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Effect of continuous milking and prostaglandin E_2 on milk production and mammary epithelial cell turnover, ultrastructure, and gene expression[1]

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ABSTRACT: Mammary epithelial cell (MEC) growth is reduced in continuously milked (CM) mammary glands, and administration of a mammogenic compound such as prostaglandin E_2 (PGE_2) at parturition might improve MEC growth in CM tissue. The objectives were to 1) compare MEC turnover, ultrastructure, and gene expression in CM and involuting mammary tissue, and 2) evaluate the effects of CM and intramammary infusion of PGE_2 on early lactation MEC turnover, ultrastructure, mammary gene expression, milk yield, and composition. First- and second-lactation cows (n = 8) were used in a half-udder model, in which one-half was dry for 60 d (CTL) and the other was CM. Udder halves (n = 16) were assigned to a postpartum (PP) treatment of PGE_2 (+PGE_2; 875 μg/10 mL of medium-chain triglyceride oil) or no PGE_2 (–PGE_2) treatment at parturition and at 72 h PP. Biopsies of CM and CTL quarters were obtained during milk stasis (MS) of the CTL half at 3 and 7 d after dry-off of the CTL half (3d-MS; 7d-MS) and postpartum (PP) at 2 and 4 d (2d-PP; 4d-PP). Milk yield was reduced ($P < 0.01$) in CM udder halves compared with CTL halves (13.2 vs. 22.1 kg/d), but reductions were less in second-lactation cows. The apoptotic index was greater ($P < 0.05$) in CTL glands than in CM glands (3d-MS, 0.52 vs. 0.11% and 7d-MS, 0.24 vs. 0.12, respectively). Proliferation of MEC was unchanged at 3d-MS, but was increased ($P = 0.01$) in CTL halves at 7d-MS compared with CM halves (3.10 vs. 0.93%). At 2d-PP, MEC proliferation was increased ($P = 0.05$) in CM halves compared with CTL halves (1.3 vs. 0.6%), but was unaffected by PGE_2 ($P > 0.2$). Apoptosis was elevated in early lactation regardless of treatment. Ultrastructure was unchanged by dry period length or PGE_2. In prepartum tissue, involution in CTL halves increased ($P < 0.05$) the expression of the proapoptotic genes Bcl-2-associated x protein (bax) and IGFBP5 and decreased ($P < 0.05$) α-lactalbumin expression compared with CM tissue. In PP mammary tissue, CTL halves expressed greater ($P < 0.05$) levels of ATP-binding cassette 1 (ABC1) and IGFBP5. Treatment with PGE_2 did not alter ($P > 0.1$) gene expression. The results confirm that CM reduced milk yield of cows with a mammary growth requirement. Reduced MEC turnover and milk yield were not alleviated by IMI of PGE_2, which indicates that peripartum PGE_2 concentrations in CM glands are not limiting mammary growth or milk synthesis.

Key words: apoptosis, dairy cow, dry period, mammary epithelial cell, proliferation, prostaglandin

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INTRODUCTION

Previous results indicate that short or omitted dry periods decrease milk yield in the subsequent lactation (Annen et al., 2004a). Negative effects of modified dry periods are believed to occur within the mammary gland (Smith et al., 1967; Swanson et al., 1967) and appear to be a result of reduced mammary epithelial cell (**MEC**) turnover (Capuco et al., 1997; Annen et al., 2007), rather than decreased MEC numbers (Swanson et al., 1967; Capuco et al., 1997). Reduced MEC proliferation and apoptosis in continuously milked (**CM**) glands has been interpreted as carryover of old MEC into the subsequent lactation rather than replacement with new MEC, as occurs in glands provided an adequate dry period (Capuco et al., 1997; Annen et al., 2007).

The use of mammogenic treatments in CM, primiparous cows may alleviate reductions in mammary growth. One such mammogen is PGE_2, present in MEC

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Table 1. Composition of the total mixed diet fed to lactating cows[1]

Ingredient, g/kg of DM	Amount
Alfalfa hay	578.2
Amino plus	32.6
Corn (steam flaked)	169.1
Dry citrus pulp	79.7
Energy II	15.6
Molasses (cane)	43.3
Supplement[2]	30.6
Whole cottonseed	50.8

[1]Diet was 48% DM and contained 19% CP, 1.74 Mcal of NE_l/kg of DM, 27.4% ADF, and 41.5% NDF based upon analysis of the total mixed diet.

[2]Contained 2.93×10^5 IU/kg vitamin A, 0.35×10^5 IU/kg vitamin D, 0.09×10^5 IU/kg vitamin E, 0.28% Cl, 0.78% K, 2.84% Mg, 11.91% Na, 5.54% P, 0.53% S, 60 mg/kg Co, 490 mg/kg Cu, 2,487 mg/kg Fe, 49 mg/kg I, 1,684 mg/kg Mn, 8 mg/kg Mo, 11 mg/kg Se, and 2,019 mg/kg Zn.

Table 2. Composition of the total mixed diet fed to close-up dry cows[1]

Ingredient, g/kg of DM	Amount
Alfalfa hay	496.6
Bermuda hay	115.1
Beet pulp	78.6
Citrus pulp (dry)	57.2
Corn (steam flaked)	198.9
Supplement[2]	30.5
Whole cottonseed	23.2

[1]Diet was 53% DM and contained 18.0% CP, 1.72 Mcal of NE_l/kg of DM, 29.7% ADF, and 44.0% NDF based upon analysis of the total mixed diet.

[2]Contained 4.08×10^5 IU/kg vitamin A, 0.44×10^5 IU/kg vitamin D, 0.07×10^5 IU/kg vitamin E, 21.20% Cl, 0.24% K, 2.62% Mg, 0.12% Na, 2.37% P, 2.63% S, 46 mg/kg Co, 449 mg/kg Cu, 817.00 mg/kg Fe, 49 mg/kg I, 1,269 mg/kg Mn, 4 mg/kg Mo, 9 mg/kg Se, and 1,322 mg/kg Zn.

(Bandyopadhyay et al., 1987; McGrath et al., 1990) and in mammary secretions [R. J. Collier, J. Byatt, M. McGrath, P. Eppard, and J. Vicini (Monsanto, St. Louis, MO); unpublished data] during the last week of gestation and first 2 to 3 d of lactation. Intramammary infusion (**IMI**) of PGE_2 increased mammogenesis in late gestation heifers (Collier et al., 2002) and increased milk yield in induced-lactation cows (Crooker et al., 2003).

The objectives of this study were to 1) compare MEC turnover, ultrastructure, and gene expression in CM and involuting mammary tissue, and 2) evaluate the effects of CM and intramammary infusion (IMI) of PGE_2 on early-lactation MEC turnover, ultrastructure, mammary gene expression, milk yield, and composition.

MATERIALS AND METHODS

Animals and Experimental Design

The University of Arizona Institutional Animal Care and Use Committee approved all animal procedures.

Cows were housed at the University of Arizona Agriculture Research Facilities in individual pens and had ad libitum access to feed and water. All diets used during the experiment were formulated to meet or exceed the NRC requirements for the given physiological state of the cow (Tables 1 and 2; NRC, 2001). Cows were fed an early lactation diet (Table 1) as long as one udder half was lactating. If the CM half underwent spontaneous dry-off, cows were fed a close-up, dry-cow diet until parturition (Table 2). Beginning at parturition, cows were fed the lactation diet for the remainder of the study. Cows were fed twice daily at 0530 and 1730 h, and orts were measured once daily at 0500 h. Lactating udder halves were milked twice daily at 0500 and 1700 h. Dry matter intake was measured daily, and milk yield was measured twice daily; samples for analysis of milk composition were obtained weekly. Colostrum was sampled at the first milking after parturition. Ration DM percentage was determined twice daily, and samples analyzed for nutrient composition were obtained weekly. For statistical analyses, daily DMI and milk yield values for each cow were combined into weekly means. Colostrum and weekly milk samples were obtained from successive a.m. and p.m. milkings, which were pooled and analyzed for fat, protein, and lactose percentage and for somatic cell count (**SCC**; Arizona Dairy Herd Improvement Association, Tempe). Milk fat, protein, and lactose were analyzed using AOAC-approved infrared analysis (Association of Official Analytical Chemists, 2007). Milk SCC was analyzed with AOAC-approved cell staining techniques (Association of Official Analytical Chemists, 2007).

