Serotonin induces parathyroid hormone-related protein in goat mammary gland

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ABSTRACT: During lactation, large amounts of calcium are exported from the mammary gland into milk to ensure skeletal growth of the offspring. Recent studies revealed that serotonin (**5-HT**) is essential to stimulate skeletal calcium resorption for milk synthesis. Our objective was to explore the correlation between circulating 5-HT and serum calcium and parathyroid hormone-related protein (**PTHrP**) concentrations around parturition in dairy goats. We also investigated the effect of 5-HT on PTHrP expression in cultured primary goat mammary epithelial cells (**GMEC**). Blood samples of multiparous Guanzhong dairy goats were collected on day -5 to 3 postpartum for analysis of serum concentrations of calcium, 5-HT, and PTHrP. Results revealed that from day -3 to 0 postpartum serum calcium and 5-HT concentrations decreased progressively, but serum PTHrP concentration only had a sharp drop in the postpartum period sampled. Correlation analysis of

circulating 5-HT and serum calcium and PTHrP concentrations on day 1 and 2 postpartum revealed that low serum 5-HT concentration was positively correlated with serum total calcium or PTHrP concentration. By knocking down tryptophan hydroxylase-1 (**TPH1**) or adding 5-hydroxytryptophan (**5-HTP**) to decrease or increase the levels of 5-HT in GMEC, we observed that 5-HTP increased PTHrP expression in a dose-dependent manner and siTPH1 decreased PTHrP protein expression. Furthermore, 5-HT increased mRNA abundance of calcium-sensing receptor (**CaSR**) in a dose-dependent manner and decreased the expression of plasma membrane Ca^{2+} ATPase-1 (**PMCA1**). Taken together, 5-HT seems to induce PTHrP expression in goat mammary cells during and after parturition. These findings suggest that increasing 5-HT biosynthesis could be a potential therapeutic target for prevention of hypocalcemia in dairy goats.

Key words: calcium, goat, PTHrP, 5-HT

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INTRODUCTION

The demand for calcium to meet the growth needs of the young increases substantially in perinatal animals, and skeletal calcium has to be released from the dam in part because the amount of dietary calcium absorbed cannot meet the demands of the neonate (Horseman and Hernandez, 2014; Kovacs, 2015). Calcium reabsorption is the primary

contributor of milk calcium in mammals, and the inability to extract sufficient calcium from the bone can cause subclinical or clinical hypocalcemia (milk fever) in perinatal dams, especially in multiparous dairy cows and goats (Oetzel, 1988; Liesegang et al., 2006; DeGaris and Lean, 2008; Kovacs, 2015). Hypocalcemia around parturition increases incidence of uterine diseases, risk of culling, and reduces reproductive and productive performance (Chapinal et al., 2012; Martinez et al., 2012).

Parathyroid hormone-related protein (**PTHrP**) plays a major role in calcium mobilization and fetal bone development (Miao et al., 2002; Kronenberg,

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2006; Martin, 2016). During lactation, calcium homeostasis is regulated by PTHrP via a feedback mechanism (Hiremath and Wysolmerski, 2014; Horseman and Hernandez, 2014). Serotonin (5-hydroxytryptamine [5-HT]), synthesized in the mammary gland, regulates various aspects of mammary gland homeostasis during lactation (Matsuda et al., 2004; Marshall et al., 2010; Laporta et al., 2014a). The processes regulated by serotonin include not only milk protein biogenesis but also calcium and glucose homeostasis (Hernandez et al., 2009; Marshall et al., 2010; Hernandez et al., 2012). A previous in vitro study demonstrated that 5-HT promotes PTHrP expression in vascular smooth muscle cells (Pirola et al., 1993). Furthermore, 5-HT induced PTHrP expression in bovine and murine mammary epithelial cells (Hernandez et al., 2012). However, the role of 5-HT and PTHrP in the regulation of calcium homeostasis around parturition in dairy goats remains largely unknown. Therefore, the objective of this study was to test whether circulating 5-HT concentration around parturition was correlated with serum concentrations of calcium and PTHrP, and also to assess the relationship between 5-HT and PTHrP in goat mammary epithelial cells (GMEC).

