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A Milk-Borne Factor Inhibits Mammotrope Differentiation in the Neonatal Rat

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Within the first few days of neonatal life in the rat, a milk-borne peptide is transferred to the neonatal circulation and transported to the pituitary gland where it acts directly to induce final differentiation of mammotropes. As we were attempting to purify this stimulatory peptide, we separated an antagonistic activity that serves as the focus of the present study. Milk obtained on days 2–3 of lactation was subjected to pH fractionation followed by acetone precipitation to yield two fractions that stimulated and inhibited, respectively, mammotrope differentiation in cultures of neonatal pituitary cells. The stimulatory agent more than doubled the proportion of prolactin secretors in those cultures, whereas the inhibitory agent exerted the opposite effect when tested alone. Moreover, the inhibitory agent severely attenuated mammotrope differentiation evoked by the stimulatory fraction or by basic FGF, an established inducer of this developmental phenomenon. The discovery of a milk-borne inhibitor, coupled with the previously described milk stimulatory factor, indicates that maternal control of mammotrope differentiation is considerably more sophisticated than previously believed.

Key Words: Prolactin; milk; neonate.

Introduction

In the rat, the differentiation of mammotropes occurs during early postnatal development. This is manifested as a rather abrupt increase in the number of prolactin-releasing cells between days 3 and 5 after birth (Hoeffler et al., 1985). There is a growing consensus that the signal for final differentiation of mammotropes is not intrinsic to the pup, but is derived from the mother's milk. Consistent with this view is the observation that mammotrope differentiation was blocked when a fostering paradigm was utilized to deprive pups of milk produced during days 1–4 of lactation (Porter et al., 1991). Further, the relative abundance of such mammotrope differentiative activity was found to be higher in "early" (day 2–3 of lactation) than in "late" (day 15–16) milk (Porter and Frawley, 1991). This change suggests that evolution of this activity was regulated. Lastly, a "switch-back" type of fostering strategy, coupled with in vitro paradigms, revealed that the stimulatory principal found in milk could be rapidly absorbed by the neonatal gut, transferred to the circulation, and delivered to the adenohypophysis of the pup where it could act directly to induce mammotrope differentiation (Porter et al., 1993). Taken together, these observations comprise a compelling case that a milk-borne agent plays an obligatory role in the development of mammotropes within the anterior pituitary gland of the neonatal rat.

After establishing the existence of this mammotrope differentiating agent, our logical next goal was to unequivocally elucidate its chemical identity. To date, we have utilized column chromatography and pepsin inactivation to demonstrate that the factor is a peptide with a relative molecular weight in the range of 2–8 kDa (Porter and Frawley, 1991; Porter et al., 1993). However, efforts to advance beyond this point have been hampered by tremendous variations in the level of stimulatory activity found in different batches of raw rat milk. One possible explanation for this phenomenon is that milk might also contain an activity that antagonizes mammotrope differentiation. In this scenario, the differentiative capacity of a given milk preparation would reflect an averaged response of the stimulatory and inhibitory components. Our ability to isolate these two components in the present study supports this contention. This was accomplished by devising an enrichment schema to separate stimulatory from putative inhibitory activities. To this end, we removed the major milk proteins from rat milk by pH fractionation, and partitioned the remaining components into two fractions based on their insolubility in either 70% or, subsequently, 95% acetone.