The study design utilized 4 first-lactation and 4 second-lactation cows in a half-udder model so that the effects of both CM and PGE_2 could be examined with fewer animals. Cows selected for the study were healthy and had an SCC <500,000 cells/mL. Within each animal, udder halves were assigned randomly to prepartum treatment of CM or 60-d dry (**CTL**) and postpartum (**PP**) IMI treatment with (**+PGE_2**) or without (**–PGE_2**) PGE_2. Treatments were balanced to have equal numbers of first- and second-lactation udder halves. Additionally, PGE_2 treatment was balanced for equal numbers of CM and CTL udder halves. Within an animal, PGE_2 treatment was administered to either a CM udder half or a CTL udder half, but not to both halves.

Cows began the study at 67 d before their expected parturition and remained on the study until 28 d PP. Throughout the study, milk yields for the right and left udder halves were collected into separate buckets using a modified, individual quarter milking unit. At 60 d before expected parturition, CTL quarters were dried by cessation of milking, treated with long-acting intramammary antibiotic (Quartermaster, Pfizer, La Jolla, CA), and sealed with Orbeseal (Pfizer). The CM quarters were milked throughout gestation and into the next lactation unless milk yield dropped below 5 kg/d for 7 consecutive days, at which time the udder-half was

considered to have spontaneously dried. Milk samples were collected every day of the spontaneous dry-off period using the sampling and analysis procedures described above. If dried off, the CM quarters were treated with long-acting intramammary antibiotic, and milk removal ceased until parturition. Milking of both udder halves resumed at parturition and continued until 28 d PP. At parturition, the cow was allowed 1 h with her calf, with supervision, to ensure that the calf did not nurse. After removal of the calf, the teats were cleaned with an iodine-based teat dip (Bovadine, West Agro, Kansas City, MO), the teat-ends were disinfected with alcohol pads, and PGE_2 was administered to each treated quarter (1 udder half per animal) by IMI using a sterile, 12-mL syringe fitted with a sterile teat cannula. Upon completion, the teat was pinched closed as the cannula was removed and the infusion massaged into the gland. A second IMI of PGE_2 occurred immediately after the milking closest to 72 h PP. Each IMI contained 875 μg of PGE_2 (Sigma, St. Louis, MO) in 10 mL of medium-chain triglyceride (**MCT**) oil; Collier et al. (2002) demonstrated that this was the maximal mammogenic dose for late-pregnant heifers. An excipient dose of MCT oil was not given to the –PGE_2 halves because it has no effect on mammary growth or milk yield (Collier et al., 2002; Crooker et al., 2003).

Tissue Samples

Mammary biopsies (Annen et al., 2007) were collected prepartum at 3 and 7 d after milk stasis (**3d-MS, 7d-MS**) in the CTL udder half and at 2 and 4 d (**2d-PP, 4d-PP**) after parturition. Because udder half was the experimental unit, both udder halves were biopsied at each time point. Rear quarters were biopsied at 3d-MS and 2d-PP, and front quarters were biopsied at 7d-MS and 4d-PP. Biopsied quarters were treated with intramammary antibiotic (Amoxi-Mast, Pfizer) immediately after biopsy and after the subsequent 2 milkings. For prepartum biopsies, penicillin (6×10^6 IU; Agri-Cillin, AgriLabs, St. Joseph, MO) was administered i.m. for 4 d beginning on the day before biopsy. Systemic antibiotic treatment across the 2d-PP and 4d-PP biopsies was provided by administration of penicillin on d 1 to 6 PP with a 2 × dose administered at 4 d PP to increase the protection for cows that had calved and had all 4 quarters biopsied within a 4-d interval. Postsurgical health was monitored by rectal temperature twice daily for 4 d after a biopsy and by continuous evaluation for fluctuations in milk yield and DMI.

Immediately after the tissue was removed from the cow, the sample was divided for immunohistochemistry, electron microscopy, and gene expression analyses. Tissue for each analysis was always obtained from the same relative area of the tissue core obtained from the biopsy. Tissue for immunohistochemistry was fixed in 10% neutral buffered formalin for 24 h at 4°C and was transferred to 70% ethanol until further processing. This tissue was subsequently dehydrated in a series of ethanol concentrations, embedded in paraffin according to standard techniques, sectioned at 6-μm intervals, and attached to sialinated slides. Tissue for electron microscopy was fixed and processed as described by Annen et al. (2007). Tissue for gene expression analyses was placed in 1 mL of RNA*later* (Ambion Inc., Austin, TX), incubated at room temperature for 24 h, and stored at –80°C until further processing.

Immunohistochemistry

The number of proliferating MEC was quantified as the number of cells that expressed the Ki-67 nuclear proliferation antigen (**Ki67**). The MIB-I antibody (Zymed Laboratories Inc., San Francisco, CA) was used to localize the Ki67 antigen (Annen et al., 2007). The slides were counterstained with hematoxylin and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Terminal deoxynucleotidyl transferase, dUTP, nick-end labeling (**TUNEL**) of free 3′-OH DNA termini was used to visualize cells that exhibited endonucleolytic degradation of DNA, a fundamental feature of apoptotic cells. The TUNEL assay was performed with a commercial kit (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Chemicon International, Temecula, CA), as modified by Annen et al. (2007). The slides were counterstained with methyl green and mounted with Permount.

Tissue sections were viewed with a light microscope to quantify Ki67- and TUNEL-positive cells. Each slide contained 2 sections of a sample from an individual cow, and each section contributed equally to cell quantification. Ten microscopic fields (500× magnification) were evaluated for each sample, and cells within a 10 × 10 ocular grid were counted. The counted fields were spread across tissue sections. At least 1,800 cells were counted per sample per cow. To eliminate experimenter bias, the reader was blind to the sample identification, and the fields were selected with the microscope out of focus.

Transmission Electron Microscopy

Thin sections for transmission electron microscopy were cut with a diamond knife into approximately 0.08-μm sections and stained with uranyl acetate (20 min) and lead citrate (3 min). The sections were viewed with a Phillips 420 Transmission Electron Microscope (University of Arizona Electron Microscopy Core).

RNA Isolation and Pooling

The RNeasy Mini Kit (Qiagen, Valencia, CA) was used to isolate total cellular RNA. Once isolated, RNA samples were divided into 3 aliquots; 2 were immediately stored at –80°C and a third, smaller aliquot was used to determine RNA concentration and integrity. Concentration of RNA was determined from photospectrometry (260/280 ratio). Integrity of 28S and 18S RNA

bands was assessed by RNA 6000 Nano Chip (Agilent Technologies, Palo Alto, CA) on an Agilent 2100 bioanalyzer (Agilent Technologies). Samples with degraded RNA were reisolated.

Within-cow pools of RNA were prepared by mixing 1 μg of RNA from 3d-MS and 7d-MS samples for prepartum and 1 μg of RNA from 2d-PP and 4d-PP samples for postpartum pools. After pooling, each cow had a prepartum and postpartum RNA sample for each udder half.

