Milking and Feed Restriction Regulate Transcripts of Mammary Epithelial Cells Purified from Milk

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ABSTRACT

Feed restriction and once-daily milking (ODM) reduce milk yield in dairy cows and the amount of glucose taken up by the mammary gland. The modulation of mammary glucose uptake may be the consequence of modifications to glucose transport, capacity for lactose synthesis, and cell death in mammary epithelial cells (MEC). The aim was to demonstrate the usefulness of a new method to purify MEC from milk somatic cells and to examine the effects of feed restriction and ODM on mammary transcripts. Five Holstein cows were subjected to a 2×2 factorial arrangement of 2 milking frequencies and 2 feeding levels, during which the cows were milked once or twice daily while fed a diet providing either 98 or 70% of requirements. The cows were equipped to study net mammary balance of glucose. On d 7 of each experimental week, milk and lactose yields and mammary glucose uptake were measured. Cells were isolated from fresh milk by centrifugation to generate total milk cell samples. Mammary epithelial cells were separated from total milk cells by using magnetic beads associated with anticytokeratin 8 antibodies. Total RNA was extracted from both total milk cells and purified MEC samples. Real-time reverse transcription PCR was performed to determine mRNA levels in purified MEC under feed restriction and under ODM. Purified MEC samples revealed higher total RNA quality (RNA integrity number = 8) and were better suited to the measurement of mammary transcripts than total milk cell samples (RNA integrity number = 4). Significant correlations were obtained between mRNA levels and net glucose balance data ($0.465 < r < 0.680$), demonstrating the validity of results obtained by using purified MEC. Feed restriction induced a significant reduction (by half) in type 1 glucose transporter mRNA levels without any effect on α -lactalbumin (α -LA), galactosyltransferase, κ -casein, bcl2, or bax mRNA levels. When compared with twice

daily milking, ODM reduced κ -casein (–86%) and α -LA (–73%) mRNA levels and up-regulated bax and bcl2 mRNA levels (7- and 9-fold). The results suggest that the regulation of glucose uptake and milk yield is dependent on the transcription of glucose transporters under feed restriction and on the transcription of α -LA under ODM. Further studies are required to confirm the suggested onset of cell death after 7 d of ODM. **Key words:** dairy cow, milk cell, mammary epithelial cell, messenger RNA

INTRODUCTION

Lactose is the osmotic agent in milk that accounts for 50% of osmotic pressure, so milk volume is partly determined by lactose synthesis. Glucose is the main precursor for lactose synthesis. Thus, the quantity of glucose taken up by the mammary gland from the glucose arterial pool is a determinant for milk synthesis. Feed restriction and once-daily milking (ODM) caused a reduction in milk and lactose yields, which were essentially associated with a drop in the amount of glucose taken up by the mammary gland (Guinard-Flament et al., 2006, 2007). Little is known of the effect of feed restriction and ODM on the cellular mechanism governing the mammary regulation of glucose uptake.

Three levels of regulation may be responsible for reductions in mammary glucose uptake. The first is transport of glucose into mammary epithelial cells (MEC) through a passive mechanism, mainly involving type 1 glucose transporter (**GLUT-1**; Zhao et al., 1996) and active mechanisms that include type 1 sodium glucose cotransporter (**SGLT-1**; Zhao et al., 2005). The amount of mammary glucose uptake may depend on the quantity of these transporters at the surface membrane of MEC. Mammary glucose uptake may be dependent on intracellular glucose, levels of which are controlled by the metabolic use of glucose in MEC. Glucose is mainly used for lactose synthesis, the catalysis of which is triggered by galactosyltransferase [**GAT-(1,4)**] and its cofactor, α -LA. Thus, regulation of this enzymatic activity may adjust the amount of mammary glucose uptake. Finally, the level of glucose uptake may vary in relation to the number of

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MEC in the mammary gland. It was shown that ODM can induce MEC death by apoptosis (Li et al., 1999) and regulate the number of MEC by the acinus (Boutinaud et al., 2004a). Therefore, the transport of glucose, its metabolic use inside cells, and the number of MEC can play a definitive role in the level of mammary glucose uptake (Guinard-Flament et al., 2006).

