Mammary core clock gene expression is impacted by photoperiod exposure during the dry period in goats

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ABSTRACT

Short-day photoperiod (SDPP) during the dry period increases milk production compared to long-day photoperiod (LDPP) in goats. Photoperiod information is sent to the master clock in the suprachiasmatic nuclei (SCN), which send temporal information to peripheral clocks located in every tissue of the body. We hypothesized photoperiod effects on milk production are mediated in part by changes in mammary clocks. Our objective was to determine the effect of photoperiod manipulation during the dry period in goats on core clock genes expression in mammary gland. Multiparous goats (n = 6) were blocked at dry off into two treatments: LDPP and SDPP. Serial mammary biopsies were taken over a 24 h period during three weeks prepartum. Total RNA was isolated, and q-PCR analysis of the core clock genes CLOCK, ARNTL, PER1, CRY1, and CRY2 found exposure to LDPP significantly increased ARNTL (P < .05) expression relative to SDPP exposure. Plasma prolactin levels were significantly increased (P < .05) after seven weeks of photoperiod treatment, but not at three weeks prepartum. The mechanism and significance of photoperiod-induced changes in mammary clock also need to be elucidated but may be related to seasonal changes in lactation.

Introduction

Lactation is regulated by homeorhetic factors that orchestrate physiological changes across the entire body around the time of birth to provide energy and nutrients to the mammary gland to support milk synthesis (Bauman and Currie 1980). Homeorhetic processes ensure animals maintain homeostasis while responding to both physiological and environmental challenges, and can affect growth rate, tissue differentiation, and metabolism (Bauman and Currie 1980; Flowers et al. 2013). In response to seasonal changes in photoperiod, the circadian timing system regulates coordinated physiological changes that impact an animal’s growth, energy balance, and reproductive capacity (Lincoln G. A. and Richardson 1998; Clarke et al. 2003; Andersson et al. 2005; Dardente H. 2007; Ebling and Barrett 2008). Growing evidence also supports a role for circadian clocks in regulating the physiological changes, including mammary specific changes, that occur during the periparturient period to prepare for lactation (Casey, 2014, p. 12070). Whereas, disruption of the circadian system negatively impacts lactation competency in rodents and cattle (Kennaway et al. 2004; Turek 2005; Williams and Schwartz 2005; Hoshino et al. 2006; Koch et al. 2010; Pan et al. 2013; Casey T et al. 2014).

The function of the circadian system is to temporally coordinate internal physiology and synchronize the organism’s physiology with the environment by generating circadian rhythms (Froy 2010). The central clock in the suprachiasmatic nuclei (SCN) acts as a master clock by integrating internal and external timing cues to coordinate circadian rhythms of physiology across the body. The immediate outflow of SCN information is to the medial hypothalamus, and here the SCN signal is translated into hormonal and autonomic signals that are received by peripheral clocks located in every tissue of the body (Kalsbeek A., Bruinstroop, et al. 2010; Kalsbeek A., Fliers, et al. 2010; Kalsbeek Andries et al. 2011). In turn, peripheral clocks drive the circadian expression of local transcriptomes and metabolomes. At the molecular level, clocks generate circadian rhythms of gene expression through a transcription–translation feedback loop. CLOCK (or its ortholog NPAS2) and ARNTL are at the core of this loop and form a transcription factor as a heterodimer. ARNTL–CLOCK heterodimers drive transcription by binding to E-box regulatory elements in promoter regions of genes (Hardin 2004). Period (PER1, PER2, PER3) and Cryochrome (CRY1, CRY2) genes are transcriptional targets of ARNTL–CLOCK, and once translated PER–CRY heterodimers function as negative regulators of ARNTL–CLOCK mediated transcription. This complex transcription–translation feedback loop has a 24 h periodicity that results in circadian rhythms of gene expression.