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Results

The effects of the 70 and 95% milk fractions on the relative abundance of prolactin-releasing cells in neonatal pituitary cultures are illustrated in Fig. 1. As indicated, 1.5 ± 0.2% of cells released prolactin under control conditions, with the two milk fractions exerting opposite effects on this percentage. Specifically, the 95% fraction augmented the number of prolactin-releasing cells more than twofold to 3.4 ± 0.4% of all pituitary cells present ($p < 0.001$ vs control). In contrast, the 70% fraction reduced the number of prolactin secretors to 0.9 ± 0.2% ($p < 0.05$ vs control). Pepsin digestion reversed the ability of the 70% fraction to retard prolactin cell differentiation (Fig. 2). This reversal indicated that the active principle was proteinaceous in nature. The capacity of the 70% fraction to inhibit the induction of mammotrope differentiation by either bFGF or the 95% fraction is illustrated in Fig. 3. As shown, treatment of neonatal pituitary cells with either bFGF or the 95% fraction increased the number of prolactin-releasing cells from control values of 1.6 ± 0.3% to 3.6 ± 0.7% ($p < 0.001$) and 2.6 ± 0.7% ($p < 0.05$), respectively. Interestingly, the 70% fraction severely attenuated mammotrope differentiation induced by either bFGF or the 95% fraction ($p < 0.05$; 1.7 ± 0.3% and 1.8 ± 0.5%, respectively).

To discern whether the effects of the 70% fraction were limited to the developing anterior pituitary gland, we also evaluated its effectiveness on anterior pituitary cells from day-3 neonatal rats and lactating adult rats. Inhibition of mammotrope differentiation imposed by the 70% fraction was even more pronounced when tested on day-3 neonatal rat anterior pituitary cells (Fig. 4A), in that the percentage of prolactin secretors decreased by 85% (from 2.6 ± 0.7% under control conditions to 0.4 ± 0.2%; $p < 0.001$). However, when anterior pituitary cells from adult lactating rats were evaluated under the same conditions, the 70% fraction had no effect on the abundance of mammotropes ($p > 0.1$; Fig. 4B).

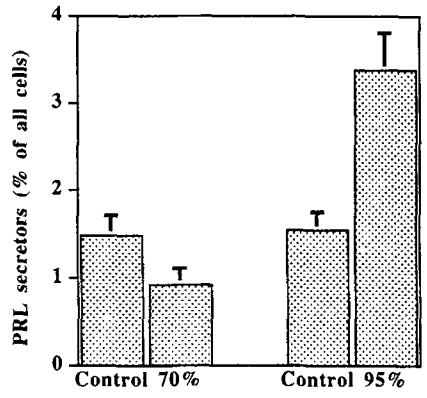

Fig. 1. Effect of the 70 and 95% fractions on differentiation of mammotropes in cultures of anterior pituitary cells from day-1 rats. Milk collected from mothers on day 2 or 3 of lactation was defatted, and the majority of milk proteins were removed by pH fractionation. The remaining proteins were divided into those precipitated in 70% acetone and the remainder that were precipitated in 95% acetone. Anterior pituitary cells were cultured for 6 d under control conditions or in the presence of 2.5 mg/mL of the 70 or 95% milk fractions. For experiments presented in this and all subsequent figures, cells were then collected and assayed for prolactin release by reverse hemolytic plaque assay. The 95% fraction induced a significant increase in the number of cells releasing prolactin ($p < 0.01$; $n = 19$ separate experiments), while the 70% fraction inhibited the number of prolactin secretors ($p < 0.05$; $n = 15$).

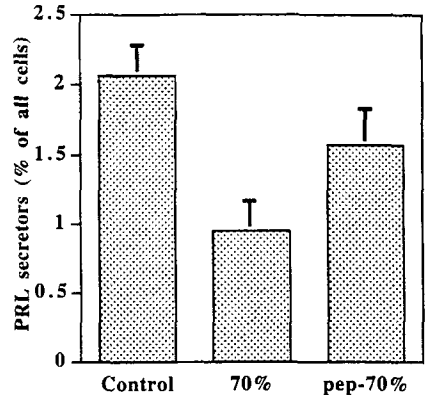

Fig. 2. Effect of pepsin treatment on the ability of the 70% fraction to inhibit differentiation of mammotropes. Dispersed anterior pituitary cells from day-1 rats ($n = 6$) were cultured for 6 d in basal medium, basal medium containing 2.5 mg/mL of the 70% fraction, or basal medium containing the 70% fraction treated with 600 U of pepsin. The 70% fraction decreased the abundance of prolactin secretors ($p < 0.01$) relative to controls. Pepsin treatment of the 70% fraction reversed the inhibitory effects of this preparation in that the proportion of mammotropes was no longer different from control values ($p > 0.1$).