Complementary DNA Synthesis and Quantitative PCR

After RNA was treated with deoxyribonuclease I (amplification grade, Invitrogen, Carlsbad, CA), complementary (c) DNA was synthesized with the Superscript III First Strand Synthesis System (Invitrogen) and cleaned (QIAquick PCR purification kit, Qiagen) as described by Annen et al. (2007). Cleaned cDNA was used for real-time quantititave PCR (**q-PCR**) assays of all samples for the 8 genes of interest [**GOI**; α-lactalbumin, adenosine 5′-triphosphate binding cassette 1(**ABC1**), bax, bcl_2, CCAT/enhancer binding protein- β (**CEBP-β**), Cyclin D1, IGFBP5, and kinase inhibitor protein p27 (**p27**)] and a housekeeping gene, 18S ribosomal RNA (**18S**) to correct for differences in RNA input. All primer sequences, the resulting products, and qPCR conditions have been validated (Annen et al., 2007). Primers were purchased from Integrated DNA Technologies Inc. (Coralville, IA). Reaction mixtures contained 10 μL of iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Hercules, CA), 4 pmol of each primer, sample or standard cDNA, and RNase/DNase-free water to a final volume of 20 μL. Master mix (SYBR green, primer, and water) was pipetted into the plate wells followed by addition of sample or standard curve cDNA. Plates were assayed on an ABI Prism 7000 (Applied Biosystems, Foster City, CA). All samples were assayed in duplicate, and all standard curve points were assayed in triplicate. Each 96-well plate contained a standard curve and a pooled cDNA sample for 18S, which were was used to calculate an intra- and interplate CV (0.60 and 0.53%, respectively). The remaining wells were used for the standard curve and samples for a GOI. Each plate contained a cDNA pool sample so that the intra- and interplate CV could be determined for each GOI. Inter- and intraplate CV were α-lactalbumin (0.61 and 0.75%), ABC1 (0.29 and 0.38%), bax (0.34 and 0.72%), bcl_2 (0.59 and 0.50%), CEBP-β (0.73 and 0.59%), Cyclin D1 (0.37 and 0.33%), IGFBP5 (0.43 and 0.63%), and p27 (0.32 and 0.45%).

Normalization of the cycle threshold (Ct) values resulted in ΔCt values ($\Delta Ct = Ct_{GOI} - Ct_{18S}$) that were used for statistical analyses. Relative changes in mRNA expression were calculated as $2^{-\Delta\Delta Ct}$ (User Bulletin No. 2; Applied Biosystems, 2001). Differential expression was declared if the ΔCt values differed ($P < 0.05$).

Statistical Analysis

All statistical analyses were performed using PROC MIXED (SAS Inst. Inc., Cary, NC). The level of significance was $P < 0.05$ for the main effects and interactions. The 305-d mature-equivalent milk yield for each cow before treatment assignment was used as a covariate for PP milk yield. Covariates for prepartum milk yield and composition were derived from milk data obtained before dry-off of the CTL udder half (i.e., during the first week of the study; d –67 to –61). Due to alterations in milk composition after mammary biopsies (i.e., blood in milk), milk was not sampled at wk –8 or +1. Milk somatic cell linear score (**SCS**) was calculated from SCC to achieve a normal distribution of the data for statistical comparisons. Statistical models for analysis of postpartum milk yield and composition, Ki67 index, and apoptotic index included the main effects of PGE_2 treatment, dry period length (CM or CTL), parity, and time (week relative to parturition, or day of biopsy relative to parturition), and their respective interactions. Time was fit as a repeated measure using a first-order autoregressive covariance structure. The variability among udder halves (nested within cow) within experimental cells was used to test the whole-plot effects of PGE_2, dry period length, and their interactions. The variability among data for dependent variables within udder half (nested within cow) was used to test for the effects of time and the interactions involving time. Statistical analysis of ΔCt values from the real-time qPCR assays included independent variables of dry period length, parity, and their respective interactions. Udder-half nested within cow and treatment was fit as a repeated measure using a first-order autoregressive covariance structure. For the postpartum samples, independent variables included previous dry period length, PGE_2 treatment, parity, and their respective interactions. Statistical analysis of milk yield and composition during spontaneous dry-off included time fit as a repeated measure. Thus, the analysis was used to determine changes over time not due to animal variation. Analysis of DMI included time, parity, and the interaction of parity by time as independent variables. Time was fit as a repeated measure using a first-order autoregressive covariance structure.

RESULTS

Dry Matter Intake

Dry matter intake remained constant ($P > 0.15$) from wk –8 to –1 of gestation, reached nadir at d 0 (parturition), and increased ($P < 0.05$) steadily during the first 4 wk PP (Figure 1). Dry matter intake is a whole-animal measurement for which effects of half-udder treatments of dry period length and PGE_2 infusion cannot be separated.

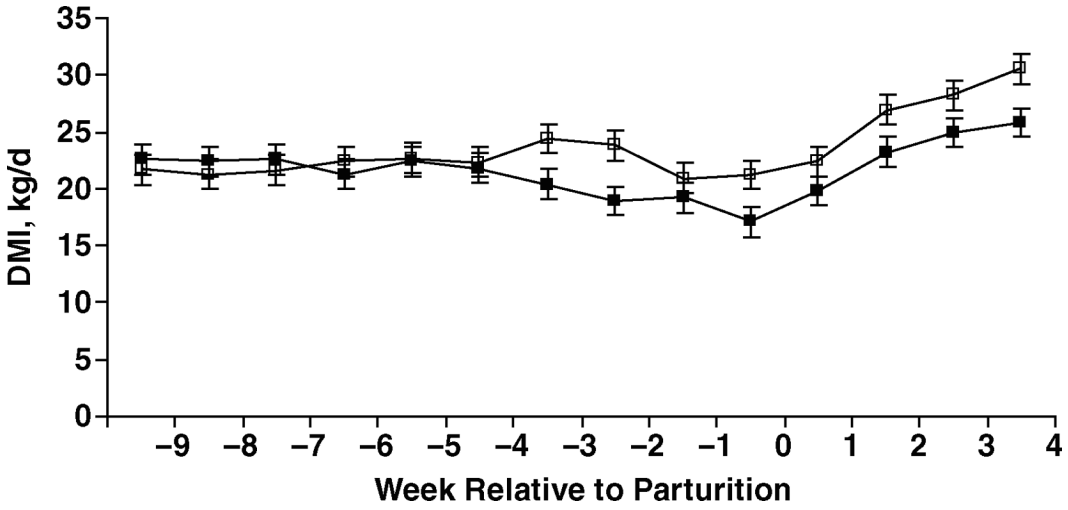

Figure 1. Dry matter intake of cows with one udder half continuously milked and one udder half dry during late gestation. Data presented are least squares means ± SEM (n = 8). Dry matter intake is a whole-animal measurement for which the effects of half-udder treatments, dry period length, and prostaglandin E_2 infusion cannot be separated. ■ = first-lactation cows; □ = second-lactation cows.