To study the cellular mechanism responsible for a reduction in mammary glucose uptake, MEC samples need to be harvested from the mammary gland. Our purpose was to develop a method to analyze the cellular mechanisms involved in modifications to the mammary uptake of glucose in animals equipped to study their net mammary nutrient balance. In such animals, mammary biopsies or the sacrifice of animals is not appropriate for the collection of MEC. Studies show that milk is a noninvasive source of viable MEC (Boutinaud and Jammes, 2002) and cells can be used to analyze mammary mRNA levels of milk protein (Boutinaud et al., 2002). Milk somatic cells provided a good reflection of the mammary gland in goats (Boutinaud et al., 2002) and cows (Hayashi et al., 2004; Murrieta et al., 2006), yet MEC constitute a minority of milk somatic cells, especially in cows (Lee et al., 1980). After preparing RNA from milk somatic cells, a large quantity of the RNA from leukocytes contaminated the RNA preparation. This rendered mRNA quantification by reverse transcription PCR problematic because of relative quantification by using a housekeeping gene, which is supposed to be expressed in all types of cells. It was possible to purify epithelial cells from somatic cells by using magnetic beads, as performed in humans (Alcorn et al., 2002). The present study thus had 2 objectives. One was to develop a new method to purify MEC from milk and to test whether this method was well suited to studying the mRNA levels of genes involved in the transport of glucose, lactose synthesis, and apoptosis. The other was to analyze some of the cellular mechanisms that might explain the effects of feed restriction and ODM on reductions in milk yield and mammary glucose uptake.

MATERIALS AND METHODS

Cows, Treatments, and Experimental Design

Five multiparous Holstein cows (657 ± 67 kg of BW) in their third or fourth lactation at the beginning of the experiment were studied. The cows were surgically prepared with an ultrasonic flow probe implanted around the right external pudic artery, and 2 permanent catheters were inserted into the right carotid artery and subcutaneous abdominal vein (Guinard-Flament et al., 2007) to estimate the mammary metabolism of nutrients.

The treatments were a 2×2 factorial arrangement of 2 milking frequencies and 2 feeding levels. Cows were milked twice daily (**TDM**, 12-h milking interval) or ODM (24-h milking interval) and fed to provide either 98 or 70% of the requirements of cows milked twice daily (Guinard-Flament et al., 2007). The experiment was divided into 2 main 3-wk periods, during which cows were fed either 98 or 70% of their needs during the first period, and diets were reversed for the next period. The first week of each period constituted a transition when cows were adapted to the feeding level and milked twice daily. During the second week, cows were either ODM or TDM, and during the third week, the milking frequency was reversed. One sequence of treatments was repeated on 2 cows to increase the amount of data.

On d 7 of each experimental week, milk and lactose yields and mammary glucose uptake were measured. On d 7, total milk cells were isolated from fresh milk and purified MEC were prepared. One cow was withdrawn from the study during the last week of the second period because of a dysfunctional catheter. No mastitis was observed during the experiment ($\text{SCC} = 147 \pm 306 \times 10^3/\text{mL}$).

Feeding

The ration provided 1.6 Mcal of NE_L and 97 g of protein truly digested in the small intestine (**PDI**) per kg of DM (Jarrige, 1989). The diet consisted of 70% corn silage, 14.5% energy concentrate, 14% soybean meal, and 1.5% minerals and vitamins (Guinard-Flament et al. 2007). The cows were fed twice daily and had access to feed for 8 h after each feeding (0715 to 1515 h and 1900 to 0300 h). The DM content of corn silage was determined daily to adjust the quantities offered. When feed was refused, the quantities were weighed.

Cell Isolation

Fresh milk (3.5 kg) was defatted by 15 min of centrifugation at $1,500 \times g$ at 4°C in several 230-mL tubes (VWR International, Fontenay sous Bois, France). The skim milk was removed and the remaining total cell pellet was resuspended and pooled in 150 mL of PBS (Gibco, Invitrogen, Cergy Pontoise, France). The cell suspension was washed twice in PBS and filtered through a 200- μm nylon membrane. After a final centrifugation ($1,000 \times g$, 10 min at 4°C), the cell pellet was resuspended in 2 mL of PBS containing 1% BSA (Sigma-Aldrich Chimie, Lyon, France). Twenty microliters of this cell suspension was analyzed with a hemacytometer (VWR International) by using light mi-

croscopy for cell count determinations. One milliliter of the 1% BSA-PBS cell suspension was used immediately for total RNA extraction of total milk cells. The remaining portion of the cell suspension was used for MEC purification with an immunomagnetic separation technique according to the method described by Alcorn et al. (2002), with some modifications.

