Parathyroid Hormone-Related Protein: A Regulated Calcium-Mobilizing Product of the Mammary Gland

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ABSTRACT

Parathyroid hormone-related protein shares similarities in sequence and function with the endocrine hormone, parathyroid hormone. However, unlike parathyroid hormone, a product of the parathyroid glands, parathyroid hormone-related protein has a wide distribution in tissues, including the mammary gland. Although during pregnancy the expression of parathyroid hormone-related protein in the mammary gland is low, following birth, protein levels rise sharply in the gland in response to elevations in serum prolactin. Large amounts of parathyroid hormone-related protein are secreted into milk, suggesting a possible role in the neonate. Transient phosphaturia and elevations of parathyroid hormone-related protein in mammary vein plasma support a possible endocrine function for parathyroid hormone-related protein during lactation. Recent evidence suggests a local function for parathyroid hormone-related protein in the lactating mammary gland, and evidence exists that parathyroid hormone-related protein stimulates calcium secretion by the goat mammary gland. Parathyroid hormone-related protein, a putative vasodilator, is produced by the external nutrient vasculature of the mammary gland, and levels within this tissue are regulated during lactation. Infusion of parathyroid hormone-related protein into the ovine mammary artery increases gland blood flow, suggesting a role for the protein in modulation of mammary gland hemodynamics. Regulation of parathyroid hormone-related protein synthesis by the lactating gland, together with the protein's actions on regional blood flow and calcium secretion, support an important function in the mammary gland during lactogenesis. (Key words: parathyroid hormone-related protein, mammary gland, calcium secretion, lactation)

Abbreviation key: PTH = parathyroid hormone, PTHrP = parathyroid hormone-related protein

INTRODUCTION

In 1941, Fuller Albright (2) at the Massachusetts General Hospital postulated that the hypercalcemia associated with certain cancers resulted from the tumoral secretion of a substance similar to parathyroid hormone (PTH), and, for nearly five decades following his initial hypothesis, scientists pursued the identity of the tumor-borne hypercalcemic factor. Taking advantage of the striking similarities between the biological activities of the N-terminal fragments of PTH and the tumor product, three independent laboratories eventually purified the protein from tumor cells and conditioned media of cultured human lung, breast, renal, and squamous carcinomas (41, 54, 55). Analysis of the N-terminal amino acid sequence of the purified tumor product revealed for the first time that tumors associated with hypercalcemia of malignancy produce a unique gene product that shared limited N-terminal sequence identity with PTH. Because amino acid sequences and biological activities were shared with PTH, this new protein was called parathyroid hormone-related protein (PTHrP).

The N-terminal amino acid sequences of PTHrP were employed for the cloning of human PTHrP cDNA (37, 56, 64), the sequences of which were subsequently used to isolate cDNA encoding rat (62, 74), mouse (35), and chicken (50, 63) proteins. As seen in Figure 1,
a comparison of the amino acid sequences of PTHrP from these species demonstrates a remarkably strong phylogenetic conservation of the PTHrP structure, which in turn emphasizes the potential biological importance of this molecule. The sequence identity between the different PTH and PTHrP molecules is highly conserved, yet restricted to the first 13 amino acids. The N-termini of PTHrP and PTH share 8 of the first 13 residues, which lend to their similar biological activities. In vivo, synthetic N-terminal fragments of both PTH and PTHrP are essentially equipotent in their calcitropic properties, including the stimulation of osteoclastic bone resorption (25, 65), renal synthesis of 25-hydroxyvitamin D 1α-hydroxylase (68), and renal calcium reabsorption (25, 29, 75). To date, N-terminal peptides of both PTH and PTHrP have been shown to stimulate the same intracellular second messenger systems (12, 16, 42) via their interaction with a common membrane-associated receptor present in traditional (bone and kidney) and nontraditional (e.g., vascular, gastric) PTH target tissues. Although PTHrP also may possibly interact with a unique membrane receptor, at this time, no direct evidence supports the existence of such a molecule.

