Inhibition of Nocturnal Prolactin Surges in the Pregnant Rat by Incubation Medium Containing Placental Lactogen (42941)

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Abstract. **Rats hysterectomized on Day 7 or 8 of pregnancy continued to have nocturnal prolactin surges 1 day later. Conditioned medium obtained from incubation of Day 11 placentas infused via the jugular vein completely blocked this nocturnal surge, indicating a negative feedback of placental secretions on prolactin. Infusion of an ultrafiltrate of the conditioned medium which only contained molecules with M_r above 10,000 also blocked the prolactin surge. Next, it was determined whether this feedback of placental secretions on prolactin may work by way of hypothalamic dopamine. Levels of dopamine in hypophysial stalk blood from pregnant rats on Day 12, a time when secretion of placental lactogen is high, were not different from those in rats in which placental lactogen was absent. It is concluded that termination of prolactin surges at midpregnancy may be due to feedback of placental secretions, possibly placental lactogen, on the hypothalamus and/or pituitary. However, these experiments do not support the hypothesis that this inhibition is mediated by alteration in hypothalamic dopamine secretion.** [P.S.E.B.M. 1989, Vol 191]

The twice daily surges in serum prolactin concentration that occur during the first half of pregnancy abruptly disappear at midpregnancy (1). This loss in prolactin surges occurs at the same time that placental lactogen-1 (PL-1) secretion is increasing (2), and results of numerous experiments suggest that PL-1 may be the primary factor responsible for this termination of prolactin secretion. Secretion of PL-1 and termination of prolactin surges have been correlated with number of conceptuses present (3, 4). A delay in blastocyst implantation, resulting in a delay in secretion of PL, prolonged the number of days the prolactin surges were present (5). Incubation of anterior pituitaries in the presence of conditioned medium containing PL resulted in inhibition of prolactin release (6, 7). Several attempts have been made to show that placental extracts given *in vivo* will inhibit prolactin (1, 8–10) and all have failed. More recently, we showed that intraperitoneal injections of placental extracts did not result in the appearance of PL in the circulation, providing a possible explanation for the lack of any effect of placental extracts of prolactin surges (11).

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The primary objective of this study was to demonstrate *in vivo* that conditioned culture medium from placental incubations could inhibit nocturnal prolactin surges. The use of conditioned medium as a source of PL was necessary because of the unavailability of purified rat PL. A secondary objective was to determine whether the elevated PL seen during the second half of pregnancy stimulated dopamine release into the hypophysial portal blood. If this is the case, it can be concluded that dopamine is a physiologic inhibitor of prolactin secretion during the second half of pregnancy.

Materials and Methods

Animals. Two sources of rats were used for these experiments. For the protocol (Experiments 1 and 2) in which conditioned medium was infused into pregnant rats, Holtzman rats from Sasco (Omaha, NE) were used. For the experiment (Experiment 3) done at Erasmus University, Rotterdam, The Netherlands, locally bred female (R × U)F_1 hybrid rats were used. All animals were kept in a temperature-controlled room with lights on 14 hr, off 10 hr daily. Food and water were always available. For mating purposes, one male was housed with one female during proestrus. The following day was designated as Day 0 of pregnancy if sperm were observed in a vaginal lavage collected that morning.

Experimental Protocols. *Experiment 1.* On Day 7 or 8 of pregnancy, all rats were hysterectomized while

anesthetized with metaphane. At the same time each rat received one cannula in the left common carotid artery and another in the right jugular vein using PE50 tubing. One day later, the jugular cannula was connected via a flow through swivel to a peristaltic pump. Infusion of incubation medium via the jugular cannula was continuous at a rate of 1 ml/hr beginning at midnight until 0700 hr. Blood samples (0.3 ml) were obtained via an extension of the carotid cannula at midnight and at 0200, 0400, 0600, and 0700 hr. In general, it was not necessary to handle or disturb the rat once the experiment began. Nine rats received conditioned medium containing placental lactogen and nine rats received unconditioned medium.