Milk Yield and Composition

Regardless of parity, milk yield from CM udder halves decreased ($P < 0.001$) throughout late gestation (Figure 2). First-lactation cows maintained numerically greater half-udder yields from wk −7 to −2, but by the final week of gestation, half-udder yields were extremely low regardless of parity. Average days dry was 9.3 d in CM first-lactation halves and 12.6 d in CM second-lactation halves, so that the low least squares mean for milk yield at −1 wk can be attributed to the fact that most CM halves (7 out of 8) had spontaneously dried and were producing 0 kg/d at this time

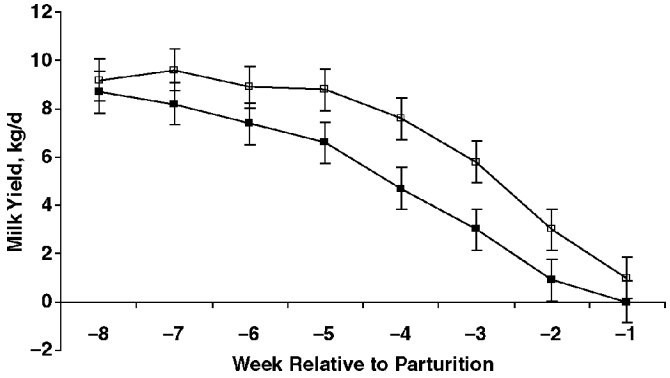

Figure 2. Milk yield of continuously milked (CM) udder halves from first- and second-lactation, late-gestation cows. Milk yields < 5 kg/d during the last 2 to 3 wk of gestation are due to zero milk yields in spontaneously dried udder halves. Data presented are least squares means ± SEM (n = 8). ■ = CM udder halves from first-lactation cows; □ = CM udder halves from second-lactation cows.

Milk yield from all udder halves increased ($P < 0.001$) throughout the first 4 wk PP (data not shown). Although temporal patterns were similar, CM halves produced less milk than CTL halves (Figure 3). This reduction ($P < 0.01$) in postpartum milk yield in CM udder halves compared with CTL halves occurred regardless of parity or PGE_2 treatment (Table 3). Treatment with PGE_2 had no effect on milk yield (Table 3). Although milk yield was reduced in all CM halves, reductions were less ($P < 0.02$) in second-lactation cows (33%) than in first-lactation cows (53%, Figure 3).

Milk composition for CM halves was measured throughout the last 7 wk of gestation and during spontaneous dry-off of CM halves. Milk fat and lactose content and SCS remained constant ($P > 0.13$) during late

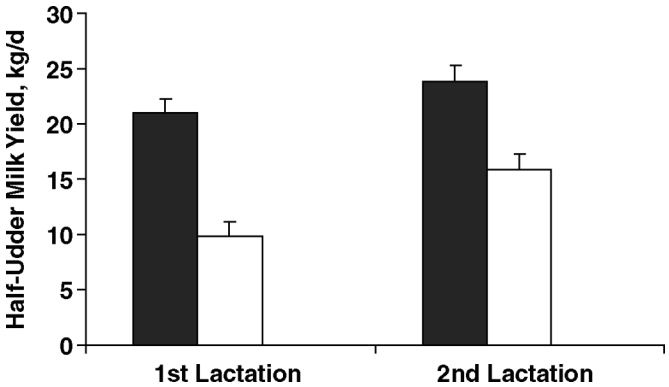

Figure 3. Postpartum average daily milk yield of control (60-d dry) and continuously milked udder-halves (n = 16) from first- and second-lactation cows. Data presented are least squares means ± SEM for milk production through 28 d postpartum. Solid bars = 60-d dry; open bars = continuously milked.

Table 3. Summary of dry period length and milk yield and composition during early lactation in 60-d dry (CTL) and continuously milked (CM) udder halves that were treated[1] (+PGE_2) or not treated (–PGE_2) with PGE_2

	–PGE_2		+PGE_2			*P*-value[2]		
Variable	CTL	CM	CTL	CM	SE	DP	PGE_2	DP × PGE_2
Actual dry period length, d	59.6	11.0	68.4	14.1	3.0	<0.01	NA	NA
Half-udder milk yield, kg/d	22.7	13.8	21.9	12.7	1.7	<0.001	NS[3]	NS
Milk composition								
Fat, %	4.1	3.9	3.9	3.6	0.14	NS	NS	NS
Protein, %	3.0	3.2	3.2	3.0	0.10	NS	NS	NS
Somatic cell linear score	0.96[a]	3.57[b]	3.07[ab]	2.41[ab]	0.73	NS	NS	0.05
Lactose, %	4.9	4.7	4.8	4.8	0.06	NS	NS	NS

[a,b]Within a treatment (row), differences over time are indicated by different superscripts ($P < 0.05$).

[1]PGE_2 intramammary infusions (875 μg) were administered at parturition and after the milking closest to 72 h postpartum. Only one udder half was treated per animal, and PGE_2 treatment was balanced for equal numbers of CM and CTL halves, as well as equal numbers of first and second lactation udder-halves. Milk yield data were collected daily through 28 d postpartum. Weekly milk composition data were collected for wk 2 through 4 postpartum. Each dry period treatment group contained 8 udder halves, 4 of which were PGE_2-treated and 4 of which were nontreated.

[2]–PGE_2 = no intramammary infusion of PGE_2; +PGE_2 = intramammary infusion of 875 μg of PGE_2; DP = dry period length; NA = not applicable.

[3]NS = no differences between means ($P > 0.10$).

gestation (data not shown). True protein content of milk increased ($P < 0.001$) during the final 2 wk of gestation compared with wk –7 to –3 (data not shown). Similar results were obtained during spontaneous dry-off, likely due to the fact that spontaneous dry-off occurs in the final 2 wk of gestation. Milk fat and lactose percentage were unchanged during spontaneous dry-off. True protein content tended ($P = 0.09$) to increase from d –6 to –1 and SCS increased ($P = 0.03$) by the last day of milk removal (d –1; data not shown).

Milk composition from wk 2 to 4 PP was not altered ($P > 0.05$) by the main effects, but the interaction of dry period length and PGE_2 treatment resulted in changes in SCS (Table 3). Milk from CM-PGE_2 halves had a greater ($P = 0.02$) SCS than milk from CTL-PGE_2 halves. There was no difference in SCS among CTL-PGE_2, CTL, and CM + PGE_2 halves or between CTL and CM + PGE_2 halves (Table 3). All milk composition variables, except lactose, decreased ($P \leq 0.01$) from wk 2 to 4 PP regardless of dry period or PGE_2 treatment (data not shown). Lactose remained constant ($P = 0.9$) during wk 2 to 4 PP. Administration of PGE_2 had not been initiated before the first milking, so only the effects of dry period length and animal variability (cow nested within udder half) on colostrum were evaluated (Table 4). Milk fat, protein, and lactose percentages and SCS were not altered ($P \geq 0.17$) by dry period treatment. Tendencies existed for increased ($P = 0.09$) milk fat percentage and decreased ($P = 0.1$) true protein percentages in colostrum from CM halves (Table 4).

A comparison of milk yield for 3 d before and 7 d after biopsies indicated yield was not adversely affected by the biopsy procedure (data not shown). These findings are similar to those reported by Annen et al. (2007) for milk yield after routine mammary biopsy.

Prepartum and Postpartum MEC Turnover and Mammary Ultrastructure

Prepartum proliferation of MEC was greater ($P < 0.01$) in CTL than CM glands (2.36 vs. 1.02%, SEM = 0.24). This difference was largely due to enhanced ($P = 0.009$) proliferation in CTL tissue at 7d-MS, because proliferation indices were similar at 3d-MS (Table 5). Prepartum MEC proliferation was not affected by any other independent variables or their respective interactions. In contrast, postpartum MEC proliferation was increased ($P = 0.04$) in CM tissue compared with CTL tissue (1.29 vs. 0.60%; SEM = 0.21) and MEC proliferation was greater ($P = 0.05$) at 2d-PP than 4d-PP (1.23 vs. 0.62%; SEM = 0.21) regardless of dry period length or PGE_2 treatment (Table 6). Treatment with PGE_2 did not alter ($P > 0.26$) MEC proliferation in CM or CTL tissue.

Prepartum MEC apoptosis was increased ($P < 0.001$) in involuting CTL tissue compared with lactating CM tissue, and MEC apoptosis was elevated ($P = 0.01$) at 3d-MS compared with 7d-MS in CTL tissue (Table 5).