Discussion

As indicated earlier, the appearance of mammotropes occurs largely around day 4 of neonatal life in the rat (Hoeffler et al., 1985), and the timing of this phenomenon is regulated precisely by a maternal signal delivered via the milk (Porter and Frawley, 1991; Porter et al., 1991; 1993). The results of the present study demonstrate that milk from early (day 2–3) lactating rats also contains a factor that inhibits mammotrope differentiation. Indeed, treatment of anterior pituitary cells from neonatal rats with this proteinaceous factor decreased the number of prolactin-releasing cells that appeared spontaneously under basal culture conditions, and also suppressed mammotrope induction by either bFGF or the as yet unidentified, stimulatory factor in milk. The discovery of a milk-borne inhibitor of mammotrope differentiation within the neonate, in addition to the previously described milk stimulatory factor (Porter and Frawley, 1991; Porter et al., 1993), indicates that maternal control may be considerably more intricate than previously understood.

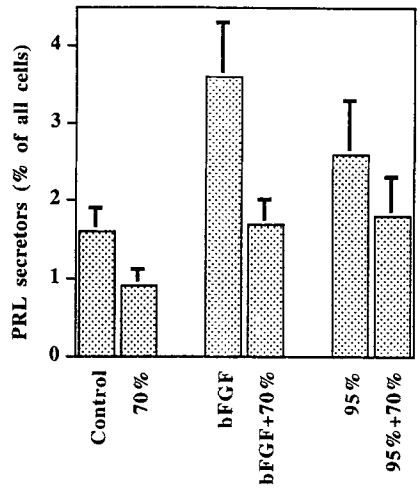

Fig. 3. Combined effects of the 70% fraction and stimulators of mammotrope differentiation. Dispersed anterior pituitary cells from day-1 rats were cultured for 6 d in control media or media containing 5 n*M* bFGF, 2.5 mg/mL of the 70% fraction, 2.5 mg/mL of the 95% fraction, or 2.5 mg/mL of the 70% fraction combined with either 5 n*M* bFGF or 2.5 mg/mL of the 95% fraction. When tested alone, bFGF and the 95% fraction significantly increased the number of cells releasing prolactin ($p < 0.001$ and $p < 0.05$, respectively; $n = 8$ separate experiments). When these treatments were combined with the 70% fraction, the induction of mammotrope differentiation by both bFGF and the 95% fraction was suppressed to values not different from that of the control ($p > 0.1$).

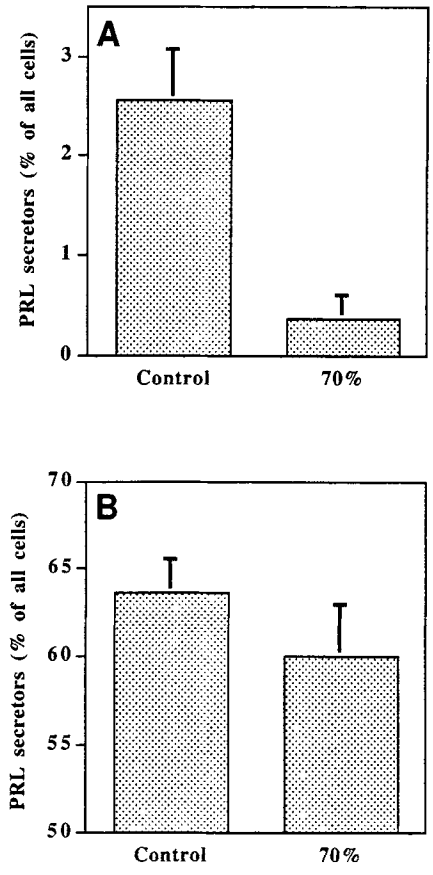

Fig. 4. Effect of the 70% fraction on the number of prolactin-releasing cells from day-3 neonatal rats **(A)** or adult lactating rats **(B)**. Anterior pituitary cells from the appropriate donors were cultured for 6 d in basal medium or in the presence of 2.5 mg/mL of the 70% fraction. The 70% fraction inhibited the percentage of day-3 neonatal cells releasing prolactin by more than 85% compared with controls ($p < 0.001$; $n = 3$), but had no effect on the percentage of prolactin cells from adults ($p > 0.1$; $n = 3$).

