Increasing serotonin concentrations alter calcium and energy metabolism in dairy cows

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Abstract

A 4×4 Latin square design in which varied doses (0, 0.5, 1.0, and 1.5 mg/kg) of 5-hydroxy-L-tryptophan (5-HTP, a serotonin precursor) were intravenously infused into late-lactation, non-pregnant Holstein dairy cows was used to determine the effects of serotonin on calcium and energy metabolism. Infusion periods lasted 4 days, with a 5-day washout between periods. Cows were infused at a constant rate for 1 h each day. Blood was collected pre- and 5, 10, 30, 60, 90, and 120 min post-infusion, urine was collected pre- and post-infusion, and milk was collected daily. All of the 5-HTP doses increased systemic serotonin as compared to the 0 mg/kg dose, and the 1.0 and 1.5 mg/kg doses increased circulating glucose and non-esterified fatty acids (NEFA) and decreased beta-hydroxybutyrate (βHBA) concentrations. Treatment of cows with either 1.0 or 1.5 mg/kg 5-HTP doses decreased urine calcium elimination, and the 1.5 mg/kg dose increased milk calcium concentrations. No differences were detected in the heart rates, respiration rates, or body temperatures of the cows; however, manure scores and defecation frequency were affected. Indeed, cows that received 5-HTP defecated more, and the consistency of their manure was softer. Treatment of late-lactation dairy cows with 5-HTP improved energy metabolism, decreased loss of calcium into urine, and increased calcium secretion into milk. Further research should target the effects of increasing serotonin during the transition period to determine any benefits for post-parturient calcium and glucose metabolism.

Key Words

- ▶ serotonin
- ▶ NEFA
- ▶ insulin
- ▶ glucose
- ▶ BHBA
- ▶ calcium
- ▶ PTHrP

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Introduction

Serotonin is an evolutionary conserved monoamine that is biochemically derived from the essential amino acid L-tryptophan. Tryptophan hydroxylase (TPH) converts L-tryptophan into 5-hydroxy-L-tryptophan (5-HTP), which is then converted into serotonin by aromatic

amino acid decarboxylase. The expression of two independent enzymes, TPH1 and TPH2 (Cote *et al.* 2003), results in two independent serotonergic systems: a neuronal system (CNS, wherein TPH2 is the rate-limiting enzyme) and a non-neuronal system (peripheral, wherein

TPH1 is the rate-limiting enzyme). Serotonin controls a wide range of biological functions. Serotonin's role as a neurotransmitter in the CNS and its participation in a variety of behavioral functions has been largely documented (Lucki 1998). However, the significance of non-neuronal serotonin is not thoroughly appreciated, despite numerous studies having been conducted on serotonin in a variety of tissues and physiological states (Berger *et al.* 2009, Amireault *et al.* 2013). Only a small percentage ($\sim 5\%$) of the body's total serotonin is found in the CNS, with the majority ($\sim 95\%$) being stored in the platelets and being derived from variety of tissues outside of the CNS (Berger *et al.* 2009).

Serotonin is synthesized by the mammary gland in several species (Matsuda *et al.* 2004, Hernandez *et al.* 2009), and it regulates several aspects of mammary gland function, including milk protein gene expression, the induction of parathyroid hormone-related protein (PTHrP) production, and mammary gland Ca pumps and transport (Matsuda *et al.* 2004, Hernandez *et al.* 2012, Laporta *et al.* 2013a, 2014a). The concept that the mammary gland acts as an endocrine organ that secretes PTHrP into the circulation as a signal to target tissues to regulate Ca supply to the milk during lactation is well accepted in rodents and humans (Wysolmerski 2010). However, these regulatory pathways have not been thoroughly explored in dairy cows. Serotonin is directly involved in bone metabolism via the decreasing of bone formation and the stimulation of bone resorption (Ducy 2011). In fact, serotonin-deficient male mice exhibit reduced bone resorption (Chabbi-Achengli *et al.* 2012). Therefore, it is possible that serotonin impacts bone directly and indirectly by stimulating PTHrP synthesis by the mammary gland during lactation to induce bone resorption (Horseman & Hernandez 2014).

Several lines of evidence suggest a role for serotonin in the regulation of energy balance (Tecott 2007), mainly through the modulation of glucose and lipid metabolism. The liver expresses TPH1 and several serotonin receptor subtypes (Papadimas *et al.* 2012). Serotonin is thought to mediate hepatic regeneration (Lesurtel *et al.* 2006) as well as glucose and insulin secretion (Sugimoto *et al.* 1990). Studies have shown that serotonin is involved in liver glucose uptake mechanisms (Moore *et al.* 2005) and glycogen metabolism (Papadimas *et al.* 2012). Additionally, serotonin has been shown to be increased in mice that are subjected to fasting conditions, which results in the stimulation of lipolysis and liver gluconeogenesis via the serotonin receptor 2b subtype in adipocytes and hepatocytes (Sumara *et al.* 2012). Furthermore, mice that were injected with

increasing doses of serotonin responded by proportionally increasing free fatty acids (Sumara *et al.* 2012).

The role of serotonin during lactation has been primarily explored in rodents, and there is limited information in dairy cows. We hypothesized that increasing systemic serotonin concentrations in lactating dairy cows would improve Ca and energy metabolism. In the present study, we therefore performed a 5-HTP dosing experiment to explore the effects of increasing serotonin on Ca and energy metabolism in late-lactation Holstein cows.