Table 4. Summary of first milking colostrum[1] composition of 60-d dry (CTL) and continuously milked (CM) udder halves

Variable	CTL	CM	SE	*P*-value[2]
Fat, %	2.5	3.5	0.39	0.09
Protein, %	14.8	11.7	1.23	0.10
Somatic cell count linear score	7.6	7.5	0.53	NS[3]
Lactose, %	3.9	3.6	0.25	NS

[1]Cows were milked and colostrum from each udder half (n = 16) was sampled 1 h after parturition.

[2]Tendency for differences between means ($P \leq 0.10$).

[3]NS = no differences between means ($P > 0.10$).

Table 5. Prepartum changes in mammary epithelial cell proliferation and apoptosis of 60-d dry (CTL) and continuously milked (CM) udder halves[1]

	Sample time point[3]			*P*-value		
Variable[2]	3d-MS	7d-MS	SEM	DP	Time	DP × Time
Proliferation index, %						
CTL	1.63[a]	3.10[bx]	0.30	0.001	0.03	0.009
CM	1.11	0.93[y]	0.30			
Apoptotic index, %						
CTL	0.52[ax]	0.24[bx]	0.04	<0.001	NS	0.01
CM	0.11[y]	0.12[x]	0.04			

[a,b]Within a treatment (row) differences over time are indicated by different superscripts ($P < 0.05$).
[x,y]Differences between treatments (column) are indicated by different superscripts ($P < 0.05$).
[1]3d-MS = 3 d after milk stasis (MS) in the CTL udder half, 7d-MS = 7 d after MS in the CTL udder half, DP = dry period length.
[2]Proliferation was measured by expression of Ki67 nuclear proliferation antigen; apoptosis was measured by in situ hybridization of DNA fragments.
[3]Samples were obtained from each udder half (n = 16) at 3 and 7 d after milk stasis (MS) of the CTL udder half.

Apoptotic levels remained constant in CM tissue at 3d-MS and 7d-MS. Postpartum apoptosis was greater ($P < 0.01$) at 2d-PP than 4d-PP (0.80 vs. 0.44%; SEM = 0.09). There was no effect of dry period length or the interaction of dry period length and PGE_2 treatment on MEC apoptosis (Table 6). There were interactions of time by PGE_2 ($P = 0.02$); apoptosis decreased at 4d-PP in $+PGE_2$ tissue compared with $+PGE_2$ and $-PGE_2$ tissue at 2d-PP (0.34 vs. 0.92 vs. 0.68%; SEM = 0.16), whereas apoptosis of MEC in $-PGE_2$ tissue from 4d-PP (0.54%; SEM = 0.16) was intermediate to and not different from other time points in $+PGE_2$ and $-PGE_2$ tissue. There were also interactions of time by parity ($P < 0.05$); MEC apoptosis increased ($P < 0.05$) in second-lactation cows at 2d-PP compared with first- and second-lactation cows at 4d-PP (1.0 vs. 0.45 and 0.42%; SEM = 0.12), and MEC apoptosis tended ($P \leq 0.06$) to be greater at 2d-PP in tissue from second-lactation than from first-lactation cows (1.0 vs. 0.61%; SEM = 0.12).

Effect of dry period length on mammary ultrastructure was consistent with what we have reported previously (Annen et al., 2007). There was no effect of PGE_2 on postpartum ultrastructure (data not presented).

Mammary Gene Expression

The housekeeping gene, 18S, was not changed ($P > 0.4$) by any of the main effects or their respective interactions. Prepartum CTL tissue had decreased ($P < 0.02$) α-lactalbumin expression, increased ($P < 0.05$) bax expression, and a tendency ($P = 0.1$) for increased IGFBP5 expression compared with CM tissue (Table 7). Expression of ABC1, CEBP-β, cyclin D1, p27, and bcl_2 were not changed ($P > 0.1$) by dry period treatment (Table 7). In postpartum tissue, PGE_2 and the interaction of PGE_2 by dry period length did not alter expression for any of the GOI (Table 8). Expression of ABC1 and IGFBP5 increased ($P < 0.05$) in postpartum CTL tissue

Table 6. Effects of dry period length (CTL vs. CM) and PGE_2 on mammary epithelial cell (MEC) proliferation and apoptosis during early lactation[1]

	Sample time point					*P*-value					
	2d-PP		4d-PP								
Variable[2]	$-PGE_2$	$+PGE_2$	$-PGE_2$	$+PGE_2$	SEM	DP	PGE_2	Time	DP × PGE_2	DP × Time	PGE_2 × Time
Proliferation index, %											
CTL	0.86[x]	0.78[x]	0.24	0.52	0.41	0.04	NS	0.05	NS	NS	NS
CM	1.73[y]	1.72[y]	0.25	0.50	0.43						
Apoptotic index, %											
CTL	0.49	0.97	0.61	0.39	0.65	NS	NS	0.01	NS	NS	0.02
CM	0.78	0.88	0.47	0.29	0.60						

[x,y]Dry period treatment altered ($P < 0.05$) MEC proliferation.
[1]Samples were obtained from each udder half (n = 16) at 2 and 4 d postpartum; PGE_2 intramammary infusions were administered at parturition and after the milking closest to 72 h postpartum; CTL = 60-d dry, CM = continuously milked; $-PGE_2$ = no intramammary infusion of prostaglandin E_2, $+PGE_2$ = intramammary infusion of 875 μg of prostaglandin E_2, 2d-PP = biopsy taken 2 d postpartum, 4d-PP = biopsy taken 4 d postpartum, DP = dry period length.
[2]Proliferation was measured by expression of Ki67 nuclear proliferation antigen; apoptosis was measured by in situ hybridization of DNA fragments.

Table 7. Summary of ΔCt[1] values in 60-d dry (CTL) and continuously milked (CM) mammary tissues during the prepartum and postpartum periods[2]

Gene of interest[3]	CTL	CM	SEM	Fold change in expression[4]
Prepartum				
α-Lactalbumin	8.7[a]	5.3[b]	0.90	–10.56
ABC1	17.6	17.6	0.47	1.00
CEBP-β	11.4	11.4	0.76	1.00
Cyclin D1	9.6	9.2	0.38	–1.32
p27	16.9	17.3	0.35	1.32
bax	11.5[a]	13.1[b]	0.39	3.03
bcl_2	11.0	11.4	0.37	1.32
IGFBP5[5]	12.9[a]	14.3[b]	0.58	2.54
Postpartum				
α-Lactalbumin	7.5	5.8	1.1	–3.75
ABC1	16.8[a]	18.2[b]	0.39	2.64
CEBP-β	10.3	10.4	0.72	0.93
Cyclin D1	8.3	9.6	0.46	2.46
p27	16.8	17.0	0.42	1.15
bax	12.9	13.1	0.56	1.15
bcl_2	11.1	11.8	0.36	1.62
IGFBP5	12.1[a]	14.3[b]	0.68	4.59

[a,b]Means within a row with different superscripts differ ($P < 0.05$).
[1]ΔCt = cycle threshold $(Ct)_{GOI} - Ct_{18S}$. GOI = gene of interest; 18S = ribosomal 18S.
[2]Samples compared were pools created from prepartum (3d-MS, 7d-MS) and postpartum (2d-PP, 4d-PP) time points for each udder half (n = 16). Intramammary infusions of PGE_2 were administered at parturition and after the milking closest to 72 h postpartum. 3d-MS and 7d-MS = 3 and 7 d after milk stasis (MS) in the CTL udder half; 2d-PP and 4d-PP = biopsy taken 2 and 4 d postpartum.
[3]Genes: $ABC1_2$ = adenosine 5′-triphosphate binding cassette 1; CEBP-β = CCAT/enhancer binding protein-β; p27 = kinase inhibitor protein p27; bax = Bcl-2-associated_X_protein gene; bcl_2 = B-cell lymphoma 2 gene.
[4]Fold changes in expression were determined by $2^{-\Delta\Delta Ct}$ calculations.
[5]Means tended to differ ($P = 0.07$).