Temporal information received by the SCN includes both photic (light) and non-photic cues, with light-dark information being the most important environmental cue for entraining...
the SCN (Reppert and Weaver 2002). Changes in day length, or photoperiod, provide a reliable and predictive indicator of seasonal change. Many mammals exhibit annual rhythms in reproduction and energy balance that are regulated, in part, by changes in a hormonal milieu which are stimulated by changes in photoperiod (Dardente Hugues et al. 2010). For example, photoperiodic information is received by the retina and transmitted to the SCN. The SCN communicates to the pineal gland and drives both circadian and seasonal rhythms of melatonin release. In turn changes in 24 h melatonin profile affect prolactin secretion, with long-day stimulating and short days inhibiting prolactin release (Lincoln Gerald A. et al. 2006). Photoperiod changes also affect peripheral tissues. Comparison of sheep exposed to short-day versus long-day photoperiod revealed significant differences in hepatic ARNTL rhythm amplitude and mRNA abundance with the phase of PER2 expression shifted (Andersson et al. 2005). Photoperiodic treatment of cattle-altered liver mRNA levels of metabolic enzymes known to show circadian patterns, including acetyl-CoA carboxylase, phosphoenolpyruvate carboxykinase, and fatty acid synthase (Connor et al. 2007).

Milk yield increases in dairy cattle exposed to long-day photoperiod (LDPP) during lactation without altering feed intake (Peters et al. 1978). Both dairy cows and goats exposed to short-day photoperiod (SDPP) versus LDPP during the dry period produce more milk in the subsequent lactation (Mabjeesh et al. 2007; Dahl 2008). The dry period, or the non-lactating period between successive lactations of pregnant animals, evolved as a dairy management tool to maximize milk production in the ensuing lactation (Capuco and Akers 1999) and is believed to be needed for factors specific to the mammary gland rather than nutrition (Swanson 1965). In dry cows and goats, a peak in epithelial cell proliferation occurs approximately three weeks before expected parturition (Capuco et al. 1997; Mabjeesh et al. 2007). Photoperiod effects on lactation are due in part to changes in mammary cell proliferation during the dry period (Dahl et al. 2002; Auchtung et al. 2003; Auchtung et al. 2005) and have been hypothesized to be mediated by light exposure induced changes in prolactin and perhaps IGF-1 levels. However, support for this hypothesis is not straightforward (Wall, Auchtung, et al. 2005; Wall, Auchtung-Montgomery, et al. 2005).

We recently reported that germ-line mutations in the CLOCK gene negatively affected mammary development in mice. Further, we found that decreased abundance of CLOCK protein in a mouse mammary epithelial cell line increased growth rates and negatively impacted differentiation of cells in culture (Koch et al. 2010). Together suggesting that the mammary clock is integral to regulating mammary development and metabolic activity, and changes in levels of its components may effectively change mammary development and lactation capacity. We hypothesize that the circadian system is a key regulator of homeorhetic and mammary-specific adaptations to lactation that occurs during the dry and periparturient periods. We envision that similar to the impact of photoperiod on the hepatic clock, changes in photoperiod exposure affect the mammary clock, and in turn are capable of affecting mammary development and lactation competence. The objective of this study was to test the first part of this hypothesis, i.e. determine the effect of photoperiod manipulation during the dry period in goats on core clock gene expression in the mammary gland.

Materials and methods

Animals

Animal experimental protocols were reviewed and approved by the ethics institutional committee (AG-14203). Multiparous Israeli Saanen goats (n = 6) were blocked at dry off (~45 d prepartum) into two treatments: LDPP (n = 3) and SDPP (n = 3) based on body weight and previous milk production. Beginning at dry off LDPP-treated animals were exposed to 16 h light: 8 h dark, and SDPP animals were exposed to 8 h light: 16 h dark. All goats were housed in metabolism chambers equipped with two separate but identical environmentally controlled rooms at a normothermic ambient temperature (22°C, 55% relative humidity), and photoperiod adjusted according to the treatment (photophase light intensity = 350 lux at eye level of the goats). Goats were fed a total mixed ration in two equal meals at 0800 and 1400 to meet nutritional demands. Goats were blood sampled weekly, serum was prepared, and stored at −80°C.