Materials and methods

Animals and experimental design

All of the experiments were performed under protocols approved by the Animal Care and Use Committee at the University of Wisconsin-Madison. Four ($n=4$) non-pregnant late-lactation (333 ± 7 days in milk; average lactation parity 3; average milk yield 21 ± 2.7 kg) Holstein dairy cows were utilized for the present experiment. Cows were enrolled in the experiment 2 weeks before the initiation of sample collection to allow for acclimation and were housed in a tie-stall barn. Cows were fed a standard lactating cow diet and given a mill grain mix (MK1283B, VitaPlus, Lake Mills, WI, USA).

The experimental design was a 4×4 Latin square. Four 5-HTP doses were used in the infusions: 0 mg/kg (saline, CON), 0.5 mg/kg (0.5 dose), 1 mg/kg (1.0 dose), and 1.5 mg/kg (1.5 dose) per body weight of the cows. Doses were i.v. infused with jugular catheters at a constant rate for 1 h for 4 days. The experiment included a 2-week acclimation period, a 5-day baseline period before the initiation of treatments, and four treatment periods (of 4 days each), with a 5-day washout period between treatment periods. The experimental design and timeline are summarized in Fig. 1. During the baseline period, blood, urine, and milk samples were collected, and milk production was recorded daily for 5 days. During each infusion period (I–IV), milk samples were collected and milk production was recorded daily, urine was collected pre- and post-infusion, and blood samples were collected pre-infusion (at 0 min) and post-infusion (at 5, 10, 30, 60, 90, and 120 min). Heart rate (HR), respiration rate (RR), and rectal temperature (RT) were recorded once pre-infusion, every 15 min during infusion, and 15 min post-infusion to monitor cow health. Manure (defecation) frequency (MF) and manure score (MS, 0=normal to 4=diarrhea) were recorded during infusion. Liver tissue biopsies were collected at the beginning of the experiment

Figure 1

Schematic representation of the experimental design. Four doses (0, 0.5, 1.0, and 1.5 mg/kg) of 5-hydroxy-L-tryptophan (5-HTP) were infused intravenously (at a constant rate for 1 h) for 4 days (1–4) into four Holstein dairy cows in a 4 × 4 Latin square design. There were a total of four infusion periods (with a 5-day washout period in between each infusion period). Only period I is shown here as an example. Before the initiation of period I, blood, urine, and milk samples were collected daily for 5 days (baseline). Milk samples were collected daily (morning milking), urine samples were

collected pre- and post-infusion, and blood samples were collected pre-infusion (at 0 min) and post-infusion (at 5, 10, 30, 60, 90, and 120 min). Animal health parameters (heart rate, respiration rate, and rectal temperature) were recorded pre-infusion, every 15 min during infusion, and 15 min post-infusion. Manure frequency and score were recorded during infusion. Liver tissue was harvested at the beginning of the baseline period and on day 2 during each of the four infusion periods. Blood, urine, and milk samples were collected daily during the washout periods as well.

for all of the cows (baseline) and on day 2 (after completion of the infusion) in each period. Blood, urine, and milk samples were collected daily in the morning and during the washout periods to monitor possible carryover effects of the applied doses between periods.

Sample collection

Before the initiation of each period, catheters (Abbocath-T Subclavian i.v. 14 g × 5 1/2' Catheter, Hospira, Lake Forest, IL, USA) were inserted into the jugular vein and sutured to skin that had been previously clipped and scrubbed. Catheters were flushed with sterile saline containing 10 IU of heparin every 8 h. Whole blood samples were collected from the jugular catheter during infusion periods or from the coccygeal vein during washout periods. To harvest the serum and plasma fractions, 10 ml BD Vacutainer Serum Plus (367820, BD, Franklin Lakes, NJ, USA) and Lithium Heparin 158 USP Units Plus Blood Collection Tubes (367880, BD) were used respectively. Samples were centrifuged at 3000 g for 20 min at 4 °C, and the serum and plasma fractions were collected and stored at –80 °C until analysis.

Urine samples were obtained by gentle massage of the area between the udder attachment and the lower portion of the vulva. Mid-stream urine samples were collected in sterile propylene tubes that were stored at –20 °C until analysis. Milk samples were collected during the morning milking in a sterile tube and kept at –20 °C until analysis. Percutaneous liver biopsy samples were collected. Cows were given an i.v. sedative and analgesic cocktail (0.02 mg/kg butorphanol, Fort Dodge Laboratories (Fort Dodge, IA, USA), and 0.02 mg/kg xylazine, AnaSed (Shenandoah, IA, USA)), and

the surgical site was then prepared with betadine surgical scrub (Veterinary, Purdue Products, Stamford, CT, USA) and 70% ethanol. Local anesthetic (10 ml of 5% lidocaine, Sparhawk Laboratories, Mission, KS, USA) was administered at the biopsy site. Liver tissue was snap-frozen in liquid nitrogen and stored at –80 °C until analysis.

Serum and plasma laboratory analysis

For all of the assays performed in the laboratory, samples were randomized and a quality control (QC) was analyzed on each plate. Blood serum serotonin was measured with a Serotonin Enzyme Immunoassay (EIA) Kit, (IM1749, Immunotech, Beckman Coulter, Marseille Cedex 9, France). Samples were diluted 1:100 in order to fall within the range of the standard curve of the assay. The intra- and inter-assay coefficient of variations (CV) were 2.3 and 7.3% respectively. Serum and urine total Ca concentrations were measured with a colorimetric Ca assay kit (no. 700550, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions; the intra-assay CV was <9.7%, and the inter-assay CV was <13% for both assays. Total milk Ca was measured by atomic absorption spectrophotometry (Perkin-Elmer Model 2280, Perkin-Elmer Corp., Norwalk, CT, USA) procedures as described previously (Rortvedt & Crenshaw 2012, Laporta *et al.* 2014a) with the addition of a hydrochloric acid digestion step. The intra-assay CV was <2.3%.