Briefly, dynabeads (Pan Mouse IgG, Dynal Biotech, Invitrogen) were first coated with a primary mouse monoclonal antibody directed against cytokeratin 8 antibody (clone K8.13, Sigma-Aldrich Chimie), which was specific to bovine epithelial cells. To purify the 5 cell samples, a 100- μ L dynabead aliquot was washed with 1 mL of 1% BSA-PBS to remove the preservative. The solution was removed after incubating the tube in a magnetic particle concentrator (MPC-S; Dynal Biotech) for 30 s. A second washing was performed and the dynabeads were resuspended in 1 mL of 1% BSA-PBS. The dynabead suspension was incubated for 30 min with 2 μ L of anticytokeratin 8 antibody on a rotary mixer at 4°C. Excess unbound antibody was removed by aspiration of the supernatant after placing the dynabead-antibody suspension on the MPC-S for 30 s. Antibody-coated dynabeads were resuspended in 1 mL of 1% BSA-PBS and distributed into each reserved milk cell sample. To each 1-mL aliquot of the washed milk cell preparation was added 250 μ L of the bead suspension, corresponding to 10^7 beads. The antibody-bead complex and cell suspension were incubated for 1 h on a rotary mixer at 4°C. Specifically bound cells were collected by placing the sample vials in the MPC-S (1 min) after aspiration of the supernatant. Cells binding to dynabeads were washed with 1 mL of 1% BSA-PBS and resuspended in 1 mL of 1% BSA-PBS. A 10- μ L aliquot was collected for hemacytometer cell count determination. The solution of purified MEC with beads was used immediately for total RNA extraction.

RNA Extraction and Reverse Transcription

Total RNA was extracted from the purified milk MEC and total milk cell samples by using Trizol reagent, according to the manufacturer's protocol (Invitrogen). Briefly, cell pellets were obtained after centrifuging fresh cell suspensions ($5,000 \times g$, 4°C, 5 min). The cell pellets were homogenized in 1 mL of Trizol reagent by pipetting and were stored at -80°C until use. After defrosting at room temperature, the cells in Trizol were homogenized with a Turrax crusher (VWR International) at room temperature. For each sample, 200 μ L of chloroform was added to the homogenate, and after 2 min of incubation at room temperature, the mixture was centrifuged at $12,000 \times g$ for 15 min

at 4°C. The aqueous supernatant containing total RNA was recovered and mixed with 500 μ L of isopropyl alcohol and incubated for 10 min at 20°C to precipitate the RNA. The RNA was made into a pellet by centrifugation ($12,000 \times g$ for 10 min at 4°C), rinsed with cold 70% ethanol, and dissolved in sterile RNase-free water (Gibco, Invitrogen). The amounts of total RNA extracted from either total milk cells or purified MEC samples were determined by the bioanalyzer (Agilent Technologies, Massy, France). Total RNA quality was assessed by using the RNA Integrity Number (RIN) generated by Agilent 2100 Expert Software, version B.02 (Agilent Technologies).

Complementary DNA was generated from total RNA for the 2 types of cell samples by using a first-strand cDNA synthesis kit (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. Total RNA (250 and 100 ng of total milk cell and purified MEC samples, respectively) was incubated for 1 h at 42°C with 10 units of AMV (avian myeloblastosis virus) reverse transcriptase, 0.8 μ g of oligo(dT)₁₅ primer, 0.5 μ L of 50 units/ μ L of RNase inhibitor, 1 μ L of 1 mM deoxynucleotide mix, 1 μ L of reverse transcriptase 10 $\times$ buffer [10 mM Tris-HCl (pH 8.3), and 50 mM KCl], and 5 mM MgCl₂ in a total volume of 10 μ L. Finally, the mixture was heated to 95°C for 5 min to inactivate the AMV reverse transcriptase, and then the temperature was reduced to 4°C. Reverse transcript products were stored at -80°C

Primer Design

Gene sequences for primer design were obtained from the gene bank of the National Center for Biotechnology Information. Primer pairs for GLUT-1, SGLT-1, GAPDH, GAT-(1,4), κ -CN, bax, and bcl-2 were designed by using Primer Express Software, version 2.0 (Applied Biosystems, Foster City, CA) to generate amplification products of between 120 and 360 bp. The intron-exon structure of the cDNA was obtained by comparison with the bovine genomic sequence or by extrapolation with multialignment to the mouse or human genomic sequence. Forward and reverse primers were chosen to bind to different exons to avoid genomic DNA amplification (Table 1). Primer pairs were chosen to achieve reliable gene quantification according to their PCR melting peaks, following a melting curve analysis powered by ABI Prism 7000 Software, version 1.1 (Applied Biosystems). To verify PCR product size, they were run (data not shown) by 2% agarose gel electrophoresis using TBE buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.8), stained with ethidium bromide, and viewed by UV transillumination. The cyclophilin and α -LA primers were as pre-