Experiment 2. This experiment was identical to Experiment 1 except the concentration of PL in the infusate was much higher and contained only compounds with M_r greater than 10 kDa. Control rats received unconditioned medium containing additional bovine serum albumin (3%) as a control for the protein in the experimental perfusate. Seven rats were used in each group.

Experiment 3. Pregnant rats were hysterectomized or sham operated on Day 6. On Day 11, the intact and half of the hysterectomized rats received an injection of the dopamine agonist, bromocriptine, subcutaneously at a dose of 3 mg/kg body wt. This agonist was used in one group of hysterectomized rats so prolactin would be reduced. Thus, the intact rats should have high PL and low prolactin levels, the hysterectomized rats should have no PL and surging prolactin levels, and the hysterectomized rats treated with bromocriptine should have no PL and low prolactin levels. The bromocriptine was dissolved in ethanol and diluted 1/1 with saline just before injection. The next day, between 1000 and 1500 hr, hypophysial stalk blood was collected for a 60-min period from rats under urethane anesthesia (ethyl carbamate, 1.1–1.2 g/kg body wt intraperitoneally; Brocades-ACF, Maarssen, The Netherlands) at a rate of 6–14 μl/min. This is similar to the method of Porter and Smith (12) with modifications (13, 14). The plasma obtained from stalk blood was mixed with an equal volume of 0.2 *M* $HClO_4$ and was used for dopamine measurement.

Media Preparation. RPMI 1640 medium was used. To each 100-ml bottle of medium, 2 ml of penicillin/streptomycin, 2 ml of L-glutamine (58 mg), and 400 mg of bovine serum albumin were added and the entire contents sterilized by filtration. Day 11 pregnant rats served as placental donors. It has been shown previously that incubation medium from Day 11 placentas contain both PL-1 and PL-2 (15). The trophoblast was separated from the decidua, cut into four sections, and added to a 10-ml incubation flask containing 2 ml of media. Two placentas were added per flask, which was then gassed with 95% air-5% CO_2, capped, and placed in a Dubnoff shaker water bath. All incubations were done at 37°C, 60 rpm for 24 hr, at which time the media were centrifuged and supernatants were decanted, pooled, and frozen. Sterile instruments and aseptic techniques were used throughout these incubation procedures.

For Experiment 1, conditioned medium prepared as described above was infused into the rats. For Experiment 2, the conditioned medium pool was subjected to ultrafiltration (Amicon) using a YM10 membrane filter, which has a 10,000 molecular weight cutoff. This procedure significantly increased the concentration of PL in the medium without increasing the salt concentration.

Assays. Blood samples were heparinized and plasma was separated and frozen for subsequent determinations of prolactin and placental lactogen. Plasma concentration of prolactin from Experiments 1 and 2 was determined by the radioimmunoassay method of Niswender et al. (16), using the assay materials provided by the NIDDK Hormone Distribution Program. The reference preparation used was rat Prl-RP1 and the limit of sensitivity was 100 pg/tube. For Experiment 3, a similar radioimmunoassay using the same reference preparation was used to measure prolactin (17). Plasma progesterone concentrations were determined by radioimmunoassay using a previously described method (18). Placental lactogen concentration in the media and plasma samples was determined using the NB_2 lymphoma cell bioassay for lactogenic hormones (19) with our modifications (2). Ovine prolactin (NIH-S-10) was used as the standard for PL assays. This assay does not distinguish between PL-1 and PL-2. Samples were assayed in duplicate at two dose levels. The limit of sensitivity of the assay is 50 pg of ovine prolactin/tube. Antibody to rat prolactin (NIADDK anti-rat-rPrl-ICF-1) was added to each culture tube to eliminate the effect of prolactin found in the plasma samples on cell growth.