Plasma glucose concentrations were measured with a glucose oxidase-peroxidase assay specific for glucose (Karkalas 1985). The intra- and inter-assay CV were 6.7 and 7.5% respectively. Plasma insulin was measured in

duplicate by RIA as described previously (Vicari *et al.* 2008), and the intra- and inter-assay CV were both <4%. Serum non-esterified fatty acid (NEFA) concentrations were measured with an enzymatic colorimetric assay (NEFA-HR (2), Wako Chemicals, Richmond, VA, USA). The intra- and inter-assay CV were 3.9 and 6.4% respectively. Plasma concentrations of beta-hydroxybutyrate (βHBA) were measured enzymatically with kit no. RB1007 from Randox Laboratories Ltd (Ibach, Switzerland). The intra- and inter-assay CV were 2.8 and 4% respectively. Plasma PTHrP was measured with a PTHrP Immunoradiometric Assay Kit (DSL8100, Immunotech, Beckman Coulter, Prague, Czech Republic) per the manufacturer's instructions, and the inter-assay CV was 15%.

Hepatic gene expression

Total RNA was isolated from liver tissue using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. Total RNA concentration and absorbance ratios were quantified by a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE, USA). One microgram of total RNA was reversed transcribed with iScript Reverse Transcription Supremix for RT-qPCR Kit (no. 1708841, BioRad, Hercules, CA, USA) and diluted (1:5) in deionized water. Quantitative PCR was conducted with the CFX96 Touch Real-Time PCR Detection System (BioRad) using SSoFast EvaGreen Supremix (no. 1725203, BioRad) as described previously (Laporta *et al.* 2013a). We evaluated the hepatic expression of genes associated with glucose, fatty acid, and ketone metabolism, including: 3-hydroxy-3-methylglutaryl-CoA synthase

(HMG-CoA), pyruvate carboxylase (PC), pyruvate dehydrogenase kinase, isozyme 4 (PDK4), glucose-6-phosphatase (G6P), carnitine palmitoyltransferase 1 (CPT1), acyl-CoA dehydrogenase, very long chain (ACADVL), peroxisome proliferator-activated receptor α (PPARα), and acyl-CoA oxidase 1, palmitoyl (ACOX1). The average of β-actin, ribosomal protein 9 (RSP9), and ribosomal protein 15 (RSP15) were used as the housekeeping (internal control) gene. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method, with the baseline day serving as the control (Livak & Schmittgen 2001). The primer sequences used are presented in Table 1.

Protein isolation and analysis

Protein was isolated from the liver tissue using radio-immunoprecipitation assay buffer (RIPA) plus 10 μl/ml of Halt Protease and Phosphatase Inhibitors Cocktail (no. 78441, Thermo Scientific, Grand Island, NY, USA). Protein concentrations were determined by a Bicinchoninic Acid Assay (no. 23227, Pierce Chemicals, Grand Island, NY, USA). Liver serotonin concentrations were determined with a Serotonin EIA Kit (IM1749, Immunotech, Beckman Coulter) using 50 μg protein per sample analyzed as reported previously (Matsuda *et al.* 2004, Laporta *et al.* 2013b, 2014a).

Statistical analysis

The experimental design was a 4 × 4 Latin square. The final model that was used to analyze the variables with R Software (R Development Core Team 2014) was:

$$y = \mu + C + R + TR + D + T + TR \times D + TR \times T + \text{error}$$

Table 1 Primer sequences for the studied genes quantified by real-time PCR. All of the primers were designed using Primer 3 (Rozen & Skaletsky 2000) and were run at an annealing temperature of 60 °C. The average of β-actin, ribosomal protein 9 (RSP9), and ribosomal protein 15 (RSP15) were used as the housekeeping (internal control) gene.

Genes	Forward primer 5'-3'	Reverse primer 5'-3'
ACADVL	CCAGCCCCTGTGGAAATACTA	GCCCCGTTACTGATCCAA
ACOX	CCATTGCCGTCCGATACAGT	GTTTATATTGCTGGGTTTGATAATCCA
CPT1	GAGACAGACACCATCCAGCA	TCGTGGTAGAGCCAGACCTT
G6P	TGATGGACCAAGAAAGATCCAGGG	TAGGGATTGACCTCACTGGCCCTCTT
HMG-CoA	TCTGGCCCATCACTCTGCC	AGTGGGGAGCCTGGAGAAGC
PC	CCACGAGTTCTCCAACACCT	TTCTCTCCAGCTCCTCGTA
PDK4	AAAGCCAGAGGACCAAAAG	GCATCCGGGTAGAAATACGA
PPARα	CGGTGTCCACGCATGTGA	TCAGCCGAATCGTTCTCTCTAAA
β-ACTIN	CACCTTCACCGTTCAGTTT	ACTTGCGCAGAAAACGAGAT
RSP9	GGAGACCCTTCGAGAAAGTCC	CTTCTCATCCAGCGTCAGC
RSP15	CGCGACATGATCATTCTACC	TTACTTGAGGGGGATGAAGC

