SEROTONIN AND THE PHYSIOLOGY OF CALCIUM HOMEOSTASIS DURING THE TRANSITION PERIOD

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INTRODUCTION

Adequate circulating Ca concentrations throughout the transition period are necessary for productive lactation, but large quantities of Ca are lost from maternal Ca pools into milk and colostrum. A rapid, large drop in maternal blood Ca causes 5-10% of cows to be afflicted with clinical hypocalcemia (CH) and an additional 50% to suffer from subclinical hypocalcemia (SCH). SCH and CH are significant risk factors of early lactation culling/premature removal from the herd (DeGaris and Lean, 2008; Reinhardt et al., 2011; Roberts et al., 2012). Furthermore, SCH increases risks of developing ketosis; displaced abomasum; and metritis; SCH depresses immune function; prolongs the interval until pregnancy is achieved; decreases pregnancy rate; and reduces overall productivity (DeGaris and Lean, 2008; Chapinal et al., 2011; Chapinal et al., 2012; Figure 1).

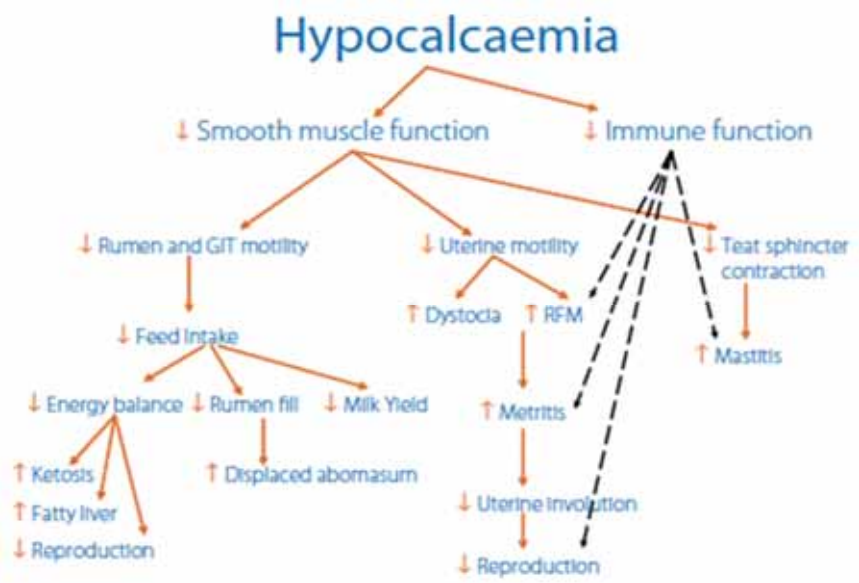

Figure 1. Hypocalcemia is a 'gateway' disease that leads to increased risks of other periparturient diseases. (DeGaris and Lean, 2008).

The transition period (3 weeks pre-calving through 3 weeks post-calving) is an extremely critical time period in the life of the dairy cow. At this time the animals are highly susceptible to a variety of disorders that negatively impact the animal's health, and hence their overall production. Of particular concern during this time is the inability of the animal to maintain adequate blood calcium concentrations due to increased

demand for calcium at the onset of lactation by the mammary gland. This increase in calcium results in decreased circulating calcium levels and can lead to the development of periparturient hypocalcemia (milk fever). Parturient paresis is one of the most common metabolic diseases of dairy cattle, with Jersey cows being more susceptible than Holsteins (Oetzel, 1988; NRC, 2001). Hypocalcemia is associated with numerous other health disorders during this time period (Oetzel, 1988). Due to inadequate blood calcium levels at the onset of lactation, animals experience a range of clinical symptoms, depending on the extent of the decreased calcium levels (Adams et al., 1996). CH is clinically defined as a total blood calcium level of less than 1.4 mmol/L, and subclinical hypocalcemia defined as total blood calcium of 1.4-2.0 mmol/L (DeGaris and Lean, 2008). Approximately 25% of heifers and 50% of older cows will succumb to SCH, and between 5-10% of animals will develop clinical hypocalcemia in the United States (Goff, 2008). Cattle that are afflicted with periparturient hypocalcemia exhibit a 14% decrease in milk production and are more susceptible to other transition disorders such as ketosis, retained placenta, displaced abomasum and muscle weakness, with the average cost of incidence of milk fever being $334/animal (Oetzel, 1988). However, should an animal succumb to additional issues due to suffering from milk fever, costs increase substantially. Subclinical hypocalcemia affects about 50% of second lactation and greater dairy cattle, and costs approximately $125/animal to treat. Overall, prevalence of milk fever and subclinical hypocalcemia are more common in Jersey cattle, likely due to their higher milk production per unit body weight (Oetzel, 1988). Typically, in order to compensate for decreased blood calcium, increased intestinal calcium absorption and/or calcium resorption from the bone must occur, however calcium resorption from the bone is the primary mode used during this time frame. Dairy cattle, in particular, exhibit a delay in calcium resorption from bone.