Dopamine was extracted from the hypophysial stalk plasma with aluminum oxide (20). Levels of dopamine were determined by a high-pressure liquid chromatographic method described previously (21, 22). The minimum amount of dopamine that could be detected using a cutoff signal:noise ratio of 2 was 10–15 pg.

Statistical Analysis. The data in Experiments 1 and 2 were analyzed by two-way analysis of variance for repeated measures. One-way analysis of variance was used for Experiment 3. Between group comparisons were made with the Neuman-Keul test.

Results

Effect of PL on Nocturnal Prolactin Surge. In Experiment 1, conditioned medium containing PL was continuously infused into pregnant rats which were hysterectomized 1 day earlier, on Day 7 or 8. The infusion began at midnight and continued for 7 hr. Figure 1 shows the plasma levels of PL obtained in these rats. Normally at midpregnancy, PL levels reach

1000–3000 ng/ml of plasma (2) which is considerably higher than that obtained in Experiment 1 (solid squares, Fig. 1). However, the nocturnal prolactin surge was significantly inhibited by infusion of this conditioned medium. As seen in Figure 2, control rats (which had no measurable PL present in their plasma) had an increase in prolactin from 50 ng/ml at midnight to a peak of 275 ng/ml at 0400 hr. Plasma prolactin in rats receiving PL remained at or below 50 ng/ml throughout the time period. This inhibition was significant at all time points except at midnight at the time of the start of infusion. In Experiment 2, conditioned medium containing a much higher concentration of PL and no compounds with M_r below 10 kDa was infused, resulting in PL levels very similar to those seen in intact pregnant rats (Fig. 1) (2). These rats also did not have a nocturnal prolactin surge, with plasma prolactin levels significantly less than controls at 0400, 0600, and 0700 hr (Fig. 3). The degree of inhibition of prolactin was

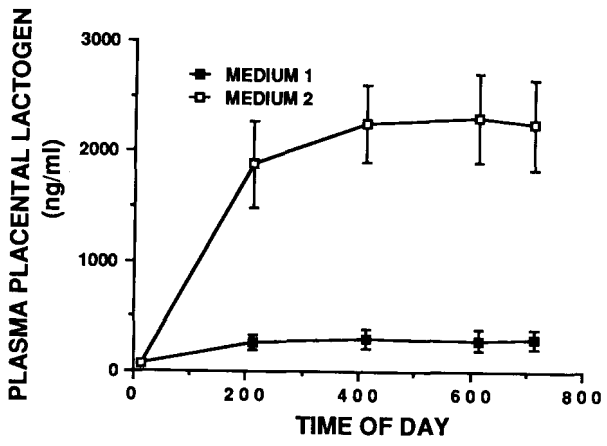

Figure 1. Plasma PL levels during infusion of conditioned medium. Medium 2 contained much higher concentrations of PL than Medium 1. The standard error bars are shown for each mean.

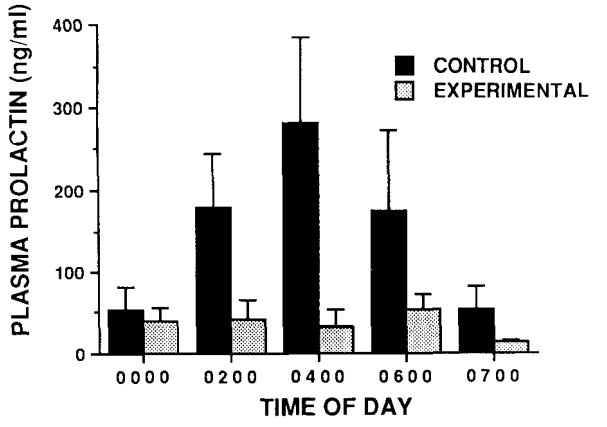

Figure 2. Plasma prolactin levels during infusion of conditioned medium containing unconcentrated amounts of PL (experimental group). Prolactin levels were significantly ($P < 0.05$) lower in the experimental group compared with the controls at all times except midnight. Data points represent means, and standard error bars are shown for each mean.