ACADVL, acyl-CoA dehydrogenase; very long chain, ACOX1, acyl-CoA oxidase 1; CPT1, carnitine palmitoyltransferase 1; G6P, glucose-6-phosphatase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA synthase; PC, pyruvate carboxylase; PDK4, pyruvate dehydrogenase kinase, isozyme 4; PPARα, peroxisome proliferator-activated receptor α.

where μ was the mean, C was the effect of the column (period 1–4), R was the effect of the row (cow), TR was the effect of the dose (categorical), D was the effect of the day of infusion (1–4, categorical), and T was the effect of time (0–120 min, categorical). All of the effects were considered fixed. For circulating Ca, PTHrP, glucose, NEFA, β HBA, and insulin, the area under the curve (AUC, according to the trapezoidal method) was calculated by the same model without the time (0–120 min) effect. Normality and outlier tests were performed for all of the variables analyzed. Transformations of serotonin (square root transformation) and Ca (natural log transformation) variables were required because of the lack of normality of the data. For all of the analyses, differences between means were considered significant at $P < 0.05$. All values are reported as LS means $\pm$ S.E.M.

Results

Animal health and milk production are not affected by 5-HTP infusion

Cow vital signs were evaluated as a measurement of cow health status during 5-HTP infusions. All of the vital signs analyzed, including HR, RR, and rectal RT, were not different among the cows that received any dose of 5-HTP as compared to the CON ($P > 0.05$; Fig. 2A, B, and C).

Additionally, milk production was measured to ensure that 5-HTP infusion had no negative effects on milk yield. Milk production was not different between cows that received any dose of 5-HTP as compared to the CON ($P = 0.15$; data not shown). Manure score was increased with increased 5-HTP dose ($P = 0.01$). The MS was increased for the 1.0 and 1.5 doses as compared to the CON (0.44 for CON vs 1.69 and $2.06 \pm .33$ for the 1.0 and 1.5 doses respectively). For the CON cows, the MF was always 0, and only cows that were infused with 5-HTP (at all doses) had an MF > 0 (1–4); the MF increased in a dose-dependent manner (Fig. 2D). Defecation frequency was increased with increased 5-HTP dose, and it was also increased with more days of treatment ($P < 0.04$). All of the cows that received 5-HTP doses defecated more frequently than the CON (Fig. 2D). Defecation frequency peaked on day 3 of 5-HTP infusion.

Circulating serotonin concentrations are increased by 5-HTP infusion

Serotonin AUC tended to be affected by 5-HTP dose ($P = 0.07$). The AUC was increased in response to the 1.5 dose as compared to the CON ($P = 0.06$; data not shown). Serotonin concentrations were affected by 5-HTP dose and days of infusion ($P < 0.0002$; Fig. 2E and F). Serotonin was elevated by all of the 5-HTP doses as compared to the CON

Figure 2

Animal health parameters and serum serotonin concentrations in response to 5-HTP infusion. (A) Heart rate (beats/min), (B) respiration rate (inhalations/min), (C) rectal temperature ($^{\circ}$ Fahrenheit, $^{\circ}$ F), (D) manure score (0=normal to 4=diarrhea) were monitored pre- (0), every 15 min (15, 30, and 45) during 1 h of continuous i.v. infusion of four doses of 5-HTP (0, 0.5, 1.0, or 1.5 mg/kg), and 15 min post-infusion (+15) into late-lactation,

non-pregnant Holstein dairy cows ($n = 4$). Defecation frequency was monitored only during 5-HTP infusion (D). (E and F) Circulating serotonin concentrations in response to (E) treatment (5-HTP dose) (E) and day of infusion (1–4) (F). (G) Hepatic concentrations of serotonin by 5-HTP dose. Different letters indicate statistical differences between group means (Tukey's $P < 0.05$). Data are presented as LS means $\pm$ S.E.M.

($P < 0.002$), and all of the doses of 5-HTP resulted in similar circulating serotonin concentrations ($P > 0.05$; Fig. 2E). Serotonin concentrations were increased on days 2, 3, and 4 as compared to day 1 of infusion ($P < 0.04$; Fig. 2F). Hepatic serotonin content was increased by the 0.5 dose ($P = 0.01$) and tended to be elevated in response to the 1.0 dose ($P = 0.09$) as compared to the CON (Fig. 2G). No statistical differences between baseline serotonin concentrations and the washout periods were observed ($P > 0.05$).

Circulating glucose concentrations are increased by 5-HTP infusion

Glucose AUC was affected by 5-HTP dose ($P = 0.02$). The AUC was increased for the 1.0 dose as compared to the 0.5 dose (data not shown). Glucose concentrations were also affected by 5-HTP dose ($P < 0.0001$; Fig. 3A) and tended to be increased with time after infusion ($P = 0.10$). Overall glucose concentrations were increased by the 1.0 dose

as compared to the 0.5 dose and the CON ($P < 0.001$) and by the 1.5 dose as compared to the 0.5 dose ($P = 0.002$; Fig. 3A). Glucose concentrations tended to be increased at 120 min as compared to 30 min after infusion ($P = 0.07$). Glucose concentrations were increased in the 1.0 dose as compared to the CON at 90 min post-infusion ($P = 0.05$), and the 1.0 and 1.5 doses tended to be increased as compared to the CON at 120 min post-infusion ($P < 0.09$). Glucose concentrations were not different across the 4 days of infusion, but there was a significant dose by day interaction ($P = 0.006$). Briefly, on day 1, glucose levels increased with the highest doses of 5-HTP as compared to the CON; however, this pattern was maintained only for the 1.0 dose on days 2 and 4 (Fig. 3B). No statistical differences between the baseline glucose concentrations and the washout periods were observed (F -test, $P > 0.05$).

