-induced PRL secretion, instead of during steady-state conditions as examined in this study, changes in PRL content would be observed. It has been reported that during the proestrous PRL surge (Haisenleder et al., 1988) and during the estrogen-induced PRL surge (Carrillo et al., 1987), periods of dynamic increases in PRL secretion, pituitary PRL content decreases and PRL mRNA levels increase. In this situation, PRL synthesis does not appear to be able to keep up with the secretion of PRL.

What factors may be involved in increasing pituitary PRL mRNA levels during lactation? One hypothesis is that suckling induces pituitary PRL secretion which in turn triggers pituitary PRL synthesis. That there may be a relationship between PRL gene expression and PRL secretory pools is supported by studies of the PRL proestrous surge. When the PRL surge is blocked by bromocriptine, the decrease in pituitary PRL content and the increase in pituitary PRL mRNA are not observed (Haisenleder et al., 1988), suggesting that PRL secretion triggers increases in PRL mRNA levels. Another hypothesis is that suckling increases the secretion of prolactin-releasing factors (PRFs), such as thyrotropin-releasing hormone (TRH) (Grosvenor and Mena, 1980; Horn et al., 1985; Rondeel et al., 1988), oxytocin (Samson et al., 1986) and vasoactive intestinal peptide (VIP) (Abe et al., 1985), which act to increase PRL synthesis and PRL secretion. In fact, the secretagogue action of TRH (Rosenfeld et al., 1983), VIP (Carrillo et al., 1985) and Ca^{2+} (Jackson and Bancroft, 1988) are associated with increased PRL gene transcription. In addition, suckling-induced suppression of dopamine secretion could increase sensitivity of the lactotrope to PRFs

(De la Escalera and Weiner, 1988) as well as increase PRL mRNA levels, since dopamine has been shown to inhibit PRL gene expression, and decrease PRL mRNA levels (Maurer, 1982). The mechanisms involved in the regulation of PRL gene expression and PRL secretion during lactation await further study.

In summary, these data show that during lactation and after withdrawal of the suckling stimulus, plasma PRL concentrations and pituitary PRL mRNA levels increase and decrease in parallel, whereas pituitary PRL content remains quite constant. In addition, increases in plasma PRL concentrations and pituitary PRL mRNA in ovarian-intact and OVX lactating rats are similar, suggesting that ovarian factors do not play a significant role in regulating these parameters during lactation.

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