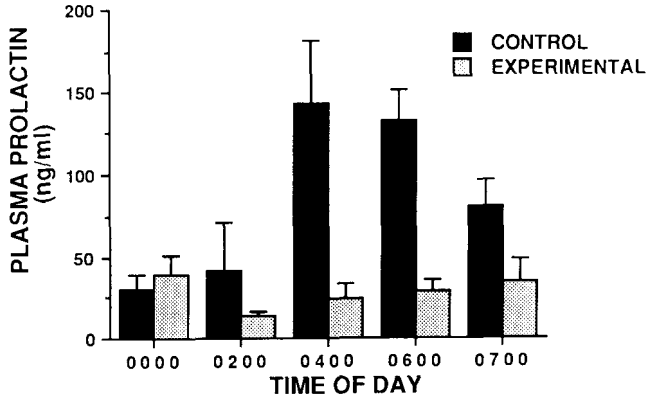

Figure 3. Plasma prolactin levels during infusion of conditioned medium containing concentrated amounts of PL (experimental group). Prolactin levels were significantly ($P < 0.05$) lower in the experimental group at 0400, 0600, and 0700 hr. Data points represent means, and standard error bars are shown for each mean.

similar in both experiments. The peak level of prolactin reached in control rats in Experiment 2 was not as high as that found in Experiment 1. There is no obvious reason for this difference in control groups, although the two experiments were done several months apart.

Hypophysial Stalk Plasma Dopamine Levels in Rats with or without Placentas Present. In Experiment 3, pregnant rats were hysterectomized on Day 6 and hypophysial stalk blood was collected on Day 12. Group 1 consisted of intact pregnant rats treated on Day 11 with bromocriptine, which was given to enable a comparison to Group 2. The second group of rats was hysterectomized (removing the source of PL) and injected with bromocriptine to prevent a prolactin surge. Group 3 rats were hysterectomized only, thus having no PL but continued prolactin surges.

Hypophysial stalk plasma dopamine levels were similar in all three groups. Measured in ng/ml stalk plasma, dopamine was 11.1 ± 2.6 in Group 1, 10.4 ± 1.9 in Group 2, and 11.4 ± 2.9 in Group 3. These levels are higher than those in intact nonpregnant females (21) or males (22), as reported by this same laboratory. At present, we have no explanation for this finding.

Discussion

These experiments demonstrate for the first time that infusion of medium containing PL can completely inhibit the occurrence of the nocturnal prolactin surge in pregnant rats. Earlier attempts to show an inhibitory effect of placental extracts on prolactin surges presumably failed due to a lack of significant amounts of PL entering the circulation and reaching the hypothalamic-pituitary axis (11). Even when medium containing PL was injected into the carotid, PL levels declined rapidly by 2 hr (11). This problem was overcome in the present experiment by infusing PL directly into the vascular system continuously, resulting in elevated PL levels for the duration of the surge time. Somewhat surprisingly, the concentration of PL in the circulation necessary to

inhibit prolactin was only about 200 ng/ml, which is reached by the 10th day of pregnancy (2), the last time a surge is present (1).

In order to gain some insight into this inhibitory effect on prolactin with plasma levels of PL at 200 ng/ml, one can look at the short-loop feedback model for prolactin. Many experiments have demonstrated that elevated prolactin is capable of inhibiting pituitary prolactin secretion. The question is what plasma prolactin level needs to be obtained before this negative feedback on further prolactin secretion can be seen. Injection of 4 mg/kg body wt of ovine prolactin inhibited prolactin release in response to stress (23), suckling, or during the proestrous surge (24). This injection resulted in a plasma level of ovine prolactin of 1000–2000 ng/ml at 2 hr, which rapidly fell and was about 100 ng/ml at 8 hr. Integrated over time, plasma ovine prolactin levels in these experiments were similar to PL levels in the present experiment. Chronically elevated plasma prolactin levels of 80–100 ng/ml due to pituitary grafts on the kidney diminished pituitary prolactin content (25) and elevated dopamine turnover in the tubero-infundibular region of the hypothalamus (26). Thus, it is highly possible that the amount of PL present in the blood during the infusion is comparable to the amount of prolactin needed to show a feedback effect on prolactin secretion.