Table 8. Summary of ΔCt[1] values in the $–PGE_2$ and $+PGE_2$ mammary tissues[2]

	$–PGE_2$		$+PGE_2$		
Gene of interest[3,4]	CTL	CM	CTL	CM	SEM
α-Lactalbumin	5.1	5.9	9.9	5.7	1.58
ABC1	17.1	18.6	16.5	17.8	0.56
CEBP-β	9.4	9.8	11.2	11.0	1.00
Cyclin D1	8.0	9.2	8.5	10.0	0.66
p27	16.9	17.6	16.7	16.4	0.60
bax	11.9	13.3	13.8	12.8	0.80
bcl_2	10.7	11.9	11.5	11.6	0.51
IGFBP5	12.0	15.0	12.2	13.6	0.97

[1]ΔCt = cycle threshold $(Ct)_{GOI} - Ct_{18S}$. GOI = gene of interest; 18S = ribosomal 18S.
[2]Pooled samples were obtained from each udder half (n = 16) for tissue taken at 2 and 4 d postpartum. Intramammary infusions of PGE_2 were administered at parturition and after the milking closest to 72 h postpartum. Only one udder half was treated per animal, and PGE_2 treatment was balanced for equal numbers of CM and CTL halves, as well as equal numbers of first- and second-lactation udder halves. CTL = 60-d dry; CM = continuously milked; $–PGE_2$ = no intramammary infusion of prostaglandin E_2; $+PGE_2$ = intramammary infusion of 875 μg of prostaglandin E_2.
[3]Genes: ABC1 = adenosine 5′-triphosphate binding cassette 1; CEBP-β = CCAT/enhancer binding protein-β; p27 = kinase inhibitor protein p27; bax = Bcl-2-associated_X_protein gene; bcl_2 = B-cell lymphoma 2 gene.
[4]For all genes of interest, no statistical differences occurred for the main effects of PGE_2 or the interactions.

compared with CM tissue (Table 8). Postpartum expression of other GOI was not affected ($P > 0.1$) by dry period length (Table 7).

DISCUSSION

A 2-part experiment was conducted to 1) evaluate effects of CM on subsequent milk yield and composition and 2) evaluate the lactogenic (increase milk yield) and(or) mitogenic activity of PGE_2 infused via IMI at parturition and 72 h PP. A half-udder model was used to study these objectives. Planned cessation of milking in CTL halves resulted in an average of 64 d dry, whereas spontaneous dry-off resulted in an average of 12.6 d dry in CM halves. Average gestation length was 283 d, which is similar to the average gestation length reported for Holsteins (279 d; Macmillian, 1992).

This study also provided the opportunity to observe DMI over the periparturient period and realize the potential of minimizing late-gestation ration changes to reduce negative energy balance and reduce health risks associated with early lactation. Others have reported improved DMI in cows given a shortened (28 or 30 d) dry period (Gulay et al., 2003; Rastani et al., 2005), and the DMI decrease during the last 3 wk of gestation was only 17% in our previous CM half-udder study (Annen et al., 2007). Results from the current study show a smaller (13.2%) decline in DMI during late gestation (from 22 kg/d at 3 wk prepartum to 19.1 kg/d at parturition) and a larger DMI during early lactation (25 kg/d by 2 wk PP). Minimizing diet changes during this period likely contributed to this effect, but effects of CM and PGE_2 on DMI could not be tested because each cow received both of these half-udder treatments. Regardless, these results suggest that development of a management scheme, such as CM for the last 8 wk of gestation, that reduced the number of dietary changes during the periparturient period might prove beneficial for the successful transition into lactation.

Milk Yield and Composition

A progressive decrease in milk yield in CM halves throughout the last 8 wk of gestation was expected, because hormonal changes in advanced pregnancy have negative effects on milk yield (Capuco and Akers, 1999). The decrease in milk yield was most rapid during the final 3 wk of gestation when progesterone and estrogen concentrations are both elevated. Reductions in subsequent postpartum milk yield from CM halves are consistent with previous research (Annen et al., 2004b, 2007; Rastani et al., 2005; Fitzgerald et al., 2007). The only report of equal postpartum milk yields in CM and 60-d dry cows was when multiparous cows were supplemented with bST throughout late gestation and the

subsequent lactation (Annen et al., 2004b). The parity sensitivity to reduced dry period length has been observed previously (Remond et al., 1997; Annen et al., 2004b), and the 53% reduction in milk yield by CM halves of primiparous cows in their subsequent lactation is also similar to reductions observed in other half-udder studies of primiparous cows (Annen et al., 2007). A 33% reduction in milk yield from CM halves of multiparous cows in their subsequent lactation is similar to results from other CM studies that used cows of varying parities (Swanson, 1965; Smith et al., 1967; Rastani et al., 2005). Although these dramatic reductions in milk yield could be caused by a combination of decreased yield by CM halves and enhanced yield by CTL halves, doubling CTL yields provided estimates of whole-udder yields of 42 kg/d for first-lactation cows and 48 kg/d for second-lactation cows through 28 d PP. These values are similar to early-lactation milk yields from second- and third-lactation cows at the University of Arizona dairy herd and indicate that the half-udder experimental model did not alter productive capacity of CTL halves. We propose that greater sensitivity of the half-udder model contributed to the ability to detect these large reductions in milk yield.

The IMI of PGE_2 did not alter milk yield, which is similar to the results obtained when IMI of PGE_2 was administered prepartum (Collier et al., 2002). The only experimental model in which milk yield was altered by IMI of PGE_2 is that of induced lactation (Crooker et al., 2003), in which PGE_2 increased milk yield dramatically. Thus, we hypothesize that mammary PGE_2 concentrations are not limiting to mammary growth or milk yield in cows that undergo parturition and initiation of lactation. However, induction of lactation (without parturition) likely does not stimulate a sufficient increase in mammary PGE_2 production.

Milk composition during late gestation was measured to determine if CM affected composition or quality of milk, which could alter saleable milk and processing characteristics. Similar to our previous results (Annen et al., 2007), milk fat and lactose concentrations were unchanged and protein tended to increase during late gestation. Other studies of CM cows have reported increased milk fat (Remond et al., 1992, 1997) and trends for increased protein (Remond et al., 1992, 1997) and decreased lactose (Remond et al., 1997) content during the last 2 mo of gestation. Milk SCS was also unchanged, although the temporal pattern appears to change at –4 wk when SCS begins to increase until parturition. Remond et al. (1997) reported a similar trend for SCC in CM cows. Milk composition during spontaneous dry-off has not been reported previously, but changes during spontaneous dry-off are likely affected more by stage of gestation and lactation than CM. Milk composition during spontaneous dry-off was similar to that during the last 2 to 3 wk of gestation, which is when spontaneous dry-off occurred most frequently.

Similar to previous research, postpartum milk composition was not affected by dry period length (Remond et al., 1997; Annen et al., 2007, Fitzgerald et al., 2007). Equal lactose content in postpartum milk from CTL and CM udder halves but reduced milk yield in CM halves indicated fewer MEC in a secretory state in CM halves, which is supported by increased populations of resting and regressing alveoli in CM tissue at 20 d PP (Annen et al., 2007). Although there have been reports of negative effects of CM on postpartum udder health (Remond et al., 1997), a series of studies in our laboratory have shown that SCS is unchanged in CM cows or udder halves (Annen et al., 2004b, 2007; Fitzgerald et al., 2007), suggesting that CM cows can be managed to maintain a low SCS.

