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The effects of milk removal or four-times-daily milking on mammary expression of genes involved in the insulin-like growth factor-I axis

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ABSTRACT

Frequent milking of dairy cows during early lactation elicits both an immediate increase in milk yield and a partial carryover effect that persists to the end of lactation. We hypothesized that the immediate response would be associated with a local increase in insulin-like growth factor (IGF)-I signaling and a consequent increase in mammary growth. Four multiparous cows were assigned at parturition to unilateral frequent milking [UFM; milking of the left udder half twice daily (2×; 0230 and 1430 h); milking of the right udder half 4 times daily (4×; 0230, 0530, 1430, and 1730 h)]. Mammary biopsies were obtained from both udder halves at 5 d in milk at 0530 h (immediately after 4× glands were milked). Incorporation of [^{3}H]-thymidine into DNA and mammary cell apoptosis were not affected by UFM. Because biopsies were obtained when udder halves were at different postmilking intervals, our results reflected both the acute, transient mammary response to milking and the sustained mammary response to frequent milking treatment. We further hypothesized that the acute, transient response involves mechanisms distinct from those regulating the sustained response to frequent milking. To test that hypothesis, mammary biopsies were obtained from UFM cows (n = 5) at 0500 h, when time postmilking was the same for both udder halves. Mammary cell apoptosis was not affected by UFM. Expression of genes involved in the IGF-I axis was analyzed to identify acute responses associated with milking, per se, versus sustained responses to frequent milking treatment. Removal of milk from 4× glands was associated with an acute increase in expression of IGF binding protein-1, -3, and -4 mRNA in 2× glands, whereas IGF-I expression was increased by frequent milking treatment. These effects, however, were significant only for expression of IGF binding protein-3. Expression of IGF-I receptor did not differ because of milking frequency but was higher in both udder halves immediately postmilking, indicating a systemic effect. We conclude that several genes of the IGF-I axis respond to milking, per se, or frequent milking treatment, via at least 3 distinct patterns. Increased milking frequency does not alter mammary cell proliferation or apoptosis at 5 d in milk; however, it may increase the bioavailability of IGF-I in the mammary gland. Moreover, the increase in local expression of IGF-I in 4× udder halves indicates a role for this gene in the immediate milk yield response to frequent milking during early lactation.
Key words: insulin-like growth factor-I, local regulation, mammary growth, milking frequency

INTRODUCTION

Frequent milking (3 or more times daily) of dairy cows during early lactation can elicit an immediate increase in milk yield and a partial carryover effect that persists through late lactation (Bar-Peled et al., 1995; Hale et al., 2003; Wall and McFadden, 2008). It is unknown whether frequent milking elicits this response by affecting mammary cell number, secretory activity, or both. Bar-Peled et al. (1995) reported that frequent milking or suckling of dairy cows during early lactation was associated with an increase in plasma IGF-I. Insulin-like growth factor-I is a potent mammary mitogen and survival factor and has been reported to increase proliferation of bovine mammary epithelial cells (McGrath et al., 1991). In addition, infusion of IGF-I into the mammary arterial blood supply of lactating goats increased milk secretion rate (Prosser et al., 1990). The action of IGF-I in the mammary gland is mediated by the IGF-I receptor (Baumrucker and Erondu, 2000), and the bioavailability of IGF is modulated by the IGF binding proteins (**IGFBP**; Plath-Gabler et al., 2001).

Whereas an increase in mammary cell activity may transiently increase milk yield, an increase in mammary cell growth could mediate both acute and persistent effects on milk yield that have been observed in response to frequent milking of dairy cows during early lactation (Bar-Peled et al., 1995; Hale et al., 2003; Wall and McFadden, 2007a). Although secretory mammary epithelial cells are considered terminally differentiated, proliferation in the lactating bovine mammary gland has

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been reported (Capuco and Akers, 1990). The effects of frequent milking during early lactation on mammary growth, however, remain unclear. Hale et al. (2003) observed an increase in cell proliferation at 7 DIM in the mammary gland of cows milked 4 times daily (**4×**) relative to cows milked twice daily (**2×**); however an increase was observed in only 1 of 2 frequent-milking treatment groups. In a similar experiment, we observed an increase in stromal cell proliferation at 5 DIM in mammary glands of cows milked 4× compared with cows milked 2×, but total cell proliferation was not affected (Wall et al., 2006). The inconsistencies in the above findings illustrate the need for a powerful animal model with which to characterize the cellular response to frequent milking during early lactation as well as the need to elucidate the mechanisms involved in the persistent milk yield response.