To determine the impact of photoperiod exposure on mammary clock serial, mammary biopsies were taken over a 24 h period from each goat during the expected third week prepartum at 4 h intervals (0900, 1300, 1700, 2100, 0100, and 0500 h). Before the biopsy was taken, local anaesthesia was administered and a skin incision was made (0.5–1.0 cm). The udder was palpated to determine the area to obtain parenchymal tissue, and the Tru Core™ II automatic biopsy instrument (Argon Medical Devices, USA) fitted with a 14-gauge needle was used to obtain 30–60 mg of parenchymal tissue per puncture. Two–three biopsies were taken to obtain ~120 mg of tissue, tissue was visually inspected for gross morphology indicative of parenchyma, rinsed with sterile saline, and snap frozen in liquid nitrogen. The skin was sutured closed. Occasionally, bleeding occurred at the biopsy site and controlled by applying pressure. When goats were first lactating blood clots were not apparent, however, milk, the first few days postpartum, was darker than typical colostrum. Any samples taken during the dark phase were done with night vision goggles and red light with <3 lux at goat eye level.

Prolactin ELISA

PRL was measured using a sandwich ELISA assay developed in our laboratory (Mabjeesh et al. 2007; Mabjeesh et al. 2013). Briefly, ELISA plates (Nunc, Thermo, Denmark) were coated with 1 µg/ml of monoclonal mouse anti-prolactin IgG1 (clone 6F11, Abnova, Taiwan) diluted in carbonate-bicarbonate buffer, pH 9.6 (Thermo, IL, USA) and incubated in a humidified chamber at 4°C overnight. Coated plates were then blocked using 0.5% skim milk (BD, Difco, Sparks, MD, USA) in phosphate-buffered saline (Thermo, IL, USA) at 37°C for 2 hours. Serum samples (diluted 1:10 in blocking solution) and a serially diluted recombinant Prolactin (Prospebio, Ness-ziona, Israel) used for standard curve were placed on plates and incubated
in a humidified chamber at 4°C overnight. Rabbit anti-ovine prolactin (Bio-Rad, Hercules, CA, USA) diluted 1:10,000 in blocking solution was then added, and plates were incubated in a humidified chamber at 37°C for 2 hours. Detection was performed using horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Jackson, PA, USA) diluted 1:10,000 in blocker at 37°C for 1 hour. Tetramethylbenzidine (TMB; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was used as a substrate. Optical absorbance was determined at 450 nm using a Bio Tek microplate reader (Bio Tek, Winooski, VT, USA). Plasma prolactin concentrations were determined by comparing absorbance values to a standard curve.

Mammary explant culture

Mammary tissue was collected at the slaughterhouse from grade Holstein cows (n = 3) that were lactating. Explants (three experimental replicates; 3 dishes/treatment/time point/cow) were prepared from mammary tissue and were cultured in Waymouth's media (pH 7.3) at 37°C in 50% O2 and 5% CO2 in media supplemented with insulin (5 µg/ml) and hydrocortisone (0.1 µg/ml) with and without prolactin (1 µg/ml). Explants were collected every 6 h over a 36 h period beginning 12 h after initiating a culture. Total RNA was isolated from tissue and reverse transcribed for q-PCR analysis of ARNTL.

Q-PCR analysis

Total RNA was isolated and reverse transcribed into cDNA. RIN scores for RNA quality was at least 7 across all samples. The q-PCR analysis was performed with SYBR green, with primers designed to measure the expression of two reference genes (ACTB and GAPDH) and the core clock genes ARNTL, CLOCK, PER1, CRY1, and CRY2 (Table 1). Primer design was done using NCBI primer blast. ARNTL was measured using in bovine mammary explant tissue was done using TaqMan expression assays (ThermoFischer, Cat. No. 4351372) using 18S as a reference gene. Relative gene expression was calculated using the delta–delta CT method (Livak and Schmittgen 2001) with mean of SDPP treatment across all circadian time points as a normalizer, or mean of expression of insulin and hydrocortisone treatment across all time points as a normalizer for explant culture. Data are expressed as the mean of log base 2 of fold change ±standard error (SE).