Circulating insulin concentrations are decreased by 5-HTP infusion

The AUC for plasma insulin was decreased in response to all of the 5-HTP doses as compared to the CON ($P < 0.005$). Circulating insulin concentrations were affected by 5-HTP dose, by time after infusion ($P < 0.0001$; Fig. 4A and B), and by dose-by-time and dose-by-day interactions ($P < 0.0005$; Fig. 4C and D). All of the 5-HTP doses decreased insulin concentrations as compared to the CON ($P < 0.0001$; Fig. 4A). Insulin concentrations decreased markedly after the first 5 min post-infusion for all of the 5-HTP doses as compared to the CON (Fig. 4B) and remained lower than the CON but began to increase at 90 and 120 min post-infusion as compared to 5, 10, and 30 min post-infusion. The decrease in insulin concentration was less dramatic for the 0.5 dose as compared to the other 5-HTP doses, particularly on days 2 and 4 (Fig. 4C). No statistical differences between the baseline insulin concentrations and the washout periods were observed ($P > 0.05$).

Circulating NEFA concentrations are increased by 5-HTP infusion

The AUC for NEFA was increased in response to all of the doses of 5-HTP treatment ($P = 0.0004$). The AUC for NEFA was greatest for the 1.0 and 1.5 doses as compared to the CON ($P < 0.001$; data not shown). Circulating NEFA concentrations were affected by 5-HTP dose, by time after infusion, and by day of infusion ($P < 0.004$; Fig. 5A, B, and C). There were significant dose-by-day ($P = 0.000$; Fig. 5D) and dose-by-time interactions ($P = 0.01$; Fig. 5E). Overall, all 5-HTP treatments increased NEFA concentrations as

Figure 3

Circulating glucose levels in response to the i.v. infusion of four 5-hydroxy-L-tryptophan (5-HTP) doses (0, 0.5, 1.0, and 1.5 mg/kg) for 4 consecutive days into late-lactation, non-pregnant Holstein dairy cows ($n = 4$). (A) Main effect of treatment (5-HTP dose). (B) Interaction between day of infusion and 5-HTP dose. Different letters indicates statistical differences between group means (Tukey's $P < 0.05$). Data are presented as means $\pm$ S.E.M.

Figure 4

Circulating insulin concentrations in response to the i.v. infusion of four 5-hydroxy-L-tryptophan (5-HTP) doses (0, 0.5, 1.0, and 1.5 mg/kg) for 4 consecutive days into late-lactation, non-pregnant Holstein dairy cows ($n=4$). (A) Main effect of treatment (5-HTP dose) and (B) time after 5-HTP infusion (0–120 min). (C) Interaction between 5-HTP dose and day of infusion, and (D) interaction between 5-HTP dose and min after infusion. Different letters indicates statistical differences between group means (Tukey's $P < 0.05$). Data are presented as LS means $\pm$ S.E.M.

compared to the CON ($P < 0.001$), and the 1.0 and 1.5 doses had higher NEFA concentrations as compared to the 0.5 dose ($P < 0.003$). This effect was primarily caused by the increase in NEFA concentrations over time after 5-HTP infusion. Circulating NEFA concentrations were elevated at 30 min as compared to 0 min and to 120 min ($P < 0.005$). On days 3 and 4 of the 5-HTP infusion, NEFA concentrations were higher than they were on day 1 ($P < 0.003$), and this effect was primarily a result of the greater concentration of NEFA in the higher 5-HTP doses. No statistical differences between the baseline NEFA concentrations and the washout periods were observed ($P > 0.05$).

Circulating β HBA concentrations are decreased by 5-HTP infusion

The AUC for β HBA was decreased in the CON as compared to the 1.0 dose ($P = 0.02$) and tended to be decreased on

day 3 as compared to day 1 of the infusion ($P = 0.06$; data not shown). Concentrations of β HBA were affected by 5-HTP dose, time after infusion, and day of infusion ($P < 0.0001$; Fig. 6A, B, and C). All of the 5-HTP doses decreased β HBA concentrations as compared to the CON ($P < 0.008$), and the 1.0 and 1.5 doses decreased β HBA concentrations more robustly than the 0.5 dose did ($P < 0.04$; Fig. 6A). Concentrations of β HBA decreased markedly the first 30 min after the 5-HTP infusion ($P < 0.001$) and then recovered to concentrations that were similar to the pre-infusion values (0 min) at 90 and 120 min after treatment (Fig. 6B). On days 2, 3 and 4 of 5-HTP infusion, β HBA concentrations were higher than they were on day 1 ($P < 0.03$; Fig. 6C). No statistical differences between baseline β HBA concentrations and the washout periods were observed ($P > 0.05$).