Recently, we validated the use of a half-udder model for experiments designed to investigate the effects of frequent milking of dairy cows during early lactation (Wall and McFadden, 2007a). Using this model, we have consistently observed a marked increase in milk yield of 4× udder halves during unilateral frequent milking (**UFM**; 2× milking of the left udder half, 4× milking of the right udder half) and a transient decrease in milk yield upon cessation of UFM that is followed by a partial rebound and a sustained increase in milk yield of 4× udder halves that persists throughout the remainder of lactation (Wall and McFadden, 2007a,b).

We hypothesized that frequent milking treatment elicits an increase in local IGF-I signaling, a consequent increase in mammary cell proliferation, and a decrease in mammary cell apoptosis. We also hypothesized that the acute, transient mammary response to milking is distinct from the sustained mammary response to frequent milking treatment. In the current study, we used a half-udder model and obtained mammary biopsies from 2× and 4× udder halves immediately after only 4× udder halves were milked to characterize the acute, transient mammary response to milking, or at 2.5 h after both udder halves had been milked to characterize the sustained response to frequent milking treatment during early lactation.

MATERIALS AND METHODS

Animals and Treatments

Experiment 1. Cows used in this study were a subset (n = 4) of animals from 2 larger studies, and details on animal management and treatments have been described (Wall and McFadden, 2007a,b). Briefly, cows were assigned at parturition to UFM beginning on d 1 of lactation. Regular milkings took place at 0230 and 1430 h, and the 2 extra milkings (during which only the right udder half was milked) took place at 0530 h and 1730 h. The University of Vermont Institutional Animal Care and Use Committee approved all animal use.

Experiment 2. Five multiparous Holstein cows were assigned to UFM beginning on d 1 of lactation. Animal management, milking routine and half-udder milking were as described for experiment 1. One animal was removed from the experiment because of IMI (SCC >1,000,000 cells/mL).

Half-Udder Milking

To quantify the response to UFM, half-udder milk yields of cows in both experiments were measured using a portable milking system with a milking claw designed to collect milk from each quarter into individual vessels. Milk from each udder half was collected into sterile 5-gallon (19-L) plastic bags (Parish Manufacturing, Indianapolis, IN) and then weighed. At the first milking post-calving, half-udder milk yields were measured to verify that udder halves produced similar amounts of milk before treatment. Cows that were unbalanced by >0.7 kg were rejected from the study. Half-udder milk yields were again measured during the afternoon milking (1430 h) at 3 DIM. Milk samples from each udder half were collected and analyzed for SCC by Vermont DHIA (White River, VT).

Mammary Biopsy

Experiment 1. Mammary tissue from both rear quarters was obtained by biopsy on d 5 of lactation as described by Farr et al. (1996). Biopsies were performed immediately after the 0530 h milking (during which only the 4× udder half was milked; Figure 1). Prior to mammary biopsy, cows were administered an epidural injection containing a cocktail of 7 mL of 2% lidocaine (0.22 mg/kg of BW; Phoenix Pharmaceuticals, St. Joseph, MO) and 1.4 mL of 20 mg/mL of xylazine (0.05 mg/kg of BW; Phoenix Pharmaceuticals). Tail heads were clipped and scrubbed with a betadine solution, then rinsed with 70% ethanol. An 18-gauge needle was introduced into the first sacrocaudal intervertebral space, and a 10-mL syringe was used to inject the lidocaine-xylazine cocktail into the epidural space. In addition, just before biopsy, lidocaine (3 mL; 0.07 mg/kg of BW) was administered in a line-block directly above the incision site. A biopsy sample of approximately 500 mg (~70 × 40 mm in diameter) was obtained, an absorbable hemostat (Surgicel, PSS, Wareham, MA) was inserted to enhance clotting, and the wound was closed with 18-mm stainless steel wound clips (Autoclip, Clay Adams, Persippany, NJ). Biopsy