Table 1. Primer sequences* for Sybr green q-PCR analysis of core circadian clock genes expression.

| Gene symbol | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| ARNTL       | GTGCAACCGGATGTCTAGCA | AAATCCATCTGCGCTTGA |
| CLOCK       | GCCCTAAGAACAGACAGAACT | TTGGCATTTCTGTGGTAG |
| CRY1        | AGACCCCTGAACTGAAACAA | ATCTGAAACATAAGCGTTT |
| CRY2        | GGCAGCTCTCTACCTAGAG | CTCTCTCTCCGCGCACAG |
| PER1        | GTACGGCCTCGGATCATG | GTCCCTGGCTGCTCCTC |
| GAPDH       | TCTACGATCATGACCAACATG | GCCCTCCATGGATGACGA |
| BETA ACTIN  | TGGACTTCGACGGAGATGG | CACCGTGTGGCATAAGGTC |

*Designed using NCBI blast.

Figure 1. The impact of photoperiod exposure during dry period on mammary core clock gene expression at three weeks before expected parturition. General linear model analysis found there was no effect of time on any gene measured. Treatment had a significant effect on ARNTL (P < .001) and tended to affect CRY1 (P = .1) expression. Goat was a significant factor (P ≤ .05) in ARNTL, CRY2, and PER1 expression. ZT: zeitgeber time, time lights were turned on. Values graphed are mean ±SE.
Results and discussion

Patterns of core clocks’ gene expression exhibited circadian patterns for each animal, however, there was no effect of time on any gene measured (Figure 1(A–E)). This was likely due to the variability of circadian phases of gene expression among the animals within treatments, as the time point that peak or trough occurred varied among all the animals and not by treatment. Photoperiod treatment had a significant effect on ARNTL (P < .001) expression (Figure 1(A)), with levels elevated in LDPP versus SDPP treated. LDPP also tended to increase CRY1 (P = .10) expression (Figure 1(C)). There was an effect of animal on expression of ARNTL, CRY2 and PER1.

Prolactin (PRL) is a potent lactogen and stimulates homeorhetic and mammary specific processes for adaptation to lactation. Circulating PRL levels are increased in ruminants exposed to LDPP (16 h of light and 8 h of dark) (Mabjeesh et al. 2007; Dahl et al. 2012; Mabjeesh et al. 2013), and this hormonal change is hypothesized to mediate the homeorhetic response to changes in photoperiod (Dahl 2008). Our previous studies using a mouse mammary epithelial cell line showed that PRL functions as an input to the mammary clock as treatment with PRL significantly increased ARNTL expression levels, and timing of application shifted phase of expression (Casey TM et al. 2014). Therefore, we hypothesized that LDPP increased PRL levels, which in turn affected BMAL1 levels in mammary tissue (Figure 2).

To test this hypothesis we measured PRL in plasma that was prepared from blood samples collected weekly from goats beginning at dry off. Prolactin concentration was significantly higher in the LDPP-treated versus SDPP-treated counterparts after seven weeks of exposure (Figure 1(E)). Concentrations were not different between photoperiod treatments at three weeks prepartum when biopsies were taken to measure mammary gene expression. Our previous studies (Mabjeesh et al. 2007; Mabjeesh et al. 2013), which were similarly designed, found photoperiod treatment increased PRL levels after two weeks of exposure (by five weeks prepartum).