In comparison with human PTHrP, amino acid sequences residues 1 to 110 of the avian and rodent molecules have been well conserved; only 2 and 17 amino acid substitutions are found between the human and rodent or avian molecules, respectively (Figure 1). Simi-

Figure 1. Comparison of the amino acid sequences of parathyroid hormone-related protein (PTHrP) and parathyroid hormone (PTH) from chicken (c), rat (r), and human (h). The top line (cPRP) represents the amino acid sequence of cPTHrP that was deduced from a cDNA isolated from a 10-d chicken embryo library (63). This sequence is compared with the corresponding sequences of the rPRP and hPRP PTHrP and the cPTH, rPTH, and hPTH. Residues that show identity with the cPTHrP are designated by an asterisk. A consensus (Con) sequence for those residues between 1 and 40 that are shared by cPTHrP, rPTHrP, and hPTHrP and cPTH, rPTH, and hPTH is presented on the bottom line.
Figure 2. Parathyroid hormone-related protein (PTHrP) encodes at least three bioactive domains. The N-terminal parathyroid hormone (PTH)-like domain lies between residues 1 and 34. Residues 67 to 86 have been found to stimulate placental calcium transfer in ovine placenta (11, 33). A pentapeptide termed "osteotstatin" (17, 18), containing residues 107 to 111, is a potent inhibitor of osteoclastogenesis and osteoclast-mediated bone resorption.

The human PTHrP gene is a complex transcriptional unit containing multiple promoter elements and alternatively spliced 3'-untranslated exons (36, 73). The human, rat, mouse, and avian PTHrP genes show a remarkable conservation of both the organization and structure of exon-intron boundaries (23). Transcription of the PTHrP gene is initiated from up to three independent promoter elements, including a GC-rich "housekeeping" promoter element (67), which is likely responsible for maintaining the levels of PTHrP mRNA seen in unstimulated tissues such as the nonlactating mammary gland (Figure 3). Multiple AU-rich motifs within the 3'-untranslated sequence of all known PTHrP mRNA may function in the regulation of PTHrP mRNA stability (51). During the postpartum period, this sequence may play an important function in the rapid decay of PTHrP mRNA in the mammary gland following removal of the suckling stimulus (Figure 3).

The expression and secretion of PTHrP are regulated by numerous factors, including peptide and steroid hormones, growth factors, interleukins, vasoconstrictor substances, and biomechanical stretch (58). In vitro and in vivo, the response of the PTHrP gene to certain stimuli generates a transient increase in both PTHrP gene transcription and PTHrP mRNA accumulation, a response indicative of an early response gene (3). Secretion of PTHrP appears to be coupled with the increase in intracellular PTHrP mRNA in cultures treated with various agents (58), and, during lactation, concentra-
Figure 3. Expression of the parathyroid hormone (PTHrP) gene in rat mammary gland during pregnancy and the postpartum period. Northern blot analysis of PTHrP mRNA in total RNA (20 μg per lane) prepared from inguinal mammary gland. The PTHrP mRNA in the inguinal mammary gland obtained on the day of gestation (gest.), at parturition (part.), 3 h postparturition (pp), or on the designated postpartum day. Postpartum samples were obtained from suckled dams (+) or from dams that were not suckled for 4 h (−).

Postpartum Day

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The expression of PTHrP secreted into the milk correlates well with the expression of PTHrP mRNA in the mammary gland (71).

Unlike PTH, which is essentially a product of the parathyroid gland, PTHrP is produced by numerous cultured cells and tissues. During development, both PTHrP mRNA and PTHrP immunoreactivity are widely distributed in fetal tissues. In the adult, PTHrP is expressed in tissues of the cardiovascular, central, and peripheral nervous systems; gastrointestinal tract; and skin, skeletal muscle, lung, spleen, long bones, kidney, reproductive tissues, urinary bladder, and mammary gland (31, 58). Both PTHrP mRNA and immunoreactivity occur in abnormal as well as normal glandular tissues such as the adrenals, parathyroids and thyroids, pituitary, and pancreas. The expression of PTHrP by keratinocytes, smooth muscle cells, and epithelial cells, including those from the mammary gland, suggests diverse functions for this protein.