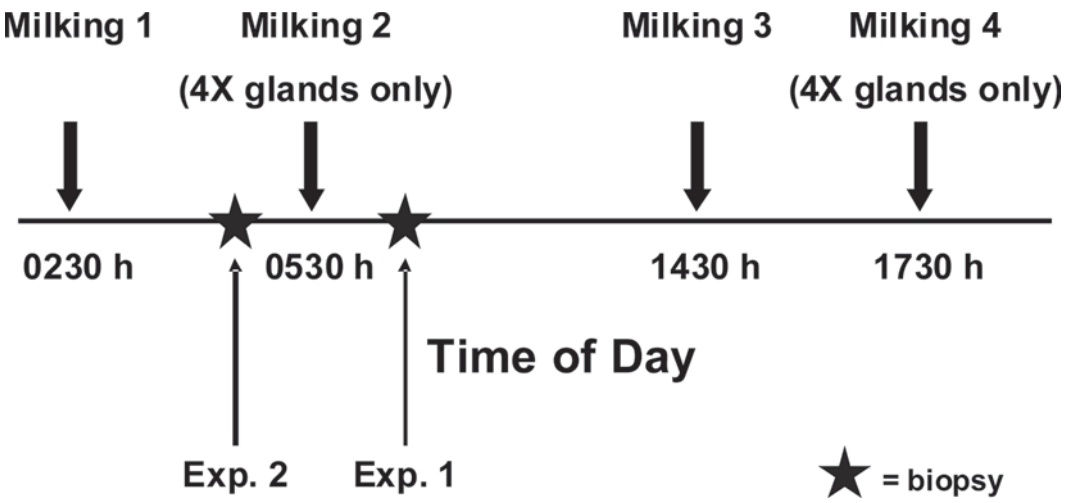

Figure 1. Milking routine and timing of mammary biopsies. In experiment 1 (Exp. 1), mammary biopsies were performed immediately after the udder half milked 4 times daily (4×) had been milked. In experiment 2 (Exp. 2), mammary biopsies were performed 2.5 h after both udder halves had been milked. Therefore, differences between udder halves observed in experiment 1 only were classified as an acute mammary response to milking, whereas differences between udder halves observed in both experiments were classified as a sustained mammary response to 4× milking treatment.

samples were trimmed of extraparenchymal tissue and a portion (~200 mg) was immediately frozen in liquid nitrogen for subsequent isolation of RNA. The remaining parenchyma was diced into explants that either were used to measure incorporation of [^{3}H]-thymidine into DNA (~100 mg) or were fixed in 10% buffered formalin for subsequent histological analysis (~5 mg).

Experiment 2. Mammary tissue from both rear quarters was obtained by biopsy on d 5 of lactation at 0500 h, approximately 2.5 h after both udder halves had last been milked (Figure 1). Prior to mammary biopsy, cows were administered an intravenous injection of 0.50 mL of 20 mg/mL of xylazine (0.01 mg/kg of BW; Phoenix Pharmaceuticals) for general sedation. The region of the udder to be biopsied was clipped, scrubbed 3 times with an iodine solution, and then rinsed with 70% ethanol. In addition, lidocaine (3 mL; 0.07mg/kg of BW) was administered in a line-block directly above the incision site just before biopsy. A biopsy sample of approximately 15 to 20 mg was obtained using a Pro-Mag Ultra automatic biopsy instrument (Medical Device Technologies, Gainesville, FL). Biopsy of the same site was repeated (~3 times) until approximately 60 mg of tissue was obtained, and the wound was closed with 18-mm stainless steel wound clips (Autoclip). Biopsy samples were trimmed of extraparenchymal tissue and a portion (~50 mg) was immediately frozen in liquid nitrogen for subsequent isolation of RNA. The remaining parenchyma (~5 mg) was diced into explants that were fixed in 10% buffered formalin for subsequent histological analysis.

Interpretation of the Mammary Response to Treatment

In experiment 1, mammary biopsies were performed immediately after only the 4× udder half had been milked (Figure 1). In contrast, mammary samples from cows in experiment 2 were obtained 2.5 h after both udder halves had last been milked (Figure 1). Therefore, differences between udder halves observed in experiment 1 only were interpreted as an acute mammary response to milking, per se, whereas differences between udder halves observed in both experiments were classified as a sustained mammary response to 4× milking treatment. During experiment 1, mammary biopsies were obtained immediately after both udder halves had been exposed to the hormones released at milking (see Figure 1). In contrast, during experiment 2, mammary biopsies were obtained 2.5 h after both udder halves had been exposed to the hormones released at milking. Therefore, if a response differed between experiments 1 and 2, but not between udder halves, it was interpreted as an acute mammary response (of both udder halves) to the systemic effects of hormones released at milking.