Circulating Ca concentrations are decreased by 5-HTP infusion

The AUC for Ca was decreased by all of the 5-HTP doses as compared to the CON ($P < 0.0001$; data not shown). Circulating Ca concentrations were decreased by 5-HTP dose, time of infusion, and day of infusion, and there was a significant interaction between dose and time ($P < 0.0001$; Fig. 7A, B, and C). All of the 5-HTP doses decreased Ca concentrations as compared to the CON ($P < 0.0001$; Fig. 7A). The largest decrease in Ca concentration occurred on day 2 of infusion as compared to days 1, 3, and 4 ($P < 0.0002$; Fig. 7B). Calcium concentrations decreased markedly at 30 min post-infusion (as compared to 0, 5, and 10 min; $P < 0.0001$) for all of the 5-HTP doses, and they continued to decrease at 60 and 90 min post-infusion. Only the 0.5 dose subsequently increased and reached initial (0 min) Ca concentrations at 120 min post-infusion (Fig. 7C). No statistical differences between the baseline Ca concentrations and the washout periods were observed ($P > 0.05$). Notably, cows on the present study had elevated Ca concentrations as a whole before the infusion of 5-HTP.

Circulating PTHrP concentrations are decreased by 5-HTP infusion

The AUC for PTHrP was lowest in the 1.5 dose as compared to the CON and 0.5 dose ($P < 0.02$; data not shown). Circulating PTHrP concentrations were decreased by 5-HTP dose ($P < 0.0001$; Fig. 7G) and time of infusion ($P = 0.014$; Fig. 7H). The two highest 5-HTP doses decreased PTHrP concentrations as compared to both the

Figure 5

Circulating non-esterified fatty acid (NEFA) concentrations in response to the i.v. infusion of four 5-hydroxy-L-tryptophan (5-HTP) doses (0, 0.5, 1.0, and 1.5 mg/kg) for 4 consecutive days into late-lactation, non-pregnant Holstein dairy cows ($n=4$). (A) Main effect of treatment (5-HTP dose), (B) time after 5-HTP infusion (0–120 min), and (C) days of 5-HTP infusion

(1–4). (D) Interaction between day of infusion and 5-HTP dose, and (E) interaction between 5-HTP dose and min after infusion. Different letters indicates statistical differences between group means (Tukey's $P < 0.05$). Data are presented as LS means $\pm$ S.E.M.

0.5 dose and the CON ($P < 0.03$; Fig. 7G). Circulating concentrations of PTHrP decreased rapidly during the first 30 min of 5-HTP infusion as compared to the pre-infusion sample ($P < 0.053$) and then began to recover (60, 90, and 120 min after infusion; Fig. 7H). No statistical differences between the baseline PTHrP concentrations and the washout periods were observed ($P > 0.05$).

Infusion of 5-HTP alters Ca secretion in the urine and milk

The percentage change between urine Ca (uCa) concentrations before (at 0 min, pre-infusion) and after (at 120 min post-infusion) 5-HTP or saline infusion was calculated as: $((\text{uCa } 120 \text{ min} - \text{uCa } 0 \text{ min}) / (\text{uCa } 120 \text{ min})) \times 100$. The percentage change in urine Ca loss was decreased by 5-HTP dose ($P = 0.04$). Cows that received the highest doses of 5-HTP (1.0 and 1.5 mg/kg) had less uCa than the CON did ($P < 0.04$; Fig. 7D). Total Ca present in the milk was increased in the cows that received the 1.5 dose as compared to the CON ($P = 0.04$; Fig. 7E). Total milk phosphorous was increased in cows that received the 0.5 and 1.0 doses as compared to the CON ($P < 0.06$; Fig. 7F).

5-HTP treatment alters hepatic gene expression

The hepatic expression of genes associated with glucose, fatty acid, and ketone synthesis were affected by the 5-HTP treatment. The mRNA expression of *PDK4* tended to increase in cows that received the 1.5 dose as compared to the CON ($P = 0.07$), and *G6P* tended to be increased by the 0.5 and 1.0 dose as compared to the CON ($P < 0.10$; Fig. 8A and B). The mRNA expression of *HMG-CoA* was increased in cows that received the highest 5-HTP doses (1.0 and 1.5 mg/kg) as compared to the CON ($P < 0.03$; Fig. 8C). The mRNA expression of both *PPAR α* and *CPT1* tended to be increased in the 1.5 dose as compared to the CON and the baseline ($P < 0.08$; Fig. 8D and E). The *CPT1* mRNA expression was increased by 5-HTP dose as compared to the CON and the baseline expression ($P < 0.02$; Fig. 8E). The expression of *ACADVL* tended to be increased by the 1.5 dose as compared to the baseline expression ($P = 0.07$; Fig. 8F).

Discussion

Our previous data in Holstein dairy cows has implicated serotonin as an indicator of positive Ca and glucose status in lactating dairy cows (Laporta *et al.* 2013c). Those

Figure 6 Circulating beta-hydroxybutyrate (βHBA) concentrations in response to the i.v. infusion of four 5-hydroxy-L-tryptophan (5-HTP) doses (0, 0.5, 1.0, and 1.5 mg/kg) for 4 consecutive days into late-lactation, non-pregnant Holstein dairy cows ($n=4$). (A) Main effect of treatment (5-HTP dose), (B) time after 5-HTP infusion (0–120 min), and (C) days of 5-HTP infusion (1–4). Different letters indicates statistical differences between group means (Tukey's $P<0.05$). Data are presented as LS means $\pm$ S.E.M.

observations prompted the hypothesis that increasing systemic serotonin in lactating cows may improve energy and Ca metabolism. Metabolites in the serotonergic pathway have been previously administered in several species to characterize various physiological responses. Particularly in bovines, serotonin was administered to

calves to characterize their cardiovascular and pulmonary responses (Linden *et al.* 1993, 1996), and L-tryptophan was administered to steers to evaluate the effects on growth hormone secretion in cattle (Kasuya *et al.* 2010). However, to our knowledge, the present study is the first in which 5-HTP was administered to lactating dairy cows. We showed for the first time that increased systemic serotonin, through the administration of its immediate precursor 5-HTP, is safe, and it alters acute energy and Ca metabolism in lactating dairy cows.