Over the past 6 yr, great strides have been made to elucidate the potential physiological functions of the N-terminus and other bioactive domains of the PTHrP molecule. For example, evidence now suggests that PTHrP inhibits keratinocyte proliferation because blockade of endogenous PTHrP synthesis via the expression of antisense PTHrP RNA induced proliferation in these cells (26). In view of the potent calcitropic property of PTHrP, regulated production of the molecule by the avian oviduct shell gland of the egg-laying hen (60, 61) and the lactating mammary gland suggests that PTHrP may modulate calcium metabolism in these tissues. In support of such an action for PTHrP, investigators have shown that N-terminal (1, 4, 33) and midmolecular (11, 33) portions of PTHrP stimulate in a dose-dependent manner the transfer of calcium by the ovine placenta. The molecule also appears to encode reciprocal biological activities: the N-terminus, which can stimulate osteoclastic bone resorption (25, 29, 65), and the C-terminus, which can inhibit this activity (17, 18).

One of the most important bioactivities of PTHrP is likely not related to calcium homeostasis, but is instead related to the contractile activity of vascular and nonvascular smooth muscle. The N-terminal fragments of PTHrP inhibit the contractile activity of smooth muscle in the gut (40), uterus (6, 43, 52), urinary bladder (72), and cardiovascular tissues (58). The production of PTHrP in response to stretching of expandable tissues (13, 59, 72) implicates PTHrP in the regulation of smooth muscle tone. Therefore, PTHrP produced by the lactating mammary gland very likely modulates the activity of mammary epithelial and myoepithelial cells as well as local vascular tissues.

**Lactating Mammary Gland**

**Production of PTHrP**

As shown in Figure 3, the initial evidence to show that PTHrP is a product of the mammary gland was the identification of PTHrP mRNA in the glands of lactating rats (57, 62).
High PTHrP mRNA in lactating rat mammary gland contrast with the low concentrations that exist in unsuckled glands or in glands obtained from pregnant animals. As seen in Figure 3, PTHrP mRNA in the mammary gland rise at parturition, and, with suckling, the expression of PTHrP increases further. The link between the synthesis of PTHrP and lactation is emphasized by the short half-life (1 h) of PTHrP mRNA in the gland following the removal of the suckling litter. On postpartum d 1 through 8, PTHrP mRNA in the lactating gland is constant and remains tightly linked to lactation. Removal of the litters for 4 h during this period dramatically reduced PTHrP mRNA content in the gland. Return of the litter for 4 h restored the concentrations of PTHrP mRNA in the gland. This rapid, reversible expression of PTHrP mRNA by the lactating gland was subsequently used to demonstrate that suckling-induced elevations in serum prolactin stimulated the expression of PTHrP mRNA in the postpartum gland (57). The response to prolactin is specific to the postpartum gland because the dose of prolactin that increased mP in unsuckled postpartum mammary gland failed to alter mammary gland expression of PTHrP mRNA in the tissue during pregnancy.

The relative expression of PTHrP mRNA by the rat mammary gland increases between postpartum d 8 and 16; highest expression occurs in the tissue at weaning (Figure 3). The accumulation of PTHrP mRNA in the lactating mammary gland at this time is also more stable because the removal of the suckling litter for 4 h failed to reduce PTHrP mRNA in the gland. The relative increase in the concentrations of PTHrP mRNA in the mammary gland during this time is reflected in higher milk content of PTHrP (71) and may be due to the temporal increase in the demand for milk. Therefore, the expression of PTHrP by the mammary gland is tightly linked to lactation but is also differentially regulated by the gland during the postpartum period. The physiological significance of the relative increase in PTHrP mRNA abundance and the apparent increase in message half-life during the week before weaning remains to be determined.