Prepartum MEC Turnover

Biopsies at 3d-MS (61 d prepartum) and 7d-MS (57 d prepartum) were obtained at key time points for MEC turnover during mammary involution in the bovine (Annen et al., 2003). In CTL glands, MEC apoptosis 3d-MS was 50% greater than at 7d-MS, indicating that the first stage of mammary involution had subsided, and progression to the second phase of tissue remodeling (Furth, 1999; Capuco et al., 2004) occurred by 7d-MS. Apoptosis of MEC remained constant in CM tissue, which demonstrates that local control of mammary involution allowed for maintenance of lactation in CM glands while normal involution occurred in CTL glands, despite the continued influence of galactopoietic and milking stimulus hormones (Li et al., 1997) due to continued milking of the CM half. Apoptotic factors present in accumulated milk (IGFBP5 and multimerized α-lactalbumin) in involuting tissue and mechanical stress due to MEC engorgement are believed to be local stimuli that initiate MEC apoptosis during the early dry period (Marti et al., 1997; Allan et al., 2004). Continuation of milk removal in CM halves prevented milk accumulation and MEC engorgement; thus, apoptotic indices remained constant. Prepartum MEC proliferation was also affected by dry period treatment. By 7d-MS, MEC proliferation was increased 3-fold in CTL tissue compared with CM tissue. To summarize prepartum MEC turnover data, CM inhibited the MEC renewal at 3d-MS and 7d-MS. These data and those of Capuco et al. (1997) and Annen et al. (2007) demonstrate reduced MEC proliferation in CM tissue throughout most the last 60 d of gestation. Accompanying reductions in apoptosis at 3d-MS and 7d-MS and at 7 d PP (Annen et al., 2007) are likely the mechanism by which total cell numbers are maintained in CM tissue despite reductions in proliferation during the last 60 d of gestation. Such reductions in MEC renewal process add further support to the hypothesis that decreased milk yield in CM glands is the result of increased carryover of old MEC with decreased secretory capacity rather than replacement of old MEC with new MEC (Capuco et al., 1997; Annen et al., 2007).

Our results indicate no compensatory milk yield or mammary growth in the CM half udder after the CTL half was dried-off and no delay in involution of the dried glands. In contrast, others have reported that inhibition of milk synthesis in udder halves or quarters by cessation of milking or colchicine treatment results in compensatory milk yield (Hamann and Reichmuth, 1990) and mammary growth (Capuco and Akers, 1990) in the glands that continued to be milked. Akers and Keys (1985) reported delayed involution in the nonlactating glands. The specific reason(s) for these discrepancies is unknown. A key difference may be the effect of late stages on concurrent pregnancy. In the current study, MS occurred during a time when the gland (whether lactating or dry) was in a state of growth. Compensatory growth beyond this pregnancy-induced growth may not occur, or may not be detectable. Other possibilities could be milk yield at the time of milk stasis and the resulting time to reach mammary engorgement, as well as the degree of engorgement and accumulation of apoptotic factors.

Postpartum MEC Turnover

In contrast to our previous report of equal MEC proliferation in CM and CTL tissue at 1 (Annen et al., 2007) and 2 d PP (Fitzgerald et al., 2007), MEC proliferation at 2d-PP in the current study was greater in CM than in CTL tissue. Reasons for these different results among our studies with equal or only a 24-h difference in time of biopsy are unknown. Further investigations on MEC growth during early lactation are needed to understand the impact of modified dry period length. The decrease in MEC proliferation from 2d-PP to 4d-PP is consistent with reports of decreased and minimal mammary growth in ruminants after parturition (Capuco et al., 2001).

Postpartum MEC apoptosis was not affected by dry period length at 2d-PP and 4d-PP, but has been reported to decrease prematurely in CM tissue by d 7 PP (Annen et al., 2007). For both CM and CTL tissue, apoptotic indices were greater at 2d-PP than at 4d-PP and were similar at 4d-PP to those observed during peak apoptosis in involuting CTL tissue (3d-MS). It was believed that mammary involution was the only period of the lactation cycle with increased MEC apoptosis, but recent research has provided consistent reports of high percentages of MEC apoptosis during early lactation (Capuco et al., 2001; Sorensen and Sejrsen, 2003; Annen et al., 2007). High apoptotic rates during initiation of lactation are likely because of increased shedding of old MEC that were replaced with new MEC generated during late gestation (Sejrsen et al., 2003). Increased apoptosis during early lactation could also be due to removal of cells with errors in DNA replication (Annen et al., 2007) or apoptosis of migrating leukocytes, which are known to increase in mammary tissue during early lactation (Sordillo and Nickerson, 1988; Annen et al., 2007).

Treatment with PGE_2 failed to stimulate mitogenic activity in mammary tissue during early lactation in CTL and CM tissue. Collectively, results from the current study and previous PGE_2 studies indicate that peripartum availability of PGE_2 does not limit pre- or postpartum mammary growth. The observed decrease in MEC apoptosis in PGE_2-treated tissue at 4d-PP compared with PGE_2-treated and nontreated tissue at 2d-PP and nontreated tissue at 4d-PP likely does not have biological significance, because PGE_2 did not alter other cellular or production variables at this time point. Greater MEC apoptosis in 2d-PP tissue from older cows indicates that their more mature glands either are able to remove more MEC or have more older MEC that need to be removed than those of younger cows.

Ultrastructure

Ultrastructural differences between involuting (CTL) and lactating (CM) tissue at 3d-MS and 7d-MS were similar to previous studies that evaluated involuting and lactating tissue (Sordillo and Nickerson, 1988; Tatarczuch et al., 1997; Annen et al., 2007). Importantly, prepartum tissue from the CTL udder halves was involuting (disorganized alveoli, engorged MEC, no MEC polarity, cellular debris in alveolar lumens) while lactation was being maintained (distinct MEC polarity, abundant cellular organelles, organized alveoli) in the CM udder halves. Postpartum ultrastructure results demonstrated that the transition into copious secretion and established lactation was not complete by 4d-PP, because MEC lacked the distinct polarity observed in lactating tissue at 20 d PP (Annen et al., 2007) and appeared disorganized. Structure of early-lactation tissue was similar for CTL and CM udder halves. Coinciding with the absence of PGE_2 effects on cell turnover or milk yield, ultrastructure was similar for PGE_2-treated and nontreated tissue.

Mammary Gene Expression

Genes evaluated by real-time q-PCR were selected to determine effects of 1) maintenance of lactation in CM halves, 2) involution of CTL halves on apoptotic and proliferative genes, and 3) effects of CM and PGE_2 on postpartum expression of apoptotic and proliferative genes. Our expression data are from prepartum (3d- and 7d-MS) and postpartum (2d- and 4d-PP) pools from CTL and CM half udders per cow.

Prepartum Gene Expression. Milk stasis of the CTL half resulted in a 10.6-fold decrease in α-lactalbumin expression compared with maintenance of lactation in the CM half. Wilde et al. (1997) utilized Northern blot analyses and reported a 99% decrease in α-lactalbumin mRNA after 7 d of MS. These differential results could be a result of different techniques, comparison of pooled vs. single samples, continued exposure of the CTL half udder in our study to galactopoietic hormones that delayed downregulation of α-lactalbumin, or a combination of these effects.