Mammary Proliferation Assay

Mammary parenchyma was diced into explants and approximately 100 mg was incubated in a shaking wa-

ter bath for 1 h at 37°C in 3 mL of medium 199 (Sigma, St. Louis, MO) supplemented with 1 μCi/mL of [^{3}H]-thymidine (33 Ci/mmol; ICN, Irvine, CA) to determine incorporation of [^{3}H]-thymidine into DNA. After incubation, explants were blotted, weighed, and frozen in liquid nitrogen. Incorporation of [^{3}H]-thymidine into DNA was determined during experiment 1 only, using methods described by Wall et al. (2005).

DNA Assay

Total DNA in tissue homogenates from experiment 1 was measured as described by Labarca and Paigen (1980), but modified for assay in a 96-well plate as described previously (Wall et al., 2005). Briefly, duplicate 2-μL aliquots of homogenate were pipetted into wells, and 98 μL of DABS-E (0.5 *M* dibasic $NaPO_4$, 0.5 *M* monobasic $NaPO_4$, 2 *M* NaCl, and 0.1 *M* EDTA) buffer and 100 μL of 2 μg/mL Hoechst 33528 dye (Sigma) were added. Fluorescence was determined using a fluorimeter spectrophotometer (KC4, Bio-Tek Instruments, Winooski, VT). The DNA concentration of homogenates was determined by comparison with a standard curve made from serial dilutions of calf thymus DNA (Sigma) and was used to calculate the total amount of DNA in the original homogenate.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Detection of apoptotic cells in situ was performed in both experiments using terminal deoxynucleotidyl transferase dUTP nick-end labeling, as described by Wall et al. (2005). Briefly, explants were embedded in paraffin, sectioned at approximately 4 μm thickness, and mounted onto silanized slides. A commercial terminal deoxynucleotidyl transferase dUTP nick end labeling kit (ApopTag Plus Peroxidase, Chemicon International, Temecula, CA) was used in accordance with the manufacturer's protocol. After the labeling assay, coverslips were mounted with Cytoseal (Thermo Scientific, Waltham, MA).

Quantification of Apoptotic Cells

Tissue sections were viewed by light microscopy with an Olympus B×41 light microscope (Olympus America Inc., Melville, NY) to quantify labeled cells. Details are described in Wall et al. (2005). Cells were classified as epithelial, stromal, labeled epithelial, or labeled stromal. All cells in one field were counted (at least 2,000 cells) and labeled brown nuclei were readily visible. A cell was classified as labeled when the nuclear staining was at least twice as intense as the background. Slides were coded for counting to prevent observer bias.

RNA Isolation and Reverse Transcription

Total RNA was isolated from mammary tissue of cows in both experiments using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Purified RNA was quantified using a Nanodrop ND1000 spectrophotometer, and quality was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Additional details on RNA processing can be found in Wall et al. (2005). Total RNA (3 μg) was primed with 1 μL of oligo dT primer (0.5 μg/μL, Invitrogen) and cDNA was synthesized using the SuperScript II reverse transcription kit (Invitrogen) according to the manufacturer's protocol.

Real-Time Quantitative PCR

Relative mRNA expression profiles were determined in both experiments by real-time quantitative PCR (**qPCR**) using a PE 7700 thermal cycler (Applied Biosystems, Foster City, CA). Additional details on PCR can be found in Wall et al. (2005). Primers (Table 1) were designed for bovine IGF-I, IGF-I receptor (**IGF-IR**), IGFBP-1, IGFBP-3, and IGFBP-4, and β-2 microglobulin using Primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). After primer design, the predicted product was BLAST searched against the bovine database to ensure specificity of the primers. All gene expression values were normalized to that of β-2 microglobulin, which was not affected by UFM. Nonnormalized gene expression values were used to calculate the ratio of IGF-I to IGFBP-1, IGFBP-3, and IGFBP-4.

Statistical Analyses

For each experiment, a 1-tailed paired *t*-test (version 9.1; SAS Institute Inc., Cary, NC) was used to determine the significance of treatment effects on milk yield, mammary cell proliferation, apoptosis, and the ratio of IGF-I to IGFBP-1, IGFBP-3, or IGFBP-4. A 2-tailed paired *t*-test was used to test for treatment effects on mammary gene expression. In addition, the MIXED procedure (version 9.1; SAS Institute Inc.) was used to compare mammary cell apoptosis and gene expression responses in each experiment and to determine the experiment by treatment interaction.

