Mammary gland serotonin regulates parathyroid hormone-related protein and other bone-related signals

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Hernandez LL, Gregerson KA, Horseman ND. Mammary gland serotonin regulates parathyroid hormone-related protein and other bone-related signals. Am J Physiol Endocrinol Metab 302:E1009–E1015, 2012. First published February 7, 2012; doi:10.1152/ajpendo.00666.2011.—Breast cells drive bone demineralization during lactation and metastatic cancers. A shared mechanism among these physiological and pathological states is endocrine secretion of parathyroid hormone-related protein (PTHrP), which acts through osteoblasts to stimulate osteoclastic bone demineralization. The regulation of PTHrP has not been accounted for fully by any conventional mammotropic stimuli or tumor growth factors. Serotonin (5-HT) synthesis within breast epithelial cells is induced during lactation and in advancing breast cancer. Here we report that serotonin deficiency (knockout of tryptophan hydroxylase-1) results in a reduction of mammary PTHrP expression during lactation, which is rescued by restoring 5-HT synthesis. 5-HT induced PTHrP expression in lactogen-primed mammary epithelial cells from either mouse or cow. In human breast cancer cells 5-HT induced both PTHrP and the metastasis-associated transcription factor Runx2/Cbfa1. Based on receptor expression and pharmacological evidence, the 5-HT2 receptor type was implicated as being critical for induction of PTHrP and Runx2. These results connect 5-HT synthesis to the induction of bone-regulating factors in the normal mammary gland and in breast cancer cells.

5-hydroxytryptamine; lactation; osteoblast; prolactin; RANK ligand; gland and in breast cancer cells.

A KEY FUNCTION OF THE MAMMARY GLANDS is to regulate the mobilization of calcium from bone. During lactation women and other mammals lose a significant portion of bone mass, which is restored after lactation ceases (2, 8, 26, 54). Failure to mobilize bone calcium extraction at the onset of lactation causes hypocalcemia in dairy cows, leading to a severe convulsive syndrome referred to as periparturient paresis or “milk fever” (17, 35). To drive calcium mobilization, the mammary glands become endocrine organs and secrete parathyroid hormone-related peptide (PTHrP) into the bloodstream (9, 27, 46, 50, 51, 53, 54). PTHrP was originally discovered as the factor responsible for humoral hypercalcemia of malignancy and is secreted from a variety of advanced soft-tissue cancers (5, 8, 28, 47). The NH2-terminal portion of PTHrP is similar to that of parathyroid hormone (PTH) and acts via the type 1 PTH receptors (PTH1R) to induce the receptor activator of NF-kB ligand (RANKL) (34).

PTHrP is undetectable in the circulation except during lactation, in advanced metastatic disease, or in patients with hyperprolactinemia (5, 6, 9, 24, 42, 48). Despite obvious correlations with states of elevated prolactin (PRL), PRL did not induce PTHrP in conventional cell cultures of mammary epithelium (29, 52), and our laboratory has done numerous experiments that confirmed that PRL does not induce PTHrP in mammary cells by a direct mechanism (unpublished results).

A previous study showed that serotonin (5-hydroxytryptamine; 5-HT) induced PTHrP expression in vascular smooth muscle cells (40). In the mammary glands, 5-HT regulates key aspects of epithelial homeostasis by autocrine-paracrine signaling. The processes regulated by 5-HT include not only specialized mammary gland functions such as milk protein and milk lipid biogenesis but also fundamental cell biological processes (i.e., apoptosis, barrier permeability, cell shedding, etc.) (16, 30, 31, 36, 38, 49). Epithelia lining other ductal/alveolar secretory organs also possess local 5-HT signaling systems, which have been implicated in various aspects of epithelial homeostasis (39).

Given the central role of 5-HT in mammary gland homeostasis and evidence that 5-HT regulates PTHrP, these studies were initiated to discover new pathways associated with the breast-bone axis. Our results implicate the 5-HT autocrine system as a critical component of the mechanisms by which breast cells regulate bone mobilization.