Animal health was monitored by the evaluation of cow heart rate, RR, and RT in response to 5-HTP administration, and the findings demonstrated no significant impacts on animal health or behavior at any dose as compared to cows that received saline infusions. Additionally, we did not observe any changes in milk production as a result of any of the 5-HTP doses. Because more than 90% of the available serotonin in the peripheral portion of the body is produced in the gastrointestinal tract and because serotonin is known to increase gastrointestinal transit (Gershon & Tack 2007), we monitored defecation frequency and consistency in response to 5-HTP administration. As expected, manure frequency and score increased in a dose-dependent manner; however, we observed no outward signs of dehydration. It is important to note, though, that we did not evaluate hematocrit in these cows. Therefore, we conclude that administering 5-HTP to cows did not impact overall animal health and well-being.

In addition to determining the safety of 5-HTP administration, we examined the effects of various doses of 5-HTP treatment on Ca and energy metabolism as well as circulating serotonin concentrations. Importantly, infusing varying doses of 5-HTP for 4 days successfully increased systemic serotonin concentrations approximately twofold (740 ± 56 ng/ml vs 1560 ± 140 ng/ml for the CON and average of serotonin doses respectively). Several lines of evidence suggest a role for serotonin in the regulation of energy balance, specifically in relation to glucose and fatty acid metabolism. Serotonin is known to induce hepatic regeneration (Lesurtel *et al.* 2006), glucose secretion from the liver, and insulin secretion by the pancreas (Sugimoto *et al.* 1990, Watanabe *et al.* 2011) as well as liver glucose uptake mechanisms (Moore *et al.* 2004, 2005) and glycogen metabolism (Papadimas *et al.* 2012). Furthermore, serotonin has been shown to regulate glucose-stimulated insulin secretion by the β cells of the pancreas during pregnancy, and it is critical for glucose-stimulated insulin secretion in an insulin-resistant state (Ohara-Imaizumi *et al.* 2013, Kim *et al.* 2015). Additionally, mice that were injected with increasing doses of

Figure 7

Serum calcium and plasma parathyroid hormone-related protein (PTHrP) concentrations in response to the i.v. infusion of four 5-hydroxy-L-tryptophan (5-HTP) doses (0, 0.5, 1.0, and 1.5 mg/kg) for 4 consecutive days into late-lactation, non-pregnant Holstein dairy cows ($n=4$). (A) Main effect of treatment (5-HTP dose), (B) days of 5-HTP infusion (1–4), and (C) interaction between 5-HTP dose and min after infusion for calcium

concentrations. (D) Percentage change of urine calcium (uCa) calculated as: $((\text{uCa } 120 \text{ min} - \text{uCa } 0 \text{ min}) / (\text{uCa } 120 \text{ min})) \times 100$ by 5-HTP dose. (E and F) Milk calcium (Ca) and phosphorus (P) concentrations by 5-HTP dose. (G) Main effect of 5-HTP dose and (H) time after infusion for PTHrP concentrations. Different letters indicates statistical differences between group means (Tukey's $P < .05$). Data are presented as LS means $\pm$ S.E.M.

serotonin were shown to have increased circulating leptin (Yamada *et al.* 1989) and free fatty acid concentrations (Sumara *et al.* 2012), which thus links serotonin with the regulation of fatty acid and energy metabolism.

At present, little is known about the involvement of serotonin in glucose and energy homeostasis during lactation. In the present study, elevated circulating serotonin concentrations resulted in increased circulating glucose concentrations and decreased β HBA concentrations, which indicates improved energy balance. Typically,

in instances of glucose deficiency, β HBA concentrations in the circulation are elevated (Bell & Bauman 1997) and can result in ketosis, which negatively impacts cow health and immunity (Zarrin *et al.* 2014). This is of particular importance during the early lactation period, at which time dairy cows are in a negative energy balance (Duffield *et al.* 2009). It is likely that the rapid decrease in insulin concentrations in response to the 5-HTP treatment was responsible for the decrease in β HBA that was also observed in the present study. High insulin concentrations have

Figure 8

Hepatic mRNA expression of genes associated with glucose (pyruvate dehydrogenase kinase, isozyme 4, *PDK4* (A); glucose-6-phosphatase, *G6P* (B)), ketone (3-hydroxy-3-methylglutaryl-CoA, *HMG-CoA* (C)), and fatty acid (peroxisome proliferator-activated receptor α , *PPAR* α (D); carnitine palmitoyltransferase 1, *CPT1* (E); acyl-CoA dehydrogenase, very long chain, *ACADVL* (F)) synthesis after the i.v. infusion of four doses (0, 0.5, 1.0, and 1.5 mg/kg) of 5-hydroxy-L-tryptophan (5-HTP) for 4 consecutive days into late-lactation, non-pregnant Holstein dairy cows ($n=4$). Liver tissue was harvested before the initiation of the experiment (baseline) and after 2 days of 5-HTP infusion. Different letters indicates statistical differences between group means (Tukey's $P<0.10$). Data are presented as LS means $\pm$ S.E.M.