Table 1. Characteristics of primer pairs for PCR gene amplifications concerning κ -CN, galactosyltransferase [GAT (1,4)], α -LA, type 1 glucose transporter (GLUT-1), type 1 sodium glucose cotransporter (SGLT-1), bcl2, bax, cytokeratin 8, GAPDH, and cyclophilin

Primer pair	Sequence, 5' → 3'	mRNA sequence localization	mRNA sequence reference	PCR product size, bp
κ CN				
Forward	TGCAATGATGAAGAGTTTTTCTCTAG	Exon 2	162810	151
Reverse	GATTGGGATATATTGGCTATTTTGT	Exon 4		
α -LA				
Forward	ACCAGTGGTTATGACACACAAGC	Exon 2	31342165	233
Reverse	AGTGCTTTATGGCCAACCACT	Exon 3, 4		
GAT-(1,4)				
Forward	TGCCCTGAGGAGTCCCC	Exon 1	381	151
Reverse	TGGCCACCTTGTGAGGAGAG	Exon 2		
GLUT-1				
Forward	GTCCTTTGAGATGCTGATCCTG	Exon 4	163104	198
Reverse	TGGTTGCCCATGATGGAGT	Exon 5		
SGLT-1				
Forward	TCCATCTTCAACAGTGCCAGC	Exon 11	21070364	267
Reverse	TCCCGGCTCATTGACTCTCTT	Exon 13		
bcl2				
Forward	TGTGGATGACCGAGTACCTGAA	Exon 2	2058667	124
Reverse	GACAGCCAGGAGAAATCAAACAG	Exon 3		
bax				
Forward	AGAAGCTGAGCGAGTGTCTGAA	Exon 3	1938370	271
Reverse	CGCTCTCGAAGGAAGTCCAA	Exon 6		
Cytokeratin 8				
Forward	AGTGGCTACGCAGGTGGACT	Exon 7, 8	480	181
Reverse	CCGCAAGAGCCTTTCACTTG	Exon 8		
GAPDH				
Forward	AAGGCCATCACCATCTTCCA	Exon 4	77404272	359
Reverse	GGGCCATCCACAGTCTTCTG	Exon 8		
Cyclophilin				
Forward	GGATTTATGTGCCAGGGTGGTGA	—	30038492	120
Reverse	CAAGATGCCAGGACCTGTATG	—		

viously reported (Bonnet et al., 2002; Schmitz et al., 2004).

Real-Time PCR

Real-time PCR was performed to measure the mRNA levels of 2 glucose transporters (GLUT-1, the principal passive glucose transporter, and SGLT-1, an active transporter), an enzyme involved in lactose synthesis [GAT-(1,4)] and its cofactor α -LA, a protein marker of MEC synthetic activity (κ -CN), and 2 proteins involved in apoptosis (bax proapoptotic and bcl2 antiapoptotic proteins).

Polymerase chain reaction was performed by using a SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. Briefly, a reaction mix was prepared by using 12.5 μ L of SYBR Green master mix (2 $\times$) with 0.5 μ M of each forward and reverse primer and RNase-DNase-free water. A total mix volume of 20 μ L was pipetted into the wells of a 96-well plate and 5 μ L of the cDNA product (diluted 25-fold) was added as a PCR template.

The real-time PCR amplification protocol consisted of a first step of 2 min of incubation at 50°C, followed

by a second step of 10 min of DNA polymerase activation at 95°C. A third step of 15 s at 95°C for cDNA denaturation, followed by 1 min at 60°C for annealing and extension reactions, was repeated 40 times. Finally, a fourth step represented a dissociation protocol during which a linear increase of 1°C/min was performed from 60 to 95°C. Continuous fluorescence acquisition was performed during this last PCR step.