MATERIALS AND METHODS

Animal studies. Mice with gene disruptions for tryptophan hydroxylase-1 [TPH1, the rate-limiting enzyme in 5-HT biosynthesis (31)], the type 7 serotonin receptor (5-HT7) (15), and corresponding wild-type control animals (C57BL/6J genetic background) were bred and maintained in our animal facility. The knockout strains were capable of lactating and rearing litters of offspring, although they were less efficient than the normal strain. All experiments were performed under protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee. Plasma was collected for PTHrP assay from nonlactating wild-type mice and both wild-type and TPH1−/− animals on day 10 of lactation. Mammary gland tissues (no. 4 glands counted from most rostral) were collected for immunostaining from TPH1−/− and 5-HT7−/− animals and wild-type controls on day 10 of lactation and were fixed in 4% paraformaldehyde before being paraffin embedded and sectioned.

PTHrP immunoradiometric assay. Plasma PTHrP levels were measured using a two-site immunoradiometric assay (IRMA) specific for PTHrP (69202, Becton-Dickinson), following the manufacturer’s instructions. The detection limit (blank serum + 1SD) was 0.3 pM under the conditions of this assay.

Cell culture. Primary bovine mammary epithelial cells (pBMECs) grown in collagen gels were induced to differentiate by release of the gel from the substratum and treating them with lactogenic hormones (prolactin 1 μg/ml, insulin 5 μg/ml, cortisol, 1 μM) as described (16, 32). The mouse mammary epithelial cells (HC11) were maintained under growth medium conditions and lactogen induced by treatment of 3-day-confluent cultures with prolactin, insulin, and cortisol as previously described (18). MDA-MB-231, MCF7, and T47D breast cancer cell lines and MCF10A (normal human ductal mammary epithelial cell lines) were obtained from the American Type
Culture Collection (ATCC) and cultured as previously described (31, 38, 49). pBMECs were obtained as a generous gift from Dr. Robert Collier, University of Arizona, and cultured as previously described (16, 32). Serotonin-HCl (5-HT, Sigma-Aldrich) was freshly prepared and diluted into the respective media at the concentrations described in the text. MC-3T3 clone E1 cells were obtained from ATCC and cultured as described (13, 14). MC3T3-E1 cells were stimulated to differentiate into osteoblasts (OB), using 50 μg/ml L-ascorbic acid.

Conditioned medium (CM) was collected following incubation of MDA-MB-231 cells for 72 h in growth medium or in growth medium plus 100 μg/ml p-chloro-phenylalanine (pCPA, Tocris), a specific and irreversible inhibitor of TPH (21). MC3T3-E1 cells were treated with 50% CM.

Quantitative real-time RT-PCR amplification. Total RNA was isolated using Tri Reagent (Molecular Research) following the manufacturer’s procedures. RNA quality was determined through spectrophotometric methods on a Nanodrop 2000 (Thermo Scientific). A total of 1 μg was reverse transcribed using a Quantitect reverse transcription kit (Qiagen). Quantitative real-time RT-PCR was performed using the Applied Biosystems Step One Plus system using fast SYBR Green Master Mix (Applied Biosystems). The following conditions were utilized: 95°C for 20 s followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Primers designed with similar amplification efficiencies, are listed in Table 1.

Immunohistochemistry and Western blotting. PTHrP fluorescent immunostaining was performed using a 1:50 dilution of goat anti-PTHRP (N-19; Santa Cruz Biotechnology) overnight at 4°C and was readily detectable during lactation (Fig. 1). Staining was observed in occasional cells that bordered the luminal epithelium, which were most likely to be myoepithelial. PTHrP immunoreactivity was markedly less in the glands of TPH1−/− lactating mice (Fig. 1A right).

Consistent with many published reports, PTHrP was below the level of detectability in the blood plasma of nonlactating females and was readily detectable during lactation (Fig. 1B, inset).