Parathyroid hormone related-protein (PTHrP) synthesized within the mammary gland has been described as the molecule responsible for mobilization of calcium from bone that occurs at the onset of lactation in mammals (Wysolmerski, 2010). Recently, we have demonstrated that mammary serotonin (5-hydroxytryptamine) regulates induction of PTHrP (Hernandez et al., 2012). Manipulation of serotonin induced PTHrP synthesis near the end of the pregnancy period could be critical in preventing the onset of hypocalcemia during the early lactation period. This is important because the early symptoms of milk fever often go undetected because they are short-lived. Data indicates that prevention of milk fever, rather than treatment, would save the dairy industry approximately $140 million per year (http://www.animate-dairy.com/dcad-nutrition/index.html).

The onset of milk production drains Ca pools in dairy cows.

Colostrum and milk synthesis rapidly depletes Ca from the maternal circulation and therefore Ca must be mobilized from maternal bone to maintain adequate circulating concentrations. Circulating Ca concentrations are tightly regulated and controlled by several hormones including: Vitamin D, calcitonin, parathyroid hormone (PTH) and parathyroid hormone related-protein (PTHrP; Figure 2). Liberation of Ca from bone stores can only be triggered when circulating Ca concentrations dip below

the animal's minimal threshold for Ca, via a classic negative feedback loop. Dietary Ca is insufficient to maintain maternal Ca homeostasis during milk synthesis. This is demonstrated by the fact that a dairy cow will lose 9-13% of her bone mass during the first 30 days of lactation. Bone loss during lactation is an evolutionary strategy of mammals used to support the cow as well as the mammary glands' demand for Ca for milk synthesis (Wysolmerski et al., 1995; Wysolmerksi, 2010; Goff, 2014).