Quantification of mRNA

A calibration curve for each studied gene and for candidate housekeeping genes was generated by using a single sample of reverse transcript product diluted several times (1:2, 1:10, 1:20, 1:100, 1:200, 1:500, and 1:1,000). The calibration curves were obtained by plotting the crossing threshold (Ct) against the log₁₀ value of serially diluted reverse transcript. Once the calibration curves had been plotted, the theoretical number of molecules (arbitrary unit) of each RNA could be calculated by using the linear regression slope for each gene determined from the calibration curve and always the same Y-intercept (arbitrary value), using a semiabsolute method as previously reported (Boutinaud et

Table 2. Mean ($\pm$ SEM) number of cells prepared, quantity and quality of total RNA (RIN), and cytokeratin 8 mRNA level in total milk cell samples and purified mammary epithelial cell (MEC) samples

Item	Total milk cells	Purified MEC
Number of cells prepared/mL of milk, 10^3	96 $\pm$ 3	1.5 $\pm$ 0.9
Total RNA quantity, μ g	32.2 $\pm$ 9.3	1.1 $\pm$ 0.4
Total RNA quantity, μ g/ 10^6 cells	0.28 $\pm$ 0.02	0.20 $\pm$ 0.02
RIN	4.1 ^a $\pm$ 0.7	8.0 ^b $\pm$ 0.4
Cytokeratin 8 mRNA level, arbitrary units	16.5 ^a $\pm$ 0.7	69.1 ^b $\pm$ 2.9

^{a,b}For $P \leq 0.05$.

al., 2004b). A nontemplate negative control was incorporated in all PCR runs. Two housekeeping genes were analyzed because the choice of standardization mode was crucial for in vivo samples. Cyclophilin (Bonnet et al., 2002) and GAPDH (Boulanger et al., 2003) were tested as the housekeeping genes classically used for mammary gland samples. The choice between the 2 housekeeping genes was made after studying the effects of treatments on their mRNA levels. The abundance of GAPDH mRNA was significantly higher and numerically higher in total milk cells and purified MEC, respectively, at the 98% feeding level when compared with the 70% feeding level (data not shown). By contrast, the abundance of cyclophilin mRNA did not differ significantly between feeding levels and milking frequencies (data not shown). Thus, the data for relative mRNA abundance were expressed as a ratio of cyclophilin mRNA levels.

Statistical Analysis

Data on mRNA levels were analyzed by using the MIXED procedure of SAS (SAS Institute, 1990), according to the following statistical model:

$$Y_{ijklm} = \mu + \text{cow}_i + \text{period}_j + \text{feeding level}_k \\ + \text{subperiod (period)}_{jl} + \text{milking frequency}_m \\ + \text{feeding level}_k \times \text{milking frequency}_m + \varepsilon_{ijklm},$$

with Y_{ijklm} as the variable dependent on cow i during period j receiving feeding level k and subperiod l and assigned to milking frequency m , and with ε_{ijklm} as the residual error associated with each $ijklm$ observation. Results were expressed as least squares means with the highest standard error of the means. The normality of relative mRNA levels was analyzed by using the Shapiro-Wilk test. Because the mRNA data were considered not normal, relative mRNA abundances were transformed to $\log_{10}$ to determine the effects of feeding level, milking frequency, and feeding level $\times$ milking frequency interaction. The data concerning relative mRNA levels are presented as model adjusted means

before log transformation. The threshold of significance was set at $P \leq 0.05$ and trends were noted at $P \leq 0.10$. Ribonucleic acid quality (RIN) and cytokeratin 8 mRNA levels in total milk cell and purified MEC samples were compared by using a t -test. Pearson correlation coefficients were determined among the data on mRNA levels, yields, and net mammary balance data for glucose (Guinard-Flament et al., 2007).

RESULTS

Number of Isolated Cells and Total RNA Quality

The number of total milk cells prepared from fresh milk was $16 \pm 10 \times 10^6$ cells, corresponding to $96 \pm 3 \times 10^3$ cells/mL of milk (Table 2). After purification by using magnetic beads coated with anticytokeratin 8 antibody, we collected 1.5×10^6 MEC/mL of milk. Purified MEC corresponded to $2.2 \pm 0.1\%$ of the total milk cells prepared. Marked variation in the number of total milk cells and purified MEC was observed, leading to variation in the percentage of MEC (from 0.09 to 9%).

Because of the number of cells prepared from each type of sample, purified MEC samples contained much less total RNA than total milk cell samples (Table 2). However, lower RNA quality was obtained from total milk cell samples than from MEC samples because RNA degradation was observed (Figure 1), and was confirmed by the low RIN values in total milk cell samples (Table 2). By contrast, no RNA degradation was observed in purified MEC samples with a higher ($P < 0.01$) RIN (Table 2 and Figure 1).