RESULTS

5-HT induces PTHrP during lactation. We examined PTHrP levels in mammary glands of TPH1−/− mice and their corresponding normal controls (TPH1+/+) at midlactation by immunostaining (Fig. 1A). In the mammary tissue in control mice (TPH1+/+), PTHrP was detected both in the cytoplasm of the lactogenic cells. Data are represented as means ± SE of log-transformed values of relative expression (n = 4 or 6, ***P < 0.001).
The conversion of 1-tryptophan to 5-hydroxytryptophan (5-HTP) is the rate-limiting step in 5-HT synthesis; therefore, we injected 5-HTP to bypass the enzyme deficiency in TPH1−/− mice and measured PTHrP plasma levels (Fig. 1B). The mice rescued with 5-HTP showed a time-dependent increase in plasma PTHrP (P < 0.0001, R² = 0.92), demonstrating that TPH1 activity was necessary for PTHrP secretion during lactation.

To determine whether PTHrP expression in mammary epithelial cells was directly responsive to 5-HT, two cell models of lactogenic mammary epithelium were studied: lactogen-treated rodent HC11 cells and pBMECs embedded in floating collagen gels. In these models, 5-HT induced PTHrP gene expression 8- and 20-fold, respectively (Fig. 1, A and C). PTHrP and other factors that may be secreted from breast cancer cells induce osteoclastic bone resorption by stimulating RANKL expression in osteoblasts (34). We confirmed that CM from MDA-MB-231 cells induced RANKL gene expression (Fig. 3A). Inhibiting TPH activity in MDA-MB-231 by treating them with pCPA blocked RANKL stimulation by MDA-MB-231 CM. In contrast, MDA-MB-231 CM suppressed osteoprotegerin (OPG, a decoy receptor for RANKL), but the inhibition of OPG was not affected by treating MDA-MB-231 cells with pCPA (Fig. 3B).

**Induction of PTHrP is mediated by 5-HT2 but not 5-HT7.** Multiple 5-HT receptor isoforms are expressed in mammary epithelium and in breast cancer cells. Of these, previous studies have implicated 5-HT7 in several aspects of epithelial homoeostasis (16, 37, 49); so we considered it possible that 5-HT7 also mediated induction of PTHrP. To address any potential role of 5-HT7, we stained glands from 5-HT7 knockout mice for PTHrP immunoreactivity during lactation. PTHrP expression was similar in glands from normal (5-HT7+/+) and knockout (5-HT7−−) mice (Fig. 4A), implying that a receptor other than 5-HT7 was involved in PTHrP induction.

Of the various 5-HT receptor isoforms, 5-HT2 (specifically 5-HT2B) was expressed in all normal mammary cells and breast cancers that we have tested (mouse, cow, and human; Table 2). Consequently, we tested the hypothesis that 5-HT2 activity regulated PTHrP in untransformed mouse mammary cells (HC11; Fig. 4B) and in human breast cancer cells (MDA-MB-231; Fig. 4C). Activating 5-HT2 with BW-723C86 [α-methyl-5-(2-thienylmethoxy)-1H-indole-3-ethanamine; Tocris, http://www.iuphar-db.org/DATABASE/] stimulated PTHrP in a concentration-dependent manner. Correspondingly, the 5-HT2 antagonist SB-204741 [1-(1-methylindol-5-yl)-3-(3-methyl-1,2-thiazol-5-yl)urea; Tocris, http://www.iuphar-db.org/DATABASE/] suppressed PTHrP expression. Moreover, Runx2 was regulated similarly in MDA-MB-231 cells by activating or inhibiting 5-HT2 receptors (Fig. 4D). Because it inhibits not only ligand-induced but also basal receptor activity, SB-204741 is best described as an inverse agonist of 5-HT2.

**DISCUSSION**

Herein, we report that 5-HT induces PTHrP secretion via an autocrine pathway in the mammary glands. A previous study...
demonstrated that 5-HT stimulated PTHrP expression in vascular smooth muscle (40); therefore, the signaling mechanisms employed by 5-HT to induce PTHrP may operate in a variety of cell types. Our results suggest that the 5-HT-PTHrP system may contribute to calcium mobilization associated with lactation and hyperprolactinemia. The presence of the autocrine 5-HT-PTHrP signaling system in breast cancer cells also indicates that this same system contributes to local bone loss and systemic hypercalcemia in metastatic cancers.