MATERIALS AND METHODS

Sample Preparation

All experimental procedures with live goats used in this study were approved by the Animal Care and Use Committee of the Northwest A&F University. Thirty-two multiparous Guanzhong dairy goats (3-yr-old and second lactation) were selected from the Xin longmen dairy goat breeding farm in Xi'an. Blood samples from the 10 goats without anticoagulant were collected from the jugular vein on day -7 before the predicted parturition until day 3 of lactation. The 10 goats' blood samples collected from day 1 and 2 postpartum were to analyze correlations among circulating 5-HT and serum calcium with PTHrP concentrations. And only four goats' blood samples were collected from day -5 to 3 postpartum to analyze difference in concentrations. These blood samples were centrifuged at $5,000 \times g$ for 15 min at 4 °C to harvest serum. Serum concentrations of total calcium were determined using a quantitative colorimetric calcium assay kit (Nanjing Jiancheng Bioengineering Institute, China). Serum concentrations of 5-HT and PTHrP were immediately analyzed by enzyme-linked immunosorbent assay (SHKXSM Co. Ltd., China) according to the manufacturer's instructions. In briefly, 50 µl of freshly prepared serum samples were

added to the appropriate wells, and adding 100 µl of enzyme conjugate to standard wells and sample wells except the blank well, cover with an adhesive strip and incubate for 60 min at 37 °C. Then the microtiter plate was washed four times. Fifty microliters of substrate A and B were added to each well and incubated for 15 min at 37 °C from light. Each well was added with 50 µl stop solution. The absorbance of each sample at 450 nm wavelength was detected using a microplate reader within 15 min.

Cell Culture and Vector Construction

The GMEC were isolated from Guanzhong dairy goats at peak lactation, and the details of cell culture were described previously (Li et al., 2014). Briefly, goat mammary epithelial cells were harvested from clinically-healthy mammary tissue collected under sterile conditions; the blood was washed away with D-Hank's solution supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin. After removing the fatty and connective tissue, the white granular acinar tissue was cut into small pieces (about 1 mm³) and cultured with complete medium. The medium was composed of Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12) medium (Invitrogen) supplemented with insulin (5 µg/ml; Sigma-Aldrich, St. Louis, MO), penicillin and streptomycin (100 U/ml; Harbin Pharmaceutical Group, Harbin, China), epidermal growth factor 1 (10 ng/ml; Invitrogen), hydrocortisone (1 µg/ml; Sigma-Aldrich), and 10% fetal bovine serum (Hyclone, Logan, UT). The fibroblast-like cells and adipocytes were separated by trypsin digestion and differential adhesion after five passages and purification. The GMEC at passage 8 to 12 were cultured to confluence in complete medium at 37 °C in a humidified atmosphere with 5% CO₂. Culture medium was changed every 24 h.

Recombinant adenoviruses for Ad-PTHrP and Ad-siPTHrP were prepared by our laboratory as described previously (Li et al., 2014). We designed and constructed three RNA interference vectors of tryptophan hydroxylase-1 (TPH1), and the siRNA with the best interference efficiency is referred as siTPH1 (upstream: GGUGCCGGCUUACUUUCUUTT; downstream: AAGAAAGUAAGCCGGCACC-TT). When GMEC grew to approximately 70% to 80% confluence they were used for cell culture as follows: 1) treatment with 0, 50, 100, or 200 µg/ml 5-hydroxy-L-tryptophan (5-HTP, Abcam Inc., USA); 2) treatment with siTPH1 (1.5 µg/well in six-well plates); 3) treatment with Ad-PTHrP (multiplicity of infection [MOI] = 200); and 4) treatment with

Ad-siPTHrP (MOI = 200). After 48 h incubation, the GMEC were used for RNA and protein extraction and intracellular calcium analysis.