RESULTS

Milk Yield

Experiment 1. The cows used in this experiment were a subset from 2 larger experiments, and the milk

Table 1. Primer sequences for bovine IGF-I, IGF-I receptor (IGF-IR), IGF binding protein (IGFBP)-1, -3, and -4, and β-2 microglobulin[1]

Target	GenBank no.	Primer	Sequence 5′ to 3′
IGF-I	AY277406	Forward	TCTCATAATACCCACCCTGACC
		Reverse	ACTGGAGAGCATCCACCAAC
IGF-IR	BM482617	Forward	TGGAGTGCTGTATGCCTCTGT
		Reverse	GGTCTCGGGCTCATCCTT
IGFBP-1	NM_174554	Forward	ACACCACCAGCCCAGAGA
		Reverse	TCCCACTCCAAGGGTAGACA
IGFBP-3	NM_174556	Forward	AAAGGTCATGCCAAGGACAG
		Reverse	TGCCCGTACTTATCCACACA
IGFBP-4	NM_174557	Forward	AATCGGGGAGGAAAACAGAC
		Reverse	CAAACGGAGGAGGAAGGAG
β-2 microglobulin	NM_173893	Forward	AAGGATGGCTCGCTTCGT
		Reverse	TTCAAATCTCGATGGTGCTG

[1]Gene-specific primers were designed using Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based on the available sequences in GenBank (accession numbers indicated). Product sequences were Basic Local Alignment Search Tool (BLAST)-searched against the National Center for Biotechnology Information (NCBI) database to confirm identity of the product.

yield and composition results have been reported (Wall and McFadden, 2007a,b). After 3 d of UFM treatment and before mammary biopsy, the subset of cows reported on herein was producing 12.7 ± 1.2 kg/d. Differential milk production from the 4× udder half was 3.5 ± 1.0 kg/d more than from the 2× udder half ($P < 0.005$). Somatic cell count averaged 175 ± 66 $\times 10^3$ cells/mL and was not affected by UFM ($P > 0.50$).

Experiment 2. After 3 d of UFM and before mammary biopsy, cows in experiment 2 were producing 13.5 ± 1.9 kg/d. Differential milk production from the 4× udder half was 2.9 ± 0.6 kg/d more than from the 2× udder half ($P < 0.003$). Somatic cell count averaged 166 ± 180 $\times 10^3$ cells/mL and was not affected by UFM ($P > 0.50$).

Mammary Cell Proliferation and Apoptosis

In experiment 1, incorporation of [^{3}H]-thymidine into DNA in vitro averaged 220 ± 56 disintegrations per minute (dpm)/μg of DNA and was not affected by UFM ($P \geq 0.50$). The percentage of epithelial and stromal cells undergoing apoptosis averaged 0.95 ± 0.20 and 0.98 ± 0.30, respectively, and was not affected by UFM ($P \geq 0.40$). In experiment 2, mammary cell apoptosis averaged 0.60 ± 0.20% in the epithelium and 0.30 ± 0.15% in the stroma and was not affected by UFM ($P \geq 0.30$).

Real-Time qPCR

Experiment 1. Mammary expression of IGF-I, IGF-IR, and IGFBP-1, IGFBP-3, and IGFBP-4 mRNA is presented in Figure 2. Expression of IGF-I ($P \geq 0.20$), IGF-IR ($P \geq 0.60$), and IGFBP-1 ($P \geq 0.20$) mRNA was not affected by UFM (Figures 2A and C), whereas expression of IGFBP-3 was higher ($P \leq 0.03$) and IGFBP-4 tended to be higher ($P \leq 0.06$) in 2× udder halves than in 4× udder halves (Figure 2C). The ratio of IGF-I to IGFBP-1 was not affected by UFM ($P \geq 0.20$), whereas the ratios of IGF-I to IGFBP-3 and IGF-I to IGFBP-4 tended to be higher in 4× udder halves compared with 2× udder halves (1.70 ± 0.50 vs. 0.38 ± 0.50 for IGF-I:IGFBP-3 and 4.30 ± 1.60 vs. 0.67 ± 1.60 for IGF-I:IGFBP-4 in 4× and 2× glands, respectively; $P \leq 0.10$).