Involution of the CTL half resulted in upregulation of proapoptosis genes (bax and IGFBP5) compared with the lactating CM half but had no effect on bcl_2 expression. Increased bax expression has been demonstrated previously in involuting mammary tissue (Heermeier et al., 1996; Li et al., 1997; Colitti et al., 1999; Metcalfe et al., 1999), and increased bax expression corresponded with increased MEC apoptosis (Heermeier et al., 1996; Colitti et al., 1999). Also corresponding with increased MEC apoptosis was a 2.6-fold increase in IGFBP5 expression in CTL tissue compared with CM tissue. The IGFBP5 protein may function independently of IGF-I or by preventing cell-survival actions of IGF-I (Allan et al., 2004). Prolactin might be involved in IGFBP5-mediated apoptosis by blocking IGFBP5 inhibition of IGF-I (Tonner et al., 1997). In the current study, IGF-I and prolactin delivery would be lower in the CM side compared with CTL because of lower blood flow associated with lower milk yield, but local production and utilization of these hormones is unknown. Despite equal systemic delivery of prolactin and IGF-I, IGFBP5 expression increased in CTL tissue. This may indicate that IGFBP5 expression is regulated locally or that interactions between prolactin, IGF-I, and IGFBP5 are affected by lactation status of the tissue. In other studies, both IGFBP5 mRNA and protein in mammary tissue (rat; Tonner et al., 1997) or culture (Marshman et al., 2003) increased by 2 to 3 d of involution, which corresponded to periods of increased apoptosis. Expression of bcl_2 was not affected by lactation status of the tissue, suggesting that suppression of bcl_2 expression was not required for increased MEC apoptosis in CTL tissue, and its elevation was not required for MEC survival in CM tissue.

None of the proliferation-associated genes (cyclin D1, CEBP-β, or p27) we evaluated were altered by dry period treatment. Proliferation of MEC was greater by 7 d in CTL tissue. However, this difference was not stimulated by cyclin D1, CEBP-β, or p 27 expression. Some possibilities are 1) expression of these genes was masked by pooling the 3d- and 7d-MS samples for gene expression analyses, or 2) regulation is posttranscriptional. Increased MEC proliferation was also not accompanied by decreased p27 expression in our previous study (Annen et al., 2007).

A stem cell population has been identified in mammary tissue: transplant studies have demonstrated that a fully developed gland can result from progeny of a single cell (Kordon and Smith, 1998). Because the dry period may be required for replenishment of mammary stem and progenitor cells (Capuco and Akers, 1999), we evaluated the effects of omitting the dry period on expression of ABC1, a proposed stem and progenitor cell marker for lactating tissue. Similar to previous reports, ABC1 was expressed at very low levels in both pre- and postpartum tissue (Annen et al., 2007). Stem cells or side populations of proliferating cells have been shown to make up less than 0.24% (Capuco, 2007) to 0.5% (Alvi et al., 2003) of the mammary epithelium, so low quantities of ABC1 transcript were expected. Prepartum ABC1 expression was not increased in CTL tissue, which indicated that stem or progenitor cell replenishment might occur later in the dry period, but expression of ABC1 was not altered by CTL vs. CM treatment during the last 20 d of gestation (Annen et al., 2007). Perhaps ABC1 is not affected by CM or this gene should be evaluated in a sample consisting of only MEC and not a sample containing all mammary cell types.

Postpartum Gene Expression. Consistent with the lack of change in milk lactose concentration and our previous results (Annen et al., 2007), postpartum expression of α-lactalbumin was not altered by the dry period treatment. Postpartum expression of cyclin D1, CEBP-β, and p27 were similar in CM and CTL tissue. Because p27 is primarily regulated at the protein level and expressed at low levels in mammary tissue, study of posttranslational modifications of p27 might lead to more valuable information on the role of p27 in mammary development. Proliferation of MEC was increased in CM tissue during early lactation with no change in expression of CEBP-β and cyclin D1. Thus, the postpartum increase in MEC proliferation must be regulated by other genes, whereas prepartum proliferation during the final stages of gestation appears to be at least partially regulated by cyclin D1 and CEBP-β (Annen et al., 2007). Neither bax nor bcl_2 was affected by dry period treatment. Differential expression of apoptotic and survival genes was not expected because early lactation apoptotic indices were similar between CM and CTL tissue. Expression of IGFBP5 was greater in CTL than CM tissue during early lactation, but this result was not observed when CM and CTL cDNA pools generated from mammary samples collected on d 1, 7, and 21 PP were compared (Annen et al., 2007). In that study, early-lactation apoptosis decreased by d 7 PP in CM tissue but remained elevated in CTL tissue (Annen et al., 2007). Although apoptosis was similar in CM and CTL tissue during the first 4 d PP in the current study, increased IGFBP5 expression in CTL tissue immediately after parturition may play a role in maintaining greater apoptotic rates in CTL tissue later in lactation (i.e., d 7 PP).

Although prepartum expression of ABC1 did not differ between CTL and CM tissue, postpartum ABC1 expression was greater in CTL than in CM tissue. Mammary expression of ABC1 increases during the dry period (A.V. Capuco, USDA-Bovine Functional Genomics Lab, Belstville, MD; unpublished data), and these results support the hypothesis that the dry period is required for stem or progenitor cell replenishment (Capuco and Akers, 1999). However, expression of ABC1 in mammary tissue from 1, 7, and 20 d PP did not differ (Annen et al. 2007), and postpartum MEC proliferation in the current study was reduced in CTL tissue despite the greater presence of this stem/progenitor cell marker. Perhaps increased proliferation in CM tissue reduces the mitotic competence of some progenitor cells,

resulting in terminal differentiation and loss of ABC1 expression in these cells. Wagner et al. (2002) reported that stem cells survived lactation and involution through at least 2 lactations and were able to rescue mammogenesis and lactation in prolactin knockout mice during their third lactation. Others have demonstrated that stem cells lose potency after several generations of serial transplantation (Smith and Boulanger, 2002). It is possible that CM hastens terminal differentiation and loss of potency of stem cells, which may decrease ABC1 expression. Further research on MEC proliferation and stem cell populations in CM and CTL mammary tissue are required to improve our understanding of these data. Identification of a MEC-specific stem cell marker would likely generate more beneficial data than obtained by the universal stem cell marker used in the current study.

Treatment with PGE_2, as a main effect or in any interactions, did not alter expression of any GOI in the current study. This result is consistent with other measures tested in this study, which indicate that PGE_2 concentrations in the mammary gland were not limiting to milk production or cellular functions and turnover in CM udder halves.

Summary and Conclusions

Milk yield was reduced in CM halves, but to a lesser degree in halves from second-lactation cows compared with first-lactation cows. The period of increased MEC renewal that occurred during involution of CTL tissue during the early dry period was eliminated by CM. The IMI of PGE_2 did not recoup reductions in milk yield or alter measures of MEC turnover associated with CM. Therefore, mammary concentrations of PGE_2 during the first 4 d PP do not limit milk yield or mammary growth in CTL or CM tissue. Dry period length did not affect milk composition or quality but effects on colostrum quality are not completely understood and require further research. Gene expression analysis of proliferating or apoptotic MEC, rather than a mixture of mammary tissue cells, may provide additional information on the regulation of these 2 processes and how they may be altered to improve mammary development or lactation performance in dairy animals.

These results indicate that cell division is enhanced in non-lactating tissue during mammary development in late gestation. We hypothesize that proliferating MEC are mammary stem or progenitor cells that require a nonsecretory, less-differentiated phenotype to divide. Because primiparous cows are more sensitive to CM than multiparous cows, we suggest that as the mammary gland matures with each lactation cycle, the requirement for mammary development in excess of normal MEC turnover during the dry period diminishes. With MEC replacement only required in older animals, decreased MEC turnover would increase the carryover of old MEC from one lactation to the next. Because these cells are still capable of milk production and can respond to bST, this likely explains why multiparous cows have smaller or no (with bST) production losses with removal of the dry period. In primiparous animals, the dry period appears to be critical for both continued mammary development and MEC turnover to occur correctly. Further research on stem and progenitor cell population dynamics in primiparous and multiparous glands and in CM and CTL glands from these parity groups are required to continue to elucidate the effects of CM and parity on the mammary gland.

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