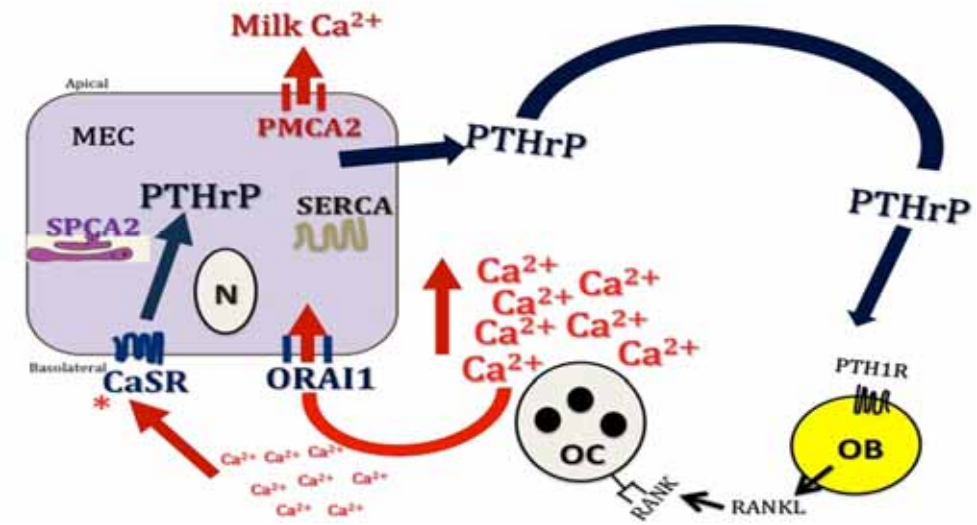

Figure 2. Maternal Ca homeostasis is regulated by the mammary gland-bone axis. During lactation, the Ca sensing receptor (CaSR) on the basolateral side of the mammary epithelial cell (MEC) during lactation detects low blood Ca concentrations due to the increased transport of Ca into the MEC by Ca release-activated Ca channel protein 1 (ORAI1). Ca is either secreted into the milk through the apical plasma membrane Ca ATPase 2 (PMCA2) or sequestered in the Golgi apparatus by secretory pathways Ca ATPase 2 (SPCA2) or endoplasmic reticulum by the sarco(endo)plasmic reticulum Ca ATPase (SERCA). Detection of systemic decreased Ca by CaSR results in parathyroid hormone related-protein (PTHrP) production. PTHrP is secreted into the circulation and will bind its receptor PTH1R on the osteoblast (OB) cell in the bone increasing production of receptor activated nuclear factor kappa B (RANKL), which binds its receptor (RANK) on the osteoclast (OC) cell in the bone tissue, activating Ca liberation from bone.

The mammary gland functions as an "accessory parathyroid gland" during lactation.

The mammary gland produces the hormone PTHrP, which binds to receptors on bone to drive bone resorption and liberate Ca into the systemic circulation (Wysolmerski et al., 1995; Wysolmerski, 2010). PTHrP is only produced by the mammary gland during lactation. The Ca sensing receptor (CaSR) present in the mammary epithelium plays a crucial role in controlling maternal Ca concentrations during lactation. CaSR is highly expressed in the mammary gland during lactation, compared to virgin and pregnant time periods (VanHouten et al., 2003). Mammary PTHrP production is

responsible for the mobilization of Ca from the bone during lactation, rather than the typical endocrine regulator of bone, PTH (Wysolmerski et al., 1995; VanHouten, 2005; Wysolmerski, 2010; Wysolmerski, 2012). Our lab made a novel discovery that serotonin is essential for the liberation of Ca from bone during lactation to sustain maternal Ca homeostasis in rodent models. This occurs through induction of PTHrP by the mammary gland (Hernandez et al., 2012; Laporta et al., 2014a, 2014b). Furthermore, we demonstrated that serotonin is critical for the expression of CaSR. This finding indicates that serotonin is crucial for mammary gland sensing of systemic Ca concentrations.

Mammary gland coordination with the skeletal system liberates Ca during lactation.

The skeletal system maintains its structural and functional roles via communication between two cell types, osteoblasts (OB), which are responsible for bone formation, and osteoclasts (OC), which are responsible for bone resorption, and thus Ca mobilization. PTH regulates this mechanism under non-lactating conditions. Research in humans and rodents has suggested the PTH action on bone is uncoupled during lactation (Wysolmerski, 2010; VanHouten and Wysolmerski, 2013). PTHrP signals through the same G-protein coupled receptor (PTH1R) that PTH does on the OB to decrease OB cell proliferation and up-regulate genes responsible for OC differentiation during lactation. In rodents and humans, the mammary gland is the main source of PTHrP found in the circulation (Thiede, 1994; Wysolmerski et al., 1995; Wysolmerski, 2010; VanHouten and Wysolmerski, 2013). Mammary-derived PTHrP, not PTH, is the critical hormone responsible for induction of bone Ca mobilization during lactation (Wysolmerski et al., 1995).

Serotonin regulates mammary gland physiology during lactation.

Serotonin is synthesized in numerous tissues throughout the body and brain and is incapable of crossing the blood-brain barrier. Serotonin is synthesized from the amino acid L-tryptophan in a two-step process. The first step is production of 5-hydroxytryptophan (5-HTP) via the rate-limiting enzyme, tryptophan hydroxylase (TPH). The second step is the conversion of 5-HTP to serotonin by aromatic amino acid decarboxylase (Wang et al., 2002). TPH1 is the rate-limiting enzyme for serotonin production in non-neuronal tissues, while TPH2 is used to produce serotonin in neuronal tissues. Our laboratory and others have shown that serotonin regulates milk protein gene expression, as well as the disassembly of tight junctions that occurs during the involution process (Matsuda et al., 2004; Stull et al., 2007; Hernandez et al., 2008; Pai and Horseman, 2008). Furthermore, we have shown that the mammary gland expresses a unique pattern of serotonin receptors in rodent, bovine, and human mammary epithelium (Hernandez et al., 2009; Pai et al., 2009). The epithelial component of the bovine mammary gland expresses at least five serotonin receptor isoforms (5-HT1B, 2A, 2B, 4 and 7; Hernandez et al., 2009). Our lab determined that the 5-HT2B receptor subtype modulates serotonin's regulation of PTHrP production within the mammary gland in a rodent model (Hernandez et al., 2012; Laporta et al., 2013a; Laporta et al., 2014a,b). We also confirmed that circulating serotonin