Experiment 2. Mammary expression of IGF-I, IGF-IR, and IGFBP-1, IGFBP-3, and IGFBP-4 mRNA was not affected by UFM ($P \geq 0.15$; Figures 2B and D). The ratios of IGF-I to IGFBP-1, IGFBP-3, and IGFBP-4 were not affected by UFM ($P \geq 0.12$).

To differentiate between the acute effects of milking per se, the sustained effect of 4× milking treatment, and the acute mammary response to milking-induced hormones, data from both experiments were combined for analysis. In both 2× and 4× glands, mammary cell apoptosis was higher in experiment 1 than in experiment 2; however, this difference was significant only in mammary stroma ($P \leq 0.03$; data not shown). The increase in IGF-I expression in 4× udder halves was consistent across both experiments (combined $P \leq 0.09$; Figures 2A and B). There was an experiment by treatment interaction for IGFBP-3 ($P \leq 0.04$) such that mRNA expression was higher in 2× udder halves than in 4× udder halves in experiment 1 but not different in experiment 2 (compare Figure 2C with Figure 2D). This interaction was not significant for IGFBP-1 or IGFBP-4 ($P \geq 0.30$). Expression of IGF-IR, IGFBP-3, and IGFBP-4 was higher in both udder halves in experiment 1 than in experiment 2 ($P \leq 0.02$; compare Figure 2A with Figure 2B and Figure 2C with Figure 2D).

DISCUSSION

Milk production is ultimately a function of mammary cell number and activity (Capuco et al., 2003; Boutinaud

et al., 2004). Because frequent milking during early lactation elicits both acute and persistent increases in milk yield, we hypothesized that this response would be associated with an increase in mammary cell number via increased proliferation, or decreased apoptosis, or both. Our results do not support this hypothesis, and there are several possible explanations for this outcome. First, the timing of tissue sampling may not have coincided with a mitogenic response to frequent milking. However, our previous observations (Wall et al., 2006) and those of Hale et al. (2003) indicated that the cellular response to frequent milking occurs by d 5 of lactation. Others have observed that increased milking frequency in goats was associated with a decrease in mammary cell apoptosis (Wilde and Knight, 1990; Li et al., 1999); however, both of those experiments compared once-daily milking with 2× or thrice-daily milking. Once-daily milking is associated with a marked decrease in milk yield, increased mammary cell apoptosis, and decreased epithelial cell integrity (Sorensen et al., 2001; Stelwagen, 2001). Therefore, although once-daily milking may elicit a more dramatic difference in milk