It has been evident that PTHrP is involved in bone mobilization during lactation and hyperprolactinemia and in hypercalcemia caused by cancers (5, 47, 48, 53). The transcription factor Runx2 is an important regulator of the metastatic and bone-homing phenotypes of breast cancer cells (41). In addition, Runx2 and PTHrP are linked in a reciprocal stimulatory network between breast cancer cells and osteoblasts (1, 11). The factors upstream of PTHrP are largely unknown (7, 10, 23, 54). One relevant hypothesis is that factors liberated from bone matrix drive a "vicious cycle" of PTHrP secretion from metastatic breast cancer cells (34). However, this vicious cycle hypothesis cannot account for the high level of PTHrP secretion from normal lactating mammary cells.

We propose that PTHrP secretion is driven by 5-HT signaling (Fig. 5). This autocrine-paracrine 5-HT hypothesis is able to account for secretion from normal mammary epithelial cells and for initiation of a vicious cycle in the bone microenvironment.

Calcium secretion into milk can place extreme pressures on calcium homeostatic mechanisms. One consequence of the onset of mammary calcium demand is periparturient hypocalcemia (aka milk fever), which is observed frequently in high-producing dairy cows and less often in humans and other mammals (17, 35). Although the metabolic consequences of milk fever are well known, the pathophysiological mechanisms responsible for the breakdown of calcium homeostasis have remained obscure, and the potential involvement of 5-HT provides a new set of mechanisms to explore.

Bone mobilization and elevated 5-HT activity. Epidemiological evidence in humans has consistently pointed to a relationship between 5-HT and bone mobilization. One common observation is that antidepressant use is associated with reduced bone mineral density (3). Another class of psychoactive drugs, the atypical antipsychotics, have well-documented negative effects on bone accrual (33). Enhanced 5-HT bioactivity leading to local PTHrP secretion may be one mechanism involved in bone loss caused by these drugs. In addition, antidepressant and antipsychotic drugs tend to cause hyperprolactinemia, and the degree of hyperprolactinemia can be substantial (4, 33). Demineralization of the bones is also commonly associated with hyperprolactinemia resulting from prolactinomas (12, 27, 44, 48). One explanation for bone loss in hyperprolactinemic patients is the antagonadal effect of PRL, which reduces estrogen secretion in females. In addition, hyperprolactinemic patients had elevated circulating PTHrP levels, and PTHrP concentrations were directly correlated with bone loss (48). Mammary gland 5-HT biosynthesis was discovered because TPH1 was highly induced by hyperprolactine-
modulating signaling downstream from 5-HT2. Other 5-HT receptor types are likely to be involved in regulating bone-related signals by activation and inhibition of 5-HT2. For the sake of simplicity, potential roles of Runx2 are omitted.

The ubiquity of 5-HT2 expression (particularly 5-HT2B) led us to hypothesize that these receptors might be responsible for induction of PTHrP and/or Runx2. Type 2B receptors are expressed in all of these systems, and types 2A and 2D are additionally expressed in certain normal and cancer cells. The ubiquity of 5-HT2 expression (particularly 5-HT2B) led us to hypothesize that these receptors might be responsible for induction of PTHrP and/or Runx2. Correspondingly, PTHrP and Runx2 were sensitive to pharmacological activation and inhibition of 5-HT2. Other 5-HT receptor types are likely to be involved in regulating bone-related signals by modulating signaling downstream from 5-HT2.

Previous studies have implicated PKC in the regulation of both PTHrP and Runx2 (11, 22, 41, 43). Therefore, the apparent involvement of type 2 receptors, which signal through $G_q$ and PKC, provides a basis for future hypotheses regarding mechanisms of regulation for PTHrP and Runx2 and for identifying other genes that may be involved in signaling from breast cells to bone cells.

**Conclusions.** Autocrine-paracrine 5-HT stimulates PTHrP gene expression and secretion and the expression of the bone-related transcription factor Runx2. The 5-HT2 type receptors appear to play an important role in the induction of bone-relevant signals. The complexity of 5-HT signaling demands cautious interpretations and the testing of new hypotheses and additional model systems. With improved knowledge, serotonergic drugs may provide novel opportunities for therapeutic interventions.

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