Using both immunohistochemistry and in situ hybridization, Rakopoulos et al. (45) investigated the cellular localization of immunoreactive PTHrP and PTHrP mRNA synthesis in the rat mammary gland during pregnancy and lactation. In those studies, both the antigen and mRNA were found to localize to epithelial cells lining alveoli in tissues of both pregnant and lactating animals, suggesting a role for PTHrP in the development and subsequent function of the gland. To support those findings, PTHrP was shown to be synthesized by cultures of mammary gland epithelial cells (19).

Secretion of PTHrP into Milk

Following the initial identification of the PTHrP structure from tumor cells, radioimmunoassays were developed for the detection of immunoreactive PTHrP in blood (8, 9, 10, 20, 46). Such assays were also employed to examine whether PTHrP was present in milk of various species. In their initial study, Budayer et al. (8) determined that PTHrP was present in milk at concentrations 5 to 10,000 times those measured in plasma. The results of other studies (20, 46) have now confirmed the presence of PTHrP in milk, and several laboratories (46, 66) have now purified milk PTHrP to show that the molecular mass of the milk proteins reflected molecules smaller than PTHrP 1 to 141. The ability of milk PTHrP to bind and to activate second messenger systems of the PTH receptor complex indicates that the biologically active N-terminus of the milk protein remains intact. As seen in Figure 3, expression of PTHrP mRNA by the lactating rat mammary gland increases during the week before weaning. Yamamoto et al. (70) demonstrated that this temporal increase in PTHrP mRNA is reflected in higher concentrations of immunoreactive and bioreactive PTHrP in rat milk. Studies have now shown a lack of correlation between the concentrations of calcium and immunoreactive PTHrP in bovine and rat milk (20, 45, 71).

Potential Biological Roles for PTHrP

The initial discovery that mammary gland production of PTHrP was tightly linked to lactation suggested the exciting possibility that this newly discovered calcium-mobilizing protein may be an important systemic factor linking lactogenesis to renal and bone calcium homeostasis (62). In the years that followed, some, but not all, studies generated evidence to
support such a function for the protein. Secretion of relatively high quantities of bioactive PTHrP into the milk suggests a possible, yet undetermined function for the protein in neonatal development. Recent studies indicate that PTHrP produced by the gland may act directly on local mammary gland physiology. Figure 4 summarizes current understanding of the potential roles for PTHrP during lactation.

**Role in Neonatal Development**

Milk is very abundant in bioactive PTHrP, being up to 5 to 10,000 times the amount in blood (8, 20, 22, 27, 30, 46). This abundance suggests either that high concentrations of PTHrP are required to act on local mammary tissues or that the milk PTHrP serves a functional significance in the neonate. To address the latter point, Kukreja et al. (32) injected anti-PTHrP antiserum into neonatal mice to examine the effect that neutralization of circulating PTHrP would have on calcium homeostasis. They found that this treatment had no measurable effect on serum calcium or the calcium content in neonatal bone. In this same study, the subcutaneous, but not oral administration, of PTHrP 1 to 34 increased serum calcium, indicating that the peptide in milk may not accumulate in the circulation to the concentrations required to alter calcium metabolism. Goff et al. (20) reported that immunoreactive PTHrP was elevated in the plasma of newborn calves following colostrum feeding. Although plasma concentrations of immunoreactive PTHrP were clearly elevated following feeding, the PTHrP in neonatal plasma was not biologically active in assays that measured N-terminal bioactivity. Perhaps these data were somewhat expected because neonatal calcium is not generally elevated following nursing. Other bioactive regions (Figure 2) within the PTHrP structure, aside from the N-terminal PTH-like domain, may possibly possess activities as yet to be determined in neonatal circulation.