One can envision a number of scenarios by which these opposing milk-borne signals might interact to orchestrate the appearance of mammotropes in the neonate. One possibility is that the concentration of milk stimulatory activity increases relative to that of its inhibitory counterpart, thus overriding a block to mammotrope maturation. Alternatively, there could be a lactational-stage-dependent diminution of inhibitory activity in the face of constant stimulatory input, thereby effecting disinhibition of the maturational process. Of course, it is equally plausible that a combination of these events might contribute to the precise timing of mammotrope appearance. Another scenario that deserves consideration is that the responsiveness of the target cell (the presumptive mammotrope) to either or both of these agents could change during the first few days after birth. Although there is a paucity of data to favor the primacy of any of the aforementioned possibilities, it is interesting to note that the concentration of the stimulatory peptide in milk appears to peak around the time that mammotrope differentiation occurs and to decline precipitously thereafter (Porter and Frawley, 1991). Moreover, our present results show that responsiveness of pituitary cells to the inhibitory activity increases during the same time frame. Given the inconsistencies of available evidence, it would be unwise to speculate further about how these opposing activities interact (physically or functionally) until each of them has been unequivocally identified and characterized.

The nature of milk factor interaction notwithstanding, it is clear that these agents must act ultimately on individual presumptive mammotropes to override a posttranscriptional block to prolactin synthesis that is present at the time of birth and persists for the first 3 d of neonatal life. Previous studies from our lab have shown that the prolactin gene is expressed at a reasonably high level on the day of birth, as evidenced by the relative abundance of prolactin mRNA (Frawley and Miller, 1989). However, polysomal profile analysis revealed that this message does not associate adequately with ribosomes at this time. It is noteworthy that translational control is regulated intracellularly by both stimulatory and inhibitory inputs in a manner conceptually parallel to the opposing influences found in early milk.

In summary, we have identified a milk-borne factor that inhibits mammotrope differentiation and thereby adds another dimension to our understanding of maternal regulation of mammotrope ontogeny in particular and of neonatal development in general. To date, almost a hundred hormones and growth factors have been identified in early

milk from a number of species (Grosvenor et al., 1992; Koldovsky, 1995), but surprisingly none has yet been shown to subserve an obligatory systemic role in postnatal development. Our studies are somewhat unique in that we have provided functional evidence for two such agents. Our obvious charge now is to complete their chemical identification.

Materials and Methods

Animals

Holtzman-Sprague-Dawley rats (Harlan, Indianapolis, IN) were provided food and watered ad libitum, and housed in an environmentally controlled room (23°C; 12-h-light: 12-h-dark light cycle). Rats were checked twice daily to determine day of birth. Litters were normalized to eight pups on the day after birth.