concentrations post-partum are positively correlated with circulating Ca concentrations on the first day of lactation in dairy cows (Laporta et al., 2013b). Furthermore, we showed that serotonin activates expression of various Ca pumps and transporters in the mammary gland to stimulate transport of Ca from blood to milk during mouse lactation (Laporta et al., 2014a). Ca transport into the mammary gland is thought to occur through the Ca^{2+} influx channel (ORAI1) and subsequent pumping into the milk by the apical plasma membrane Ca^{2+} ATPase (PMCA2; Cross et al., 2014).

Current research in humans and rodents implicates PTHrP in the regulation of maternal Ca homeostasis during lactation. Our laboratory has demonstrated the necessity of serotonin for regulation of Ca transport in the mammary gland during lactation. Furthermore, we have demonstrated that serotonin is necessary for the production of mammary PTHrP during lactation. Mammary PTHrP production is critical to the mobilization of Ca from bone tissue to support lactation. Therefore, delineation of the mechanisms regulating the mammary gland serotonin-PTHrP axis in the dairy cow could lead to development of novel therapeutic interventions to reduce the incidence of SCH and CH in the U.S. dairy cow population.

The following model for the regulation of Ca mobilization from bone by the mammary gland during the transition period has been proposed by our laboratory.

New ideas about calcium and serotonin

Our laboratory recently demonstrated that serotonin is necessary for mammary PTHrP synthesis in lactating rodents and mammary epithelial cells grown in lactogenic culture (Hernandez et al., 2012; Laporta et al., 2013a; Horseman and Hernandez, 2014). We also demonstrated that supplementation of a serotonin precursor, 5-HTP, to rats during the transition from pregnancy to lactation increased the post-parturition circulating serotonin, PTHrP, and Ca concentrations, and also increased total Ca content in milk (Laporta et al., 2013a). Furthermore, we observed increased OC numbers in the femurs collected from rats supplemented with 5-HTP, indicating this response was due to bone Ca mobilization. These findings led us to perform several experiments in dairy cows in order to evaluate the utility of these findings in rodents to dairy cows.

In order to evaluate the utility of the mammary serotonin-PTHrP axis in Holstein dairy cows, we performed several observational studies. We have observed that serotonin concentrations are dynamic over the course of a given lactation, and decrease around the time of calving (d 0-2 lactation), rebounding by approximately ten days into lactation (Moore et al., 2015). The overall average serotonin concentration in dairy cows is approximately 1700 ng/ml. However, it should be noted, that the concentrations fluctuate dependent on stage of lactation. These results combined with our rodent data support our hypothesis that serotonin and PTHrP are critical players in the regulation of Ca homeostasis in Holstein dairy cows.

Intravenous (IV) infusion of 5-HTP in late lactation, non-pregnant, multiparous Holstein dairy cows increases circulating serotonin concentrations and alters Ca dynamics.

In order to demonstrate the role of serotonin in Ca homeostasis in dairy cows, we performed a preliminary experiment in which we infused 5-HTP IV for one hour daily for four days in late-lactation dairy cows at varying doses (0, 0.5, 1.0, or 1.5 mg/kg) to determine an optimum dose of 5-HTP necessary to produce significant changes in Ca. All three doses of 5-HTP significantly increased circulating serotonin concentrations (Laporta et al., 2015) to a similar extent in the two hours after dosing, with concentrations returning to baseline concentrations observed in the saline controls by two hours after infusion. In addition to serotonin concentrations, we measured circulating total Ca concentrations following the same time course post infusion. While initially counter-intuitive, our data demonstrated that total Ca concentrations decreased in immediate response to 5-HTP treatments (Laporta et al., 2015). In order to determine where the circulating Ca was going after 5-HTP infusion, we measured urine Ca concentrations prior to the start of infusion and two hours after the end of the infusion. Our results indicate that there was a decrease in urine Ca output with higher doses of 5-HTP treatment. This suggests that Ca is not being lost into the urine. Therefore, we measured total Ca concentrations in the milk during the infusion periods and observed that the highest dose of 5-HTP increased total milk Ca concentrations. This supports the hypothesis that serotonin causes transient hypocalcemia by increased Ca transport into the mammary gland and subsequently into milk. Increased Ca transport into the mammary gland during lactation is critical for the stimulation of bone Ca mobilization by PTHrP because transient systemic hypocalcemia.