Figure 4. Potential functions for parathyroid hormone-related protein (PTHrP) produced by the mammary gland during lactation. GI = Gastrointestinal.
Endocrine Factor

The calciotropic property of PThrP, together with the tight regulation of its synthesis by the mammary gland during lactation, suggests that PThrP may be a calcium-mobilizing endocrine factor released into the systemic circulation during lactation. The PThrP released into the circulation during suckling could interact with receptors in bone and kidney to increase the movement of calcium into blood. Interestingly, even before it was structurally identified as PThrP, Brommage and DeLuca (7) suggested that the tumoral hypercalcemic factor may be involved in this process. Once sensitive radioimmunoassays for PThrP were developed, the concentrations of PThrP in the blood of nursing women and animals could be assessed. Initial studies (8, 30) showed that PThrP concentrations in forearm venous blood were generally very low and did not change following the initiation of suckling, indicating that the mammary gland does not release measurable amounts of PThrP into blood. Further evidence not in support of an endocrine function for PThrP during lactation was presented by Melton et al. (38) in their studies of lactating mice. In their studies, passive immunization to PThrP via the injection of an anti-PThrP antiserum into lactating mice was used to show that PThrP neutralization did not alter maternal calcium, milk calcium, or pup weight gain.

Two studies have recently compiled indirect and direct evidence in support of an endocrine function for PThrP. Yamamoto et al. (70) studied the effects of lactation on renal function and found that the initiation of lactation was associated with transient increases in renal excretion of phosphate and cyclic AMP. They found that these responses were similar to that generated following a single injection of PThrP 1 to 34 and could be measured in lactating rats following removal of their parathyroid glands. Additional indirect evidence for endocrine activity for PThrP during lactation has been provided in preliminary data by Miller et al. (39) in their study that showed a correlation between the increase in bone resorption and the high concentrations of PThrP synthesis during early lactation. The only direct evidence to support release of PThrP by the gland during lactation has been provided by Ratcliffe et al. (47), using lactating goats. In those studies, lactation was associated with a measurable increase in PThrP 1 to 86 in blood obtained from the mammary gland vein during the early postpartum period. Measurable elevations of PThrP in venous plasma were blocked by treatment with bromocriptine, suggesting that the release of PThrP into the venous blood is dependent on serum prolactin. These results suggest that the failure of others (8, 30) to identify elevations in PThrP in plasma from forearm veins may be due to the rapid clearance of PThrP in plasma. Whether the PThrP released during lactation has any direct consequences on calcium metabolism of bone and kidney remains to be determined.

Local Transfer of Calcium

Although synthetic N-terminal peptides of PThrP stimulate the reabsorption of renal calcium and release of calcium from bone, the first evidence implicating PThrP in the translocation of calcium across biological membranes was presented by Rodda et al. (49). Using an ovine model of placental calcium transfer, they showed that both synthetic PThrP and PThrP extracted from the fetal parathyroid glands and placenta could restore the maternal-fetal calcium gradient that was lost as a result of fetal thyroid-parathyroidectomy. These initial findings, along with the data from a recent study by Barlet et al. (4), suggest that the N-terminus of PThrP possesses a portion of this activity. Experimental results of Care et al. (11) and MacIsaac et al. (33) now strongly suggest that this activity is encoded in PThrP 67 to 86 and not PThrP 1 to 34 (Figure 2). Whether PThrP 67 to 86 interacts with the same plasma membrane receptor as the N-terminus of PThrP remains to be determined.

In keeping with the model described, Figure 5 suggests the possible autocrine or paracrine actions of PThrP on mammary epithelial cells during lactogenesis. In view of the ability of PThrP to stimulate placental calcium transfer, Barlet et al. (5) studied the effect of synthetic PThrP fragments on calcium secretion into milk during early lactation in the goat and found that PThrP 1 to 34 and PThrP 1 to 86, but not PThrP 140 to 173, increased the calcium, magnesium, and inorganic phosphate...
content in milk in a time-dependent manner. None of these peptides altered milk production during the experiment. These data support an association between the concentrations of PTHrP in milk and the secretion of minerals into milk. However, the arterial hypercalcemia induced by infusion of PTHrP 1 to 34 and PTHrP 1 to 86 suggests that the action of PTHrP delivered in this manner may not be directed solely to the mammary epithelium, but may also alter systemic calcium homeostasis. Because PTHrP 67 to 86 stimulates placental mineral transfer but does not induce hypercalcemia, it is important to examine the effect of this midmolecular fragment of PTHrP on mineral secretion by the mammary gland.

