Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7183–7185, September 1989
Medical Sciences

High levels of a parathyroid hormone-like protein in milk

(malignancy-associated hypercalcemia/lactation/neonate/mammary gland)

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Communicated by Hector F. DeLuca, June 26, 1989

ABSTRACT **Expression of a parathyroid hormone-like protein (PLP), which is associated with hypercalcemia in malignancy, has recently been localized to normal lactating mammary tissue. We examined the possibility of an extramammary role of PLP by measuring its levels in serum and milk of lactating women. The levels of PLP by radioimmunoassay in serum of lactating and nonlactating women were indistinguishable [4.2 ± 1.8 and 3.6 ± 1.0 pg equivalents (eq) of PLP-(1–34) amide per ml, respectively]. As PLP was undetectable in some serum samples, this result does not exclude the possibility that lactation results in a small increase in serum levels of PLP. In contrast, high concentrations of immunoreactive PLP [40,000–75,000 pg eq of PLP-(1–34) amide per ml] and correspondingly high concentrations of bioactive PLP were found in human, rat, and bovine milk. A variety of processed bovine milk products had a PLP content similar to that of fresh bovine milk, whereas infant formulas had lower concentrations, ranging down to undetectable. Although the physiological role of PLP in lactation is unknown, the data establish the presence of PLP in milk and suggest the possibility that PLP may be important in neonatal calcium homeostasis.**

Parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D are considered to be the major regulators of extracellular calcium homeostasis in humans. Recently, a variety of human tumors have been shown to secrete a 16-kDa protein (1–3) that has amino-terminal sequence homology with PTH (4–6). Synthetic peptides of this PTH-like protein (PLP) corresponding to PLP-(1–34) reproduce all of the major physiologic effects of PTH, including PTH receptor binding (7, 8), accelerated bone resorption, phosphaturia, and hypocalciuria (9–11). Secretion of PLP probably accounts for most instances of hypercalcemia associated with solid malignancies. The striking evolutionary conservation of the PLP sequence between the rat and human genomes [two conservative changes in PLP-(1–110) (12)] is consistent with a significant physiological function in mammals, but this function is unknown.

The existence of a unique calciotropic hormone of lactation has been postulated, based on findings that adaptive increases in maternal bone resorption and intestinal calcium absorption to meet calcium losses during lactation do not require PTH or 1,25-dihydroxyvitamin D (13, 14). That PLP could fulfill this role is suggested by the observation that, in the rat, lactating mammary tissue is the site of greatest expression of PLP (12). To determine whether PLP could have a systemic role in the mother during lactation or a role in the neonate, we measured PLP levels in maternal serum by radioimmunoassay (RIA) and in milk by RIA and bioassay.

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MATERIALS AND METHODS

Materials. [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(3–34) amide (Nle = norleucyl; b = bovine) was obtained from Peninsula Laboratories. Synthetic PLP-(1–34) amide was a gift from M. Rosenblatt (Merck Sharp & Dohme). The clonal rat osteosarcoma cell line UMR-106 was provided by T. J. Martin (Melbourne, Australia).

Radioimmunoassay of PLP. Details of the RIA methodology will be described elsewhere. Briefly, the RIA used rabbit antiserum to synthetic PLP-(1–34) (provided by M. Shimizu, Peninsula Laboratories) with ^{125}I-labeled PLP-(1–34) amide as radioligand and PLP-(1–34) amide as standard. Immunoreactive PLP (iPLP) in serum was measured after partial purification and concentration (15- to 25-fold) by immunoaffinity chromatography with a polyclonal rabbit antibody to synthetic PLP-(1–37) bound to Sepharose. When tumor-derived PLP was added to normal serum to give 48 pg equivalents (eq) of PLP per ml, 58 ± 13% (mean ± SD) of iPLP was recovered in the immunoextracts. The within-assay coefficient of variation was 7.5%, and the between-assay coefficient of variation was 19%. No cross-reactivity was seen with human PTH or PTH fragments. Normal serum contained <10 pg eq of PLP-(1–34) amide per ml, and hypercalcemic patients with cancer had levels up to 100 pg eq/ml (unpublished data). The limit of detection was 5 pg of PLP-(1–34) amide per tube, or 50 pg eq of PLP(1–34) amide per ml in unextracted milk (to convert to pM, multiply by 0.25). The limit of detection in serum varied with the fold concentration afforded by the immunoaffinity extraction and was 1–4.5 pg eq of PLP-(1–34) amide per ml. Multiple serum samples from the same subject were always assayed in the same assay. When PLP-(1–34) amide was added to milk or milk products and assayed, the recovery was 100 ± 10% (n = 4).