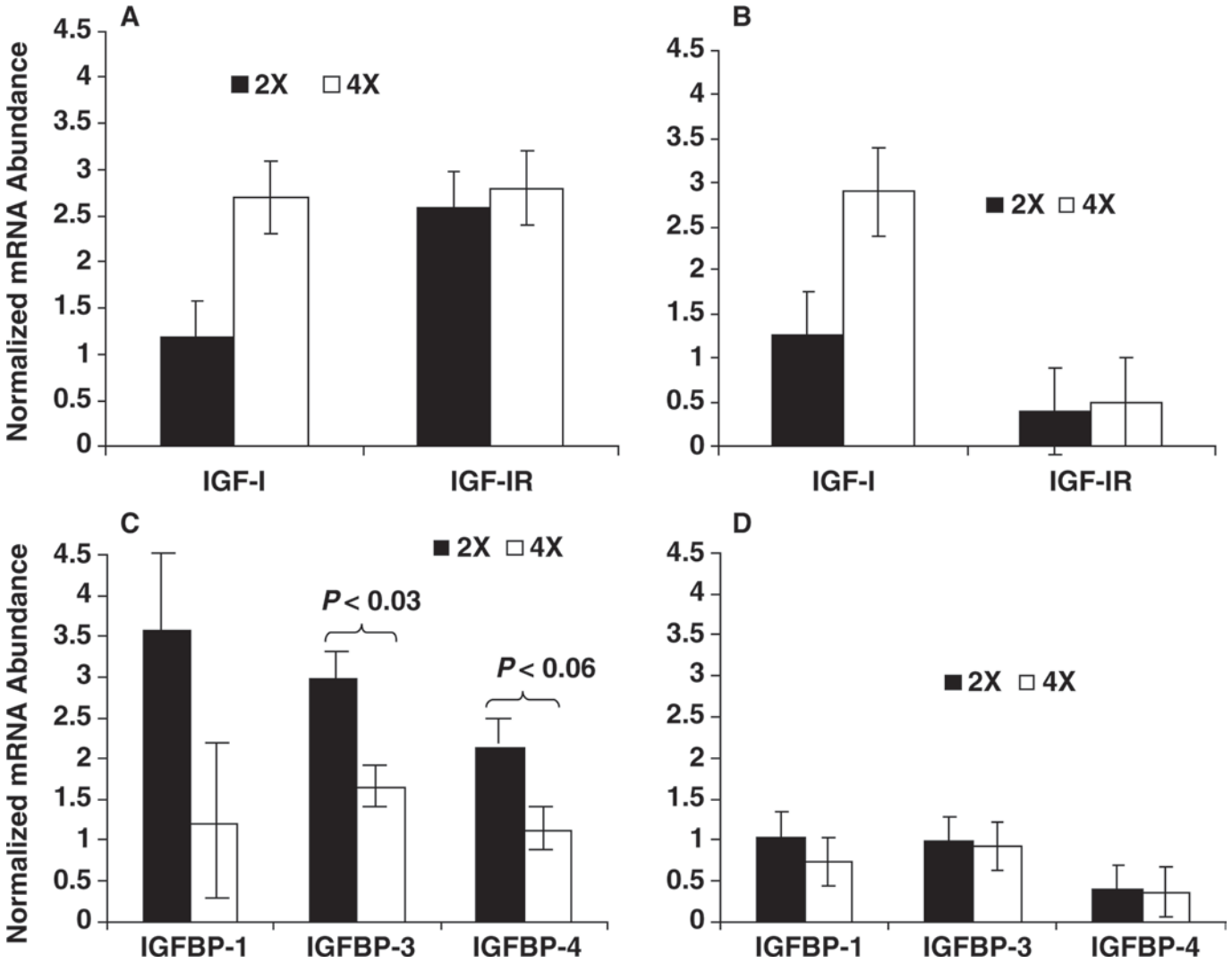

Figure 2. Effects of unilateral frequent milking (UFM) during early lactation on expression of IGF-I and IGF-I receptor (IGF-IR), or IGF binding protein (IGFBP)-1, -3, and -4 mRNA in bovine mammary gland. Cows were assigned to unilateral frequent milking [milking twice daily of the left udder half (2×) or milking 4 times daily of the right udder half (4×)] starting on d 1 of lactation. Mammary biopsies from both rear quarters were obtained on d 5 of lactation. In panels A and C (experiment 1; n = 4 cows), biopsies were obtained at 0600 h, which was immediately after the 4× udder half was milked but 3.5 h after the 2× udder half had last been milked. In panels B and D (experiment 2; n = 4 cows), biopsies were obtained at 0500 h, which was 2.5 h after both udder halves had last been milked. Each bar represents LSM ± pooled SE mRNA expression normalized to β-2 microglobulin. Expression of IGF-I, IGF-IR, and IGFBP-1 mRNA was not affected by milk removal or UFM treatment ($P \geq 0.20$). Expression of IGFBP-3 and IGFBP-4 mRNA was decreased with milk removal ($P \leq 0.06$) but not affected by UFM treatment ($P \geq 0.30$).

yield and a marked mammary response, it may not represent the biology underlying the stimulatory effect of thrice-daily or 4× milking relative to 2× milking.

Of the publications that reported the effects of frequent milking of dairy cows on mammary cell proliferation and apoptosis, either no effect was observed (Nørgaard et al., 2005; Wall et al., 2006) or only a very modest effect that was marginally significant and inconsistent was observed (Hale et al., 2003). It is possible that even very small changes in rates of mammary cell proliferation or apoptosis, if maintained over time, could significantly affect mammary cell number. Another possible explanation is that frequent milking stimulates an increase in mammary cell activity (Hillerton et al., 1990) or mammary cell hypertrophy (Knight et al., 1990), which is maintained (at least partially) through the entire lactation. Alternatively, frequent milking may change the cellular dynamics of the gland and shift the proportions of quiescent and active epithelial cells. Any of these changes could permanently alter the secretory capacity of the gland and mediate a long-term increase in milk yield. Indeed, contraction of alveoli associated with frequent milk removal has been predicted to cause reactivation of quiescent epithelial cells (Stelwagen and Knight, 1997; Shorten et al., 2002; Capuco et al., 2003; Vetharaniam et al., 2003). Although we did not measure alveolar area or number of cells per alveolus, both have been shown to increase during frequent milking of dairy cows (Hillerton et al., 1990). Our measurements of mammary cell proliferation, apoptosis, and expression of genes involved in the IGF-I axis would not have captured either of those types of responses to frequent milking. We did, however, have the ability to detect differences in expression of genes associated with milk synthesis and mammary cell activity, but the preliminary results of microarray analysis did not indicate such a response (Wall et al., 2008a).