We wondered if PRL effects on the mammary clock were distinct between mice and ruminants. To test this possibility, mammary tissue was collected at the slaughterhouse from cows that were lactating and cultured in media supplemented with insulin and hydrocortisone with and without prolactin treatments. ARNTL expression found to be similar to mouse mammary epithelial cells in culture with the continuous presence of hydrocortisone (Casey TM et al. 2014). The addition of PRL into culture significantly increased ARNTL (P < .05) mRNA levels (Figure 3). Thus, similar to mice, PRL appears to induce BMAL1 expression in ruminant mammary tissue. However, in our study differences in ARNTL levels at three

Figure 2. Plasma prolactin levels (ng/ml ± SE) beginning at seven weeks (W-7) before expected parturition through the week of expected parturition (W0). Student t-test analysis of plasma prolactin levels measured in each week prepartum found levels significantly (P < .05, *) greater in LD treated animals at week-1 (W-1) and week of expected parturition (W0).

Figure 3. Effect of prolactin treatment on ARNTL expression in bovine mammary explant cultures. Mammary tissue was collected from three cows (three different experiments) that were lactating at time of slaughter, and placed in Waymouth’s media supplemented with insulin (5 μg/ml) and hydrocortisone (0.1 μg/ml) and transported to lab on ice. Tissue was minced into 1 mm size pieces, divided into two treatments: insulin (Ins, 5 μg/ml) and hydrocortisone (Hyd, 0.03 μg/ml), grey line; and Ins, Hyd, and PRL (1 μg/ml). At each time point (6, 12, 18, 24, 36, 48 h) from start of culture 3 dishes of explants per treatment were collected and snap frozen in liquid nitrogen. Total RNA was isolated and q-PCR analysis of ARNTL and reference gene 18S was performed using TaqMan assays. Delta=delta CT method of relative expression was calculated using the mean of Ins and Hyd treatment across all time points as the normalizer. Two-way ANOVA found that treatment had a significant effect (P < .05) on ARNTL expression, but there was no effect of time. Values graphed are mean ±SE.
weeks prepartum between LDPP- and SDPP-treated goats occurred before a measurable rise in plasma PRL in long-day exposed animals. Therefore, a mechanism other than changes in prolactin maybe mediating photoperiod alterations in the mammary clock and will need to be identified in future studies.

Studies will also be needed to understand the significance of photoperiod alterations in the mammary clock. It is likely that alterations in mammary clock affect gland development as well as metabolic output, i.e. milk synthesis. The idea of circadian clocks driving mammary gland development is supported by photoperiod effects on mammary function, as exposure to SDPP (8 h light:16 h dark) versus LDPP during the dry period increases milk production in the subsequent lactation, in part, by increasing mammary cell proliferation (Mabjeesh et al. 2007; Dahl 2008). Further, genes that exhibited circadian oscillation in lactating mammary were found to be involved in regulation of cell development, growth, proliferation, and cell morphology (Maningat et al., 2009). Our studies demonstrated that reducing levels of CLOCK protein in mouse mammary epithelial cells using shRNA caused higher rates of growth compared to wild-type cultures (Koch et al. 2010). In vivo studies of CLOCK mutant mice found the Clock–Δ19 mutation had minimal effects on growth and development of pups during gestation, however, litter growth and survival was significantly decreased postnatal (Kennaway, et al. 2004; Dolatshad et al. 2006; Hoshino et al. 2006). Studies in our lab showed that alveolar differentiation in mammary glands from late pregnant Clock–Δ19 mice was impaired compared to wild type. Thus suggesting that circadian clocks affect lactation competency in part through regulation of mammary development (Koch et al. 2010).

Conclusion

To our knowledge, this is the first time that dry period photoperiod manipulations were shown to cause an alteration in mammary core clock genes. In particular, ARNTL levels were significantly increased in LDPP versus SDPP exposed goats. The mechanism and significance of photoperiod-induced changes in mammary clock still needed to be elucidated. Although, previous studies in our lab and others support a role for the mammary clock in regulation of gland development, and thus changes in these dynamics may be responsible for differences in mammary parenchyma content between animals exposed to different photoperiods.

Disclosure statement

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