**Regulation of Mammary Gland Hemodynamics**

In addition to the calciotropic actions of PTHrP, infusion of N-terminal fragments of the protein can increase cardiac output and regional blood flow (48) via their interaction with receptors present in the heart, aorta, and peripheral vascular tissues. The vasodilatory action of PTHrP on peripheral blood vessels results from the ability of the peptide to inhibit the contractile activity of vascular smooth muscle (61, 69). The production of both PTHrP and its receptor throughout the cardiovascular system and in cultured vascular smooth muscle cells (24, 44) indicates that PTHrP and its receptor represent an autocrine-paracrine ac-

Figure 5. Parathyroid hormone-related protein (PTHrP) may facilitate the translocation of calcium from serum to milk by mammary epithelial cells. The PTHrP produced by epithelial cells may interact with receptors on the surface of either the cell or adjacent cells. Because PTHrP is concentrated in the milk, the protein may possibly have never left the cell but acted on signaling pathways present on the cytoplasmic face of the plasma membrane or within other intracellular compartments of the expressing cell.
that PTHrP likely modulates local activities of cells by a number of vasoconstrictor substances (44) further emphasize the potentially important role for PTHrP in the regulation of vascular tone.

Lactation is associated with increased cardiac output and blood flow to the mammary gland and other tissues (21). The presence of PTHrP and its receptor in the nutrient vasculature of the rat inguinal mammary gland and the regulation of PTHrP mRNA during lactation (60, 62) support a potential role for the protein in the regulation of mammary gland hemodynamics. Recently, Davicco et al. (15) presented evidence to support a direct role for PTHrP in increasing blood flow to the mammary gland of sheep. Using flow probe analysis, they found that infusion of PTHrP 1 to 34 into the mammary artery rapidly increased blood flow to the gland in a dose-dependent manner. The local action of the infused peptide was supported by the localization of the response to the infused gland and the lack of hypercalcemia following peptide infusion. Thus, PTHrP can quite possibly modulate blood flow to the mammary gland via its local expression and action in both the nutrient vasculature as well as vascular beds of the gland.

Discussion

It has been approximately 5 yr since PTHrP was first shown to be produced by the lactating mammary gland. During this time, the results of numerous studies have advanced the understanding of the potential physiological function that the protein might play during lactation. Although a small body of evidence supports an endocrine activity for the protein, most do not; thus, further studies are needed to determine whether the PTHrP produced by the mammary gland is responsible for the responses measured in kidney (70) and bone (39). The accumulation of evidence in support of a local action of PTHrP in cells and tissues supports the view that PTHrP likely modulates local activities of the mammary gland, including mineral secretion and gland blood flow during lactation. Although studies have shown that PTHrP present in skin (14) can regulate proliferation and differentiation of cultured cells, such as keratinocytes (26), the effects of PTHrP on mammary gland epithelial cell proliferation or function have not been reported. The presence of relatively high concentrations of PTHrP in milk suggests that this protein may function in neonatal development; however, positive experimental results to support such a role for PTHrP in neonatal calcium homeostasis have not been forthcoming.

A powerful in vivo model to test the importance of PTHrP in mammary gland biology would be a genetically modified mouse in which the PTHrP gene is rendered nonfunctional via homologous recombination. Such a mouse has recently been engineered (28), but unfortunately, the homozygous offspring die at birth. These results emphasize the biological importance of this gene product, but the limited lifespan of these mice makes it impossible to determine the effect or effects that this gene removal will have on the lactating mammary gland. An alternative approach would be to engineer a mouse that expresses the anti-sense RNA in the mammary gland via a tissue-specific promoter element. Such an approach has been successful in vitro to demonstrate the anti-proliferative action of the protein in keratinocytes (26). The continued application of creative approaches to this field of study will result in an even greater understanding of the biological role of this calciotropic protein in mammary gland biology in the next 5 yr.

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