Quantitative Real-Time PCR and Western Blot

Total RNA was isolated using Trizol reagent (Takara Bio Inc., Japan) according to the manufacturer's instructions. The first-strand complementary DNA was synthesized using the PrimeScript RT kit (Takara Bio Inc., Japan). Quantitative real-time polymerase chain reaction (qPCR) primer sequences are shown in Supplementary Table S1. Ubiquitously expressed transcript (*UXT*) and *GAPDH* were used as internal control genes. The qPCR was run in triplicate in a Bio-Rad master cycler using the SYBR Green PCR Master Mix (Takara, Japan) according to the manufacturer's protocol. The qPCR data were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Western blot was performed as described previously (Li et al., 2016). Cells were collected from different treatment groups, pelleted them by centrifugation, and lysed them in radio immunoprecipitation assay (RIPA) buffer. Total protein was prepared and protein concentration was determined using the Bradford method. Proteins were then separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes and blocked with milk powder solution for 1.5 h at room temperature and overnight incubation with the primary antibody. We incubated membranes overnight with the primary antibody. Anti-PTHrP, anti-TPH1, and anti- β -actin were purchased from Abcam, Cambridge, MA. Then the membranes were washed with PBS-tween and incubated for 1.5 h with horseradish peroxidase-conjugated secondary antibodies (Abcam). β -actin was used as a house-keeping protein. Protein bands were detected after treatment with SuperSignal West Femto agent of Thermo (Thermo Scientific, Karlsruhe, Germany).

Measurement of Intracellular Calcium

Intracellular calcium trafficking was detected using Rhod-2 AM (2 μ M, Molecular probes, Life Technologies) by a laser scanning confocal microscopy (Becton Dickinson, Inc.), and cells were processed using previously described protocols (Zheng et al., 2014; Sawant et al., 2016).

Statistical Analysis

Results are expressed as means $\pm$ standard error of the means (SEM) for at least three independent

experiments, and the statistical significance for RNA expression was analyzed by one-way analysis of variance (ANOVA) and Tukey's test using SPSS 19.0 software (Chicago, USA). Nonparametric test was used to analyze serum concentrations of calcium or 5-HT or PTHrP on day -5 to 3 postpartum. The Pearson's correlation test was used to examine relationships between serum calcium, 5-HT, and PTHrP concentrations. Statistical significance was declared at $*P < 0.05$.

RESULTS

Correlation Between Serum Calcium and 5-HT and PTHrP

The serum calcium and 5-HT concentration were not changed significantly from -5 to +3 days postpartum ($P > 0.05$, Fig. 1A and B, Supplementary Tables S2 and S3), but concentration of PTHrP had a sharp drop in the immediate postpartum period ($P < 0.01$, Fig. 1C and Supplementary Table S4). Correlations of circulating 5-HT and serum calcium with PTHrP concentrations were analyzed on day 1 and 2 postpartum in the 10 goats. We found that the concentrations of 5-HT or calcium or PTHrP between day 1 and 2 postpartum were not different ($P > 0.05$), thus we put the two days of data together for analysis of correlations (Supplementary Table S5). The relationship between PTHrP concentration and serum 5-HT concentration may be expressed by a regression equation $Y = -0.0008 + 1.2704X - 0.3848X^2 + 0.0451X^3 - 0.0018X^4$ ($r = 0.7705$, $P < 0.01$, Fig. 1D and Supplementary Table S5). The regression equation of serum total calcium concentration to 5-HT concentration may be expressed by the equation $Y = 0.1090 + 1.4393X - 0.3560X^2 + 0.0354X^3 - 0.0012X^4$ ($r = 0.8286$, $P < 0.01$, Fig. 1E and Supplementary Table S5), and serum total calcium concentration to PTHrP concentration may be expressed by the equation $Y = -0.0001 + 2.8852X - 0.9914X^2$ ($r = 0.88$, $P < 0.01$, Fig. 1F and Supplementary Table S5).

Results revealed that the correlation between 5-HT and serum calcium with PTHrP concentrations was positive when the concentration of 5-HT was 1.7–2.9 ng/ml ($P < 0.01$, Fig. 1D–F and Supplementary Tables S2–5). Their correlation may be negative or positive when the concentration of 5-HT was more than 2.9 ng/ml. It revealed that the regulatory relationships may be regulated by multiple mechanisms.

We also found that with the increase of 5-HT concentration (1.7–3.8 ng/ml), the first peak of

Figure 1. Correlation between serum calcium and serotonin (5-HT) and parathyroid hormone-related protein (PTHrP) concentrations around parturition in dairy goats. Concentrations of serum calcium (A), 5-HT (B), and PTHrP (C) from day -5 to 3 postpartum. (D-F) Correlation between serum total calcium and 5-HT and PTHrP concentrations on day 1 and 2 postpartum. Goat mammary gland epithelial cells (GMEC) were transfected with Ad-PTHrP or Ad-siPTHrP for 48 h, then *PTHrP* mRNA expression was detected by quantitative real time PCR (G), and intracellular calcium levels were measured in GMEC loaded with Rhod-2 AM using a confocal laser scanning microscope (H-J). Scale bar indicates 20 μm . Values are means $\pm$ SEM. Different lowercase letters (a and b) indicate significant ($P < 0.05$) differences. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. 5-HT, serotonin; PTHrP, parathyroid hormone-related protein; Ad-PTHrP, PTHrP overexpression recombinant adenoviruses; Ad-siPTHrP, PTHrP interference recombinant adenoviruses.