Local expression of IGF-I and its receptor as well as the IGFBP is physiologically regulated, and these genes play a key role in mammary development and function (Plath-Gabler et al., 2001). Our preliminary data from microarray analysis implicated several of the IGFBP in the response to frequent milking (Wall et al., 2008a). Based on microarray, expression of IGFBP-2 and IGFBP-5 followed a similar pattern to that of IGFBP-3 and IGFBP-4, but real-time qPCR did not confirm those results (data not shown). Nevertheless, both of those IGFBP, especially IGFBP-5, have been shown to be involved in the mammary response to milk stasis in rodents as their expression is upregulated upon cessation of milk removal (Travers et al., 1996; Tonner et al., 1997). Paradoxically, we (Wall et al., 2005) and others (Plath-Gabler et al., 2001) have shown that IGFBP-5 expression in bovine mammary gland is upregulated during early lactation when milk yield is increasing rapidly, suggesting a marked difference in function between species, or physiological state, or both.

Because we obtained biopsies when 2× and 4× glands were at different postmilking intervals (experiment 1; Figure 1) or when they were at the same interval after the previous milking (experiment 2; Figure 1), we were able to distinguish between the acute, transient response of the mammary gland to milking and the sustained response of the mammary gland to UFM treatment. Our results show that expression of IGFBP-1, IGFBP-3, and IGFBP-4 is increased in response to milk removal (experiment 1 only) but only in 2× glands. Therefore, local expression of these genes appears to be acutely regulated, with the response gone by 2 h postmilking. Perhaps the responsiveness of the 2× glands reflects heightened sensitivity to acute signals in comparison with the 4× glands. That would be consistent with maintaining a set point or threshold with respect to milk synthesis, that set point having been altered by frequent milking treatment in 4× glands. Because oxytocin is secreted during extra milkings (Bar-Peled et al., 1995), it is probable that ejection of milk from alveoli occurred in both 2× and 4× udder halves when only 4× udder halves were milked. Still, the significant increase in milk yield of 4× udder halves is consistent with speculation by Morag (1973a,b) that the milk yield response to UFM does not depend on the presence of residual milk. In contrast to the transient response of the IGFBP, IGF-I appears to be responsive to the long-term effect of frequent milking treatment. That is, frequent milking treatment may elicit an increase in the bioavailability of IGF-I in the mammary gland, causing a sustained increase in cellular activity. Interestingly, Prosser and Davis (1992) reported that hourly milking of goats before mammary infusion with IGF-I completely attenuated the increase in milk yield and mammary blood flow that had previously been observed after IGF-I infusion. Considering the results of the current experiment, it is possible that increased milking frequency enhances IGF-I signaling and mammary cell activity and that in their experiment (Prosser and Davis, 1992), infusion of IGF-I into frequently milked glands failed to elicit an additional effect in an already optimized system. Others have observed an increase in mammary enzyme activity during increased milking frequency (Wilde et al., 1987; Hillerton et al., 1990; Wilde and Knight, 1990). Such a response could support the persistent increase in milk production that has been observed after frequent milking during early lactation (reviewed by Wall and McFadden, 2008). Intensive sampling of mammary tissue over time, both during and after frequent milking treatment, is needed

to clarify the mechanisms and biological processes involved. Such experiments are currently in progress (Wall et al., 2008b).

CONCLUSIONS

We conclude that the milk yield response to frequent milking during early lactation is not associated with changes in mammary cell proliferation or apoptosis at 5 DIM. Our results reveal distinct acute but transient responses to milking and sustained responses to frequent milking treatment. Several genes of the IGF-I axis are acutely responsive to milking. Moreover, a sustained increase in the bioavailability of local IGF-I may mediate both the immediate and the persistent milk yield responses to frequent milking during early lactation.

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