Use of 5-HTP before calving to prevent hypocalcemia, is it possible and are breed differences present?

In order to determine if elevating serotonin concentrations in pre-fresh dairy cows would result in increased post-calving Ca concentrations, we treated multiparous Holstein cows with daily IV infusions of 1.0 mg/kg of 5-HTP beginning 7 d before the estimated calving date until calving. Our data demonstrates that IV infusions of 5-HTP pre-calving increased post-calving total Ca concentrations compared to saline treated controls (Weaver et al., 2016). Furthermore, we measured deoxypyridinoline (DPD), a marker of OC activity and therefore bone resorption, in the urine. These data demonstrate that cows receiving 5-HTP before calving have increased bone resorption on at calving. These results support demonstrate that 5-HTP treatment pre-calving can potentially improve post-calving Ca concentrations by increasing bone Ca resorption. Furthermore, we also tested the same hypothesis in multiparous Jersey cows. Interestingly, Jersey cows responded to 5-HTP differently than the Holstein cows. Jersey cows had significantly decreased calcium concentrations prior to parturition, and then began to increase calcium concentrations at calving. This was in contrast to the control Jersey cows who did not reach their total calcium concentration nadir until 1 day post-partum (Weaver et al., 2016). Furthermore, Jersey cows treated with 5-HTP had higher concentrations of calcium in their milk compared to the saline treated cows, which was opposite to what was seen in the Holstein cows. These data indicate that

serotonin positively impacts calcium homeostasis in both Holstein and Jersey cows, but the mechanisms underlying this appear to be different and should be further investigated.

Interrelationship of a negative DCAD and serotonin.

Given that 5-HTP treatment pre- calving was capable of increasing post-calving Ca concentrations, we wanted to determine if a common preventative treatment for SCH and CH, negative DCAD, controls Ca homeostasis via a serotonergic mechanism. To this end, we fed Holstein dairy cows a positive DCAD (+130 mEq/kg) diet for 21 days prior to calving or a negative DCAD (-130 mEq/kg) diet for 21 days prior to calving. Upon analysis of circulating serotonin concentrations from 9 days before calving through 6 days post-calving, we determined that a negative DCAD diet increased circulating serotonin concentrations pre-calving ($P=0.05$). This suggests the resulting improvement in post-calving Ca concentrations in the cows receiving a negative DCAD diet pre-calving could be due to serotonin's control of Ca homeostasis. We have preliminary results from a study testing the hypothesis that 5-HTP and negative DCAD diets have a synergistic effect on post-calving calcium concentrations. Our preliminary results indicate that the combination of 5-HTP treatment with a negative DCAD diet has the largest increase in post-calving ionized calcium concentrations.

CONCLUSION

In conclusion, we have demonstrated that serotonin plays a critical role in regulation of maternal Ca transport, maternal Ca homeostasis and mammary PTHrP production in the rodent. Additionally, our data demonstrate that mammary gland Ca transporter expression and induction of PTHrP production by the mammary gland during lactation are key regulators of maternal Ca homeostasis in rodent models. Furthermore, our rodent models indicate that the mammary gland is a significant source of serotonin during lactation. Our observational data in Holstein cows suggests that serotonin, PTHrP, and Ca are interrelated during the early days post-partum. Furthermore, our initial experiment exploring the effects of 5-HTP on maternal Ca homeostasis in late-lactation dairy cows supports the hypothesis that serotonin induces transient hypocalcemia by shuttling Ca into the mammary gland in order to stimulate mammary production of PTHrP, and the elevated PTHrP is critical to stimulate bone Ca resorption. Treating pre-partum Holstein dairy cows with 5-HTP resulted in improvement of post-partum Ca concentrations. It also appears that Jersey cows respond differently to 5-HTP treatment and further research should be directed to understanding their physiology as compared to Holstein cows. Using a current therapeutic intervention for prevention of SCH and CH in the dairy industry, feeding of a negative DCAD diet pre-partum, resulted in the increase of circulating serotonin concentrations. Our preliminary data examining the interaction of 5-HTP and negative DCAD suggests that two treatments together have a synergistic effect on increasing post-calving ionized calcium concentrations.

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