Bioassay of PLP. The cAMP response in UMR-106 rat osteosarcoma cells was determined after a 10-min incubation of UMR-106 monolayers at room temperature with dilutions of milk samples in the presence of 1.0 mM isobutylmethylxanthine and 100 nM forskolin as described (15). In the rat osteosarcoma cell bioassay, the recovery of PLP-(1–34) amide added to human milk was 115% (range, 105–125%). Adenylyl cyclase activation in canine renal plasma membranes by immunoextracts of milk was assayed as the conversion of [α-^{32}P]ATP to [^{32}P]cAMP (16). Both assays used PLP-(1–34) amide as standard.

Milk and Serum Samples. Human subjects gave informed consent according to the guidelines of the Human Research Committee of the University of California at Berkeley. Milk and serum samples were obtained between 10 and 14 weeks postpartum. Maternal serum was obtained approximately 4

Abbreviations: PTH, parathyroid hormone; PLP, parathyroid hormone-like protein; iPLP, immunoreactive PLP; eq, equivalent(s); Nle, norleucyl.

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hr after the last nursing and again in midnursing. Milk was obtained in midnursing. Control sera were pooled serum samples from nonlactating young women.

Animal experiments were conducted under a protocol approved by the Animal Studies Committee of the Veterans Administration's Medical Center (San Francisco). Milk was obtained from rats 14 days postpartum and from nonpregnant dairy cows (Avia Dairy Farms, Sonoma, CA) during their regular milking routine. Dairy products were obtained from retail sources and assayed after preparation per manufacturers' instructions. In each case, samples from at least three different animals or lots were assayed at multiple dilutions, and data shown are means ± SD. In no case did levels in comparable dairy products of different suppliers differ more than 2-fold.

RESULTS AND DISCUSSION

Serum iPLP levels in five lactating women ranged from <3 pg eq/ml to 8.4 pg eq/ml and could not be distinguished from those in nonlactating women (Table 1). Serum PLP was undetectable (<3.2 pg eq/ml) in one pool from nonlactating women. Comparison of samples obtained before nursing (Basal) and during nursing did not show a nursing-induced increase. As some subjects had undetectable levels and others had barely detectable levels, these data do not exclude the possibility that maternal serum PLP levels are modestly increased during lactation and that PLP may play a role in the adaptation of maternal calcium homeostasis.

The concentration of iPLP in human milk was about 10,000 times higher than its concentration in serum, ranging from 40,000 to 80,000 pg eq of PLP-(1–34) amide per ml (Table 1). PLP levels in human milk were also measured by bioassay as the increase in cAMP in UMR-106 rat osteosarcoma cells. PLP levels by bioassay were approximately half the levels of iPLP (Table 1), suggesting the presence in milk of a form(s) of PLP with a reduced ratio of bioactivity/immunoactivity compared with the synthetic PLP-(1–34) amide. We have observed that the sensitivity of the RIA to recombinant PLP-(1–141) is about one-fifth of its sensitivity to PLP-(1–34) amide, indicating that different molecular forms of PLP are not equally immunoreactive (unpublished data). The molecular form of PLP in milk is unknown but may differ from the assay standard in its potency in either the RIA or the bioassay. In contrast to their high content of PLP, human milk samples had levels of PTH (Allegro PTH kit, Nichols Institute) at or below the detection limit of 15 pg/ml (data not shown).

Parathyroid hormone-like protein in milk samples was also measured after partial purification by immunoaffinity chromatography. Of the iPLP present in milk, 83–93% was immunoextractable; as in unextracted milk, biologically active PLP levels in the immunoextracts were about half of iPLP levels in the same extracts (data not shown). Extracts of milk also displayed bioactivity in the canine renal adeylyl cyclase assay. Activation of adenylyl cyclase by milk extracts was blocked by the PTH/PLP receptor antagonist [Nle^8, Nle^{18}, Tyr^{34}]bPTH-(3–34) amide (17), indicating that activation was the direct result of occupation of PTH receptors (Fig. 1).