calcium concentration appeared later than PTHrP. This suggested that 5-HT may regulate serum calcium levels by affecting PTHrP concentration (Fig. 1D and E). These data indicate that 5-HT and PTHrP play important roles in regulating calcium homeostasis in dairy goats.

5-HT-induced PTHrP Expression in GMEC

Mammary gland epithelial cells, which secrete milk calcium, are a suitable model to study mechanisms of calcium homeostasis in a more

controlled fashion. Having established that the serum 5-HT level was correlated with PTHrP concentration, we next set to determine whether the expression of PTHrP was directly responsive to 5-HT in GMEC. Ad-PTHrP markedly increased the expression of PTHrP, and PTHrP expression reduced significantly in GMEC transfected with interference recombinant adenoviruses Ad-siPTHrP ($P < 0.05$; Fig. 1G). Overexpression of PTHrP led to a transient increase in the calcium levels (Fig. 1H-J). Conversely, Ad-siPTHrP decreased the mitochondrial calcium fluorescent

intensity (Fig. 1 H–J). After GMEC were transfected with siTPH1, the expression of TPH1 decreased significantly compared with the control group at both the mRNA ($P < 0.05$) and protein levels (Fig. 2A). In addition, PTHrP expression decreased significantly in the GMEC transfected with siTPH1 ($P < 0.05$; Fig. 2B and D). Furthermore, 5-HTP increased PTHrP expression in GMEC in a dose-dependent manner (Fig. 2C and D).

Previous studies have shown that PTHrP stimulates calcium resorption from the bone after which it can activate calcium-sensing receptors (CaSR) on the basolateral membrane leading to calcium entry into cells and utilization for milk

synthesis. Subsequently, activated CaSR inhibits PTHrP production and secretion into milk (VanHouten et al., 2004; Ardeshirpour et al., 2006). Secretion of calcium into milk depends greatly on CaSR and calcium transporters: plasma membrane Ca^{2+} ATPase-1 (PMCA1), sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase-2 (SERCA2), secretory pathway Ca^{2+} ATPase-1 (SPCA1), and SPCA2 in mammary epithelial cell membranes. Knockdown of TPH1 inhibited the expression of *CaSR* ($P < 0.05$) and induced the expression of *PMCA1* ($P < 0.01$; Fig. 2E). The 5-HTP increased mRNA abundance of *CaSR* in a dose-dependent manner and decreased the expression of *PMCA1* ($P < 0.01$; Fig. 2F). No significant

Figure 2. Serotonin (5-HT)-induced parathyroid hormone-related protein (PTHrP) expression in goat mammary gland epithelial cells (GMEC). The GMEC were transfected with *siTPH1* for 48 h or treated with 5-HTP for 24 h, then the expression of *TPH1* and *PTHrP* was analyzed by quantitative real-time polymerase chain reaction (qPCR) and western blot (A–D). The mRNA expression of *CaSR* and mammary gland calcium transporters (*PMCA1*, *SERCA2*, *SPCA1*, and *SPCA2*) was also analyzed by qPCR (E and F). Values are means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NC, mock-vehicle for control; siTPH1, RNA interference vector of TPH1; 5-HTP, 5-hydroxytryptophan; CaSR, calcium-sensing receptor; PTHrP, parathyroid hormone-related protein; PMCA1, plasma membrane Ca^{2+} ATPase-1; SERCA2, sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase-2; SPCA1, secretory pathway Ca^{2+} ATPase-1; SPCA2, secretory pathway Ca^{2+} ATPase-2; TPH1, tryptophan hydroxylase-1.

difference was detected for *SERCA2*, *SPCA1*, or *SPCA2* mRNA expression in GMEC treated with 5-HTP or *siTPH1*.