been previously reported to suppress ketogenesis (Hayirli *et al.* 2002, Li *et al.* 2013). Interestingly, in an experiment conducted in wethers that were fasted for 24 h, i.v. treatment with serotonin, not 5-HTP, increased both glucose and insulin concentrations for 1 h post-treatment (Watanabe *et al.* 2014). This indicates that serotonin may act differently depending on the stage of the energy balance of the animal. Additionally, in the present study, we observed increased circulating NEFA concentrations; however, the NEFA concentrations were below the threshold that is considered to be unhealthy for a lactating dairy cow (Grummer 1993, LeBlanc 2010). The increase in NEFA could possibly improve milk fat synthesis without placing the lactating animal in a severe negative energy balance; however, this should be more thoroughly explored. I.v. administration of serotonin to wethers also increased NEFA concentrations 1 h post-treatment, but concentrations then returned to baseline (Watanabe *et al.*

2014). This suggests that even though we administered a serotonin precursor and not serotonin itself in the present study, the effects on NEFA concentrations would not have been long-lasting either way. The hepatic expression of key enzymes involved in gluconeogenesis (*G6P* and *PDK4*) and fatty acid metabolism (*PPAR* α , *ACADVL*, and *CPT1*) accompanied the observed increase in circulating glucose and NEFA circulations in response to 5-HTP. It is plausible that the increase in NEFAs in the circulation may be attributed to increased adipose tissue mobilization; however, in the present study, we did not directly test the effects of 5-HTP treatment on adipose tissue. The marked decrease observed in insulin and β HBA concentrations after 5-HTP administration might trigger the hepatic mRNA expression of *HMG-CoA* as part of a negative feedback loop; however, this requires further exploration. Overall, 5-HTP treatment appears to alter energy metabolism in late-lactation dairy cows.

Infusion of all doses of 5-HTP acutely decreased circulating Ca and PTHrP concentrations, while also decreasing Ca excretion in urine and increasing Ca secretion into milk. This implies that 5-HTP treatment shifts Ca from maternal circulation to other tissues, for example, the mammary gland, seeing as it is not being excreted in the urine. Our previous work in mice demonstrated that serotonin is responsible for increasing Ca pumps in the mammary gland and secretion into milk (Laporta *et al.* 2013a, 2014a). The decrease in circulating Ca concentrations observed in the present study is in contrast to previous work in rodents where dietary 5-HTP supplementation increased circulating Ca and PTHrP concentrations (Laporta *et al.* 2013a). One plausible explanation for this is that in the present experiment, blood samples were collected during the 2 h post-infusion period, whereas in the previous rodent experiments, blood samples for measuring Ca and PTHrP in response to 5-HTP administration were collected after several days and up to 2 weeks after treatment (Laporta *et al.* 2013a, 2014b). This suggests that physiological responses to increased serotonin concentrations are different depending on the time point that is evaluated post-treatment. We have previously shown that PTHrP reached a maximal concentration in the circulation in response to 5-HTP in mice 3 h after treatment (Hernandez *et al.* 2012). Moreover, serotonin has been shown to cause physiological changes to tight junctions in the mammary epithelium in a biphasic manner in the mammary gland (Pai & Horseman 2008). Therefore, the blood collection interval should be extended beyond the 2 h post-infusion period to establish if serotonin acts in a biphasic manner in the bovine.

Classically, Ca is regulated in a negative feedback manner, which means that decreased circulating Ca concentrations are necessary for inducing Ca mobilization from bone, and this process is necessary for supporting maternal Ca homeostasis during lactation (Kovacs 2005, Kovacs & Fuleihan 2006). It is the decrease in circulating Ca concentrations that allows for PTHrP production in the mammary gland. This mechanism is supported by rodent experiments that have indicated that the trigger for PTHrP production in the mammary gland is the decrease in Ca concentrations that occur as a result of milk synthesis, as detected by the Ca sensing receptor located on the basolateral side of the mammary gland epithelium (Wysolmerski 2010, Kovacs 2011). It is also possible that the observed decrease in Ca may also contribute to changes in energy metabolism. Previous research has indicated that hypocalcemia can depress insulin concentrations in the circulation (Martinez *et al.* 2014). Therefore, we hypothesized that serotonin may increase Ca transport from the blood into the mammary gland and milk in order to initiate PTHrP production by the mammary gland. This hypothesis must be further studied in the bovine. Further research should focus on the examination of PTHrP and Ca concentrations at time points beyond the initial 2 h post-treatment as well as the specific effects of serotonin on Ca transport mechanisms within the mammary gland, and it should investigate whether 5-HTP treatment affects Ca homeostasis differentially during various stages of lactation. Finally, the interaction between serotonin's effect on Ca and glucose homeostasis during lactation should be further explored in cows.

In summary, the present data demonstrate that the administration of 5-HTP to lactating dairy cows was safe and increased systemic serotonin concentrations over time. Increased serotonin concentrations in turn impacted glucose, ketone, and fatty acid metabolism and increased Ca transport from the circulation into milk. We propose that serotonin is a key regulator of Ca and glucose metabolism and homeostasis in lactating dairy cows and suggest that the lactation stage specific physiological mechanisms should be further explored. It is possible that understanding serotonin's regulation of maternal metabolism during lactation will be important for the health and productivity of the dairy cow.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J L and L L H designed, wrote, and analyzed the experiment. S A E M, S R W, C M C, B P S, A P P, and M O helped carry out the experiments. F P performed statistical analysis. F P, T D C, and R M B aided with the experimental design and analysis and contributed to the writing of the manuscript.

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