Table 1. Concentrations of PLP in human serum and milk. Each milk sample was assayed at multiple dilutions by RIA and by UMR-106 cell bioassay

	PLP-(1–34) amide, pg eq/ml		
		Serum	
Subject	Milk	Basal	Nursing
1	38,300 (17,000)	5.6	<2.9
2	44,200 (21,000)	3.2	<4.5
3	53,000 (24,000)	6.6	8.4
4	74,700 (38,000)	2.9	4.2
5	39,800 (17,000)	2.8	3.1
Mean ±SD	50,000 (23,400 ±14,900 ±8,700)	4.2 ±1.8	4.6 ±2.2
Nonlactating women		3.6 ± 1.0	

*Values in parentheses are PLP levels measured by bioassay.

The concentration of iPLP was also measured in rat and bovine milk, in milk products, and in several bovine milk-based formulas (Table 2). Data are expressed in terms of the PLP-(1–34) amide standard, which gave parallel immunodilution curves to the samples (Fig. 2). Rat milk and both unprocessed and pasteurized homogenized bovine milk had iPLP levels comparable to those in human milk. The biological activity of PLP in rat and bovine milk was confirmed in the UMR-106 bioassay. As in human milk, rat milk had higher levels of immunoactive than bioactive material (91 ± 45 vs. 45 ± 13 ng eq/ml, respectively). In bovine milk, measured levels of bioactive material were comparable to levels obtained by RIA (92.5 ± 32.4 vs. 82.8 ± 21.0 pg eq/ml, respectively). Compared to unprocessed milk, some milk products had greatly reduced iPLP levels. A number of infant formulas based on bovine milk had iPLP levels ranging from undetectable to about one-third the concentration in milk, and a representative selection is shown in Table 2. The wide variation in iPLP levels present in the processed milk products was not attributable to differences in shelf storage time. In soy-based infant formulas, PLP was not detectable. Samples of infant formulas and milk products with low levels of iPLP (Enfamil, Enfamil 24, chocolate milk) were also assayed for PLP-like bioactivity, and in each sample levels of bioactive PLP were <35% of iPLP.

The level of PLP in milk is more than 100 times higher than the serum level associated with hypercalcemia in malignancy. It is known that in the lactating rat, mammary tissue expresses PLP in response to suckling (12). PLP could potentially have a local role in the regulation of the mammary gland and/or a systemic role in maternal adaptation to lactation (13, 14). Alternatively, the finding of high levels of

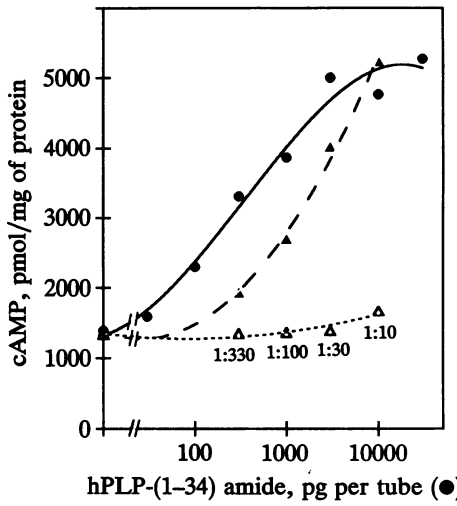

FIG. 1. Stimulation of adenylyl cyclase by milk extract. A 10-μl sample of human (h) milk was immunoextracted, and the extract was lyophilized and reconstituted in 100 μl of standard vehicle (10 mM acetic acid/0.1% bovine serum albumin). Adenylyl cyclase activity in canine renal plasma membranes was assayed in the presence of PLP-(1–34) amide (●) or dilutions (1:10 to 1:330) of the immunoextract of human milk in the absence (▲) or presence (△) of the PTH/PLP receptor antagonist [Nle^8, Nle^{18}, Tyr^{34}]bPTH-(3–34) amide (10 μg/ml).