DISCUSSION

The PTHrP protein is normally only detectable during lactation and is secreted into the bloodstream to mobilize bone calcium for secretion into milk; hence, this protein plays a central role in calcium homeostasis (Kovacs, 2001; VanHouten et al., 2003; Horseman and Hernandez, 2014). PTHrP deficiency (mammary-specific knockout) reduces circulating PTHrP and bone turnover, and decreases the milk calcium level (VanHouten et al., 2003). The concentration of 5-HT, synthesized in the mammary gland, is highest during lactation and induces PTHrP (Hernandez et al., 2012; Laporta et al., 2013a, 2013b). Supplementation of 5-HTP increased circulating 5-HT and PTHrP concentrations and milk calcium concentration around parturition in mice and rats (Hernandez et al., 2012; Laporta et al., 2013b). Knockout of TPH1 in mice, resulting in 5-HT deficiency, reduces the expression of mammary PTHrP during lactation and the expression can be rescued by restoring 5-HT synthesis (Hernandez et al., 2012). During lactation, calcium homeostasis is regulated by PTHrP by a feedback mechanism (Hiremath and Wysolmerski, 2014; Horseman and Hernandez, 2014). In this study, correlation analysis of circulating 5-HT and serum calcium and PTHrP concentrations on day 1 and 2 postpartum revealed that low serum 5-HT concentration was positively correlated with serum total calcium or PTHrP concentration (Fig. 1). We suspect that 5-HT-PTHrP system is regulated by this feedback mechanism when the 5-HT concentration is low. And when 5-HT increases at high concentration, more skeletal calcium is resorbed for milk production via activating calcium sensing receptor (CaSR) on the basolateral membrane, then activated CaSR inhibits 5-HT and PTHrP production and followed by the decreased calcium resorption.

Using a *TPH1* knockout mouse model, resulting in 5-HT synthesis deficiency, led to a decrease in expression of mammary PTHrP during lactation, and an injection of 5-HTP rescued the expression of PTHrP (Hernandez et al., 2012). Using the same mouse model, it was observed that 5-HT alters DNA methylation of the sonic hedgehog (SHH) gene leading to transcriptional initiation at an alternate start site and induction of *SHH* mRNA expression, which induces the expression of PTHrP and mobilizes calcium from bone to milk (Laporta et al., 2014b).

In mouse or cow primary mammary epithelial cells, 5-HT induced PTHrP expression (Hernandez et al., 2012). In cultured human breast cancer cells, 5-HT induced both PTHrP and the bone-regulating factor Runx2/Cbfa1 (Hernandez et al., 2012). The mammary alveolar epithelial cells act as an accessory endocrine gland, secreting PTHrP, which enters the circulation and stimulates calcium resorption from the bone. The released calcium is transported via the bloodstream to the mammary gland, where it activates CaSR on the basolateral surface to promote calcium entry into the cells for subsequent secretion into milk and to inhibit PTHrP production and secretion into milk (Hiremath and Wysolmerski, 2014). In the present study, the positive correlation between blood calcium and blood 5-HT and PTHrP, the high level of intracellular calcium stimulated by PTHrP overexpression, and the 5-HT induced PTHrP and *CaSR* expression. All these results suggested that serotonergic control of PTHrP may control calcium release from the skeleton by activating serotonin signaling, and CaSR activated by PTHrP mediates feedback inhibition of PTHrP to maintain calcium balance (Hiremath and Wysolmerski, 2014; Horseman and Hernandez, 2014).

In the present study, we designed and constructed *siTPH1* and observed that *siTPH1* decreased the expression of 5-HT and PTHrP significantly (Fig. 2). When 5-HTP was added to GMEC to increase the levels of 5-HT, the expression of PTHrP increased markedly. It is noteworthy that we detected an increase in intracellular calcium levels in response to PTHrP in GMEC, and this was consistent with the conclusion that serum total calcium concentration was correlated with low serum PTHrP concentration on day 1 and 2 of lactation (Fig. 1).

In conclusion, this experiment provides preliminary evidence that low circulating 5-HT is correlated with circulating calcium or PTHrP concentration around parturition in goats, and 5-HT induces PTHrP expression in GMEC. These findings suggest that increasing 5-HT biosynthesis could be a potential therapeutic target to explore for prevention of hypocalcemia in dairy goats.

SUPPLEMENTARY DATA

Supplementary data are available at *Animal Frontiers* online.

Conflict of interest statement. The authors have declared that no competing interests exist.

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