In the second experiment, the level of PL in the plasma obtained during infusion was comparable to that normally present by midpregnancy. This level was possible because the amount of PL in the conditioned medium was greatly concentrated by the use of ultrafiltration. This technique also removed molecules with M_r below 10 kDa. Prolactin also was inhibited during this infusion. It is not possible to conclude that it was either PL-1 and/or PL-2 that exerted a negative feedback on prolactin. There are other factors found in the placenta that could contribute to prolactin inhibition. The rat placenta synthesizes significant amounts of androgens by Day 11 (27, 28). Even though ultracentrifugation would have removed the free androgens, it is possible that significant amounts of androgens remained bound to proteins which were subsequently infused. The placenta also secretes many proteins which have homology to prolactin. In addition to PL-1 and PL-2, proliferin is expressed by the placenta by midpregnancy (29). Another prolactin-related protein, PLP-A (prolactin-like protein) is first expressed by Day 13 or 14 (30). Very recently, PLP-B was isolated and characterized, and it also is first expressed by Day 13 or 14 (31). Thus, a growing number of prolactin or placental lactogen-like genes and proteins are present in the placenta. There likely are more to be discovered. Thus, it is possible that proteins other than PL-1 or PL-2 play a role in continuously inhibiting prolactin secretion during the last half of pregnancy. However, until it is possible to obtain significant amounts of these proteins in pure form, it is not possible to delineate fully what factors are inhibitory. The conclusion which can be made is that the placenta secretes an inhibitor of prolactin.

The second question asked in this study was whether placental secretions caused this prolactin inhibition via modulation of hypothalamic dopamine release. Placental lactogen and the other prolactin-like proteins found in the placenta are similar to prolactin in biologic functions and possess structural homology with prolactin (32). It is reasonable to propose that PL exerts a negative feedback on prolactin, and the mechanism for this feedback is similar or the same as that for prolactin. Many experiments have been reported in which it was shown that prolactin increased the release of dopamine from neurons terminating in the median eminence, presumably inhibiting further prolactin release (33). McKay *et al.* (34) found that dopamine neuronal activity, estimated by measuring the rate of DOPA accumulation in the median eminence, was chronically elevated after midpregnancy. Pregnant rats hysterectomized during the first half of pregnancy, thus having no PL present but prolactin surges present, did not have chronically elevated DOPA accumulation (35). The dopamine agonist, bromocriptine, did not reduce DOPA accumulation in pregnant rats, but was effective if the pregnant rats were previously hysterectomized. It was concluded from these experiments that at midpregnancy a uterine-placental factor stimulates dopamine release, and thus terminates prolactin surges. The present study was designed to test whether the release of dopamine into hypophysial stalk blood leading to the pituitary was stimulated when endogenous PL levels were high. Three groups were used so that both variables, prolactin and PL, were controlled. Results of the present experiment do not support the hypothesis of a feedback of PL on prolactin via dopamine. However, our approach may not be appropriate, since anesthetized, surgically stressed rats were used.

It is possible that the major or exclusive site of PL feedback is at the pituitary prolactin cell directly. Two separate reports (6, 7) indicate that placental secretions were very effective in inhibiting prolactin release from either dispersed cell cultures or pituitary fragments.

We have been able to show for the first time that infusion of medium containing multiple factors secreted from the placenta, including PL-1 and PL-2, was able to inhibit the nocturnal prolactin surge *in vivo*. This classical approach of removing a gland or organ suspected of secreting a hormone which regulates a second hormone, observing the effect, and then counteracting the effect by infusing material containing the first hormone is limited. However, until purified placental prolactin-like proteins are available, this approach is necessary.

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