Table 2. Levels of iPLP in human and nonhuman milk and in infant formulas

Sample	*n*	PLP-(1–34) amide, mean ng eq/ml ± SD
Unprocessed milk		
Human	5	50.0 ± 14.9
Bovine	6	95.6 ± 34.0
Rat	6	75.7 ± 42.8
Commercial milk products		
Whole milk*	5	81.4 ± 17.9
Lowfat milk*	3	88.9 ± 8.7
Nonfat milk†	3	118.3 ± 18.9
Dried milk†	3	76.2 ± 32.7
Chocolate milk†	3	6.8 ± 6.3
Buttermilk†	3	4.5 ± 4.2
Milk-based infant formulas		
Similac (Ross)	3	32.8 ± 8.9
Enfamil (Mead Johnson)	3	6.8 ± 1.9
SMA (Wyeth)	3	5.9 ± 2.1
Enfamil Premature 20	3	7.3 ± 0.9
Similac 60/40	3	1.1 ± 0.8
Enfamil Premature 24	3	13.7 ± 4.4
Soy-based infant formulas*‡		Und§

*Three suppliers.
†Two suppliers.
‡Nursoy (Wyeth), i.Soyalac (Loma Linda), Prosobee (Mead Johnson).
§Undetectable (<0.1 ng eq/ml).

PLP in milk from multiple species raises the possibility that PLP has a role in the neonate, either in the alimentary tract or systemically. Other hormones, such as epidermal growth factor, are also present at high concentrations in milk. Epidermal growth factor in milk can exert local effects on growth and function of the neonatal intestine (18). Systemic effects of milk-derived hormones are also possible, since the neonatal mammalian small intestine can absorb macromolecules by an endocytotic mechanism (19).

Infants fed soy-based formula do not receive PLP, yet are able to regulate mineral metabolism, within physiologic limits (20). The serum levels of 1,25-dihydroxyvitamin D, however, are higher in soy formula-fed than in breast-fed infants (20),

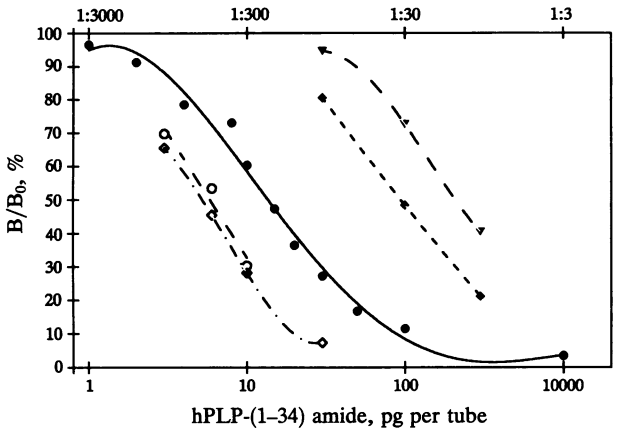

FIG. 2. RIA of PLP in milk products, showing the binding of ^{125}I-labeled PLP-(1–34) amide to antiserum in the presence of PLP-(1–34) amide (●) or dilutions (shown at the top) of human breast milk (◇), processed whole milk (○), chocolate milk (◆), or Similac Special Care infant formula (▼). hPLP-(1–34) amide, human PLP-(1–34) amide; B, bound/free; B_0, bound/free in tubes with no added sample or standard.

suggesting that normal mineral metabolism requires milk-derived factors not present in soy formula. Furthermore, human neonates experience a transient fall in serum calcium after birth that is exaggerated in formula-fed as compared with breast-fed infants (21, 22). Many factors could be responsible for differences in calcium homeostasis between breast-fed and formula-fed infants; further investigation will be necessary to determine whether PLP is one such factor. In this regard, it is of interest that in sheep, partially purified PLP has been reported to stimulate placental calcium transport (23), which serves an analogous role to intestinal calcium transport in the newborn. The postulated neonatal role of PLP may also be unrelated to calcium metabolism. Nonetheless, the broad hypothesis that high levels of PLP in milk subserve a function in neonatal development is deserving of further investigation.

Assays of PTH by R. F. Klein and the skilled technical assistance of R. Bruce are gratefully acknowledged. This work was supported by National Institutes of Health Grants CA34738 and DK35323 and by the Research Service of the Veterans Administration. Dr. Nissenson is an Associate Research Career Scientist of the Veterans Administration.

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