Milk Processing

Milk was collected as previously described (Porter and Frawley, 1991) from rats at day 2 or 3 of lactation, diluted to twice the volume with H_2O and centrifuged twice at 6000*g* for 20 min at 4°C to remove lipids and precipitates. The infranate was then subjected to pH fractionation to eliminate the majority of major milk proteins. Specifically, milk was first adjusted to pH 4.6 with glacial acetic acid to remove milk caseins (1 h at 4°C followed by centrifugation), in accordance with the protocol of Kaetzel and Ray (1984). The supernate obtained was adjusted to pH 7.0 with 10*N* NaOH (1 h at 4°C). After a subsequent centrifugation, the resulting supernate was adjusted to pH 3.5 with 6*N* HCl (1 h at 4°C). Centrifugation of this preparation precipitated the vast majority of milk proteins. The remaining milk factors in the supernate were then fractionated based on their solubility in acetone. First, the supernate was diluted with acetone to achieve a final concentration of 70% acetone. This preparation was subsequently incubated for 12 h at 4°C, after which the insoluble proteins (70% fraction) were collected after centrifugation and resuspended in H_2O. The remaining factors in the supernate were precipitated by increasing the acetone concentration to 95% (12 h at 4°C), with the ensuing precipitate (95% fraction) resuspended in H_2O. Both the 70 and 95% fractions were then exhaustively dialyzed against H_2O. The molecular weight cutoff of the dialysis tubing (Spectrum Medical Industries, Houston TX) was 500 Daltons. After dialysis, both fractions were lyophilized and stored at –20°C until used for bioassay purposes.

Milk Bioassay

The bioassay used to test the effects of the 70 and 95% fraction on anterior pituitary cells was described previously in detail (Porter and Frawley, 1991). In short, anterior pituitaries from either adult lactating rats or neonatal rats (at least two litters per replicate) were dispersed by typsinization and cultured at 500,000 cells per well in a six-well Petri dish (Becton-Dickinson, Franklin Lakes, NJ). Basal media used in the bioassay was a 1:1 dilution of medium 199 and Ham's F-12 nutrient mixture, supplemented with 5 μg/mL insulin, 5 μg/mL transferrin (all from Gibco-BRL, Grand Island, NY), 0.1% bovine serum albumin, 5 m*M* HEPES and antibiotics (all from Sigma Chemical Co., St. Louis, MO). Cells were cultured for 6 d under the various treatments that were replenished every other day. After 6 d, cells were retrypsinized, collected by centrifugation, and then tested for prolactin secretion by the reverse hemolytic plaque assay (RHPA). The RHPA was identical to the previous study (Porter and Frawley, 1991) except the prolactin antibody was used at a 1:60 dilution and the incubation time was 8 h. A minimum of 500 cells per slide (with two replicate slides per treatment) were quantified under light microscopy to determine the percentage of cells secreting prolactin. Prolactin secretors were defined as nuclear stained cells surrounded by at least two rows of lysed erythrocytes.

The direct effects of either the 70 or 95% fractions on the percentage of prolactin secretors were tested on anterior pituitary cultures from day-1 neonates. Neonatal cells were cultured in basal medium alone, or in the presence of either the 95% fraction (2.5 mg/mL) or the 70% fraction (2.5 mg/mL). To determine the proteinaceous nature of the 70% fraction, 30 mg were dissolved in control buffer (5 m*M* acetic acid) or buffer containing pepsin (600 U; Sigma). The solutions (at pH 3.5) were incubated at 37°C for 2 h and then 12 mL of basal media were added to stop pepsin digestion by dilution and adjustment of pH. The solutions were sterile filtered and then tested on anterior pituitary cells from day-1 neonates ($n = 6$). Interactions between the 70% fraction and factors that induce mammotrope differentiation were tested on the same type of cultures. These treatments included basal medium, the 95% fraction (2.5 mg/mL), 5 n*M* of basic fibroblast growth factor (bFGF, a known inducer of mammotrope differentiation [Porter et al., 1994]), the 70% fraction (2.5 mg/mL), and a combination of the 70% fraction (2.5 mg/mL) with either bFGF (5 n*M*) or the 95% fraction (2.5 mg/mL). Lastly, anterior pituitary cells from both day-3 neonates and adult rats at day 3 of lactation were cultured in either basal medium or basal medium containing the 70% fraction (2.5 mg/mL) to evaluate responsiveness to the 70% fraction.

Statistical Analysis

The effects of the treatments on cells derived from various pituitary donors (day-1, day-3, or adult) were analyzed separately by way of predetermined paired t-tests. Data are reported as the means ± the standard error of the mean (SEM).

Acknowledgments

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