Cytokeratin 8 gene expression was analyzed by reverse transcription PCR to verify the efficiency of the purification method in both types of cell samples. As expected, cytokeratin 8 mRNA expression was higher ($P < 0.001$) in purified MEC samples than in total milk cell samples (Table 2). Only purified MEC were used to analyze the effect of treatments. Therefore, the results presented are only mRNA levels in purified MEC.

Effects of Treatments on DMI, Milk Yields, and the Mammary Glucose Balance

Results concerning DMI, milk yield and composition, and the mammary balance of glucose were reported

Figure 1. Quality of total RNA prepared from (A) total milk cell samples and (B) purified mammary epithelial cell samples (2 representative samples are shown, corresponding to 2 samples obtained from the same milk) analyzed by using the Agilent 2100 bioanalyzer (Agilent Technologies, Massy, France).

previously (Guinard-Flament et al., 2007). A summary of these results is presented in Table 3. During the last 3 d of treatment, the 98% feeding level provided, respectively, 19.9 kg/d of DMI, 29.8 Mcal/d, and, respectively, 1,912 g/d of net energy and PDI intake (Table 3). The 70% feeding level treatment provided 15.3 kg/d of DMI, 22.9 Mcal/d, and, respectively, 1,463 g/d of net energy and PDI intake (Table 3). The experimental conditions reduced the intakes of DM by 4.7 kg, of net energy by 23%, and of PDI by 24%.

Feed restriction and ODM provoked significant reductions in milk yield (–13 and –19%) associated with significant reductions in protein, fat, and lactose yields. Mammary glucose uptake decreased under both treatments (–22% with both treatments) as a result of depressed mammary blood flow (–15 and –12%, respectively, with feed restriction and ODM) and a reduced extraction rate (–12%) with ODM only. The ratio between glucose converted into lactose and glucose taken up by the mammary gland remained unchanged under ODM and feed restriction.

Effects of Treatments on Mammary Transcripts

Feed restriction reduced GLUT-1 mRNA levels (–53%, $P = 0.03$) and yielded a tendency ($P = 0.07$) to reduce SGLT-1 mRNA levels (Table 4). No significant effect of ODM was observed regarding glucose transporter mRNA levels.

κ -Casein and α -LA mRNA levels were reduced by –86 and –73%, respectively ($P < 0.001$), under ODM compared with TDM (Table 4). The GAT-(1,4) mRNA levels did not vary under either feed restriction or milking frequency (Table 4).

Feed restriction had no effect on the expression of genes related to apoptosis. By contrast, ODM had a stimulating effect on bcl2 and bax gene expression (9-fold up-regulation, $P = 0.010$, and 7-fold up-regulation, $P = 0.037$, respectively) resulting in no significant variation in the bcl2:bax mRNA ratio attributable to milking frequency.

Correlations Between Transcripts, Milk Yield, and Mammary Balance Data for Glucose

To validate the use of purified MEC to analyze mammary transcripts, consistency between transcript data and the other measurements performed was studied. κ -Casein, α -LA, GLUT-1, and SGLT-1 mRNA levels were significantly correlated with milk yields, lactose yields, and CN yields (Table 5). These relationships were involved directly in milk synthesis. No significant correlations were observed between GAT-(1,4), bcl2, and bax mRNA levels and milk yields and mammary balance data for glucose.

Moreover, significant correlations were observed between some mammary transcripts and mammary balance data for glucose. κ -Casein, α -LA, and GLUT-1, but not SGLT-1, mRNA levels were correlated with mammary blood flow, the arterial supply of glucose to the mammary gland, and mammary glucose uptake. Interestingly, a correlation was observed between α -LA mRNA levels and the glucose extraction rate ($P = 0.002$), whereas GLUT-1 mRNA levels were not correlated with the glucose extraction rate.

DISCUSSION

Evaluation of the MEC Purification Method

The cell purification method allowed the specific selection of epithelial cells from total milk cells. Before purifying epithelial cells, total milk cells were prepared as previously reported in the goat (Boutinaud et al., 2002) and cow (Murrieta et al., 2006). Approximately 96,000 total milk cells were prepared per milli-

Table 3. Effect of milking frequency (MF)¹ and feeding level (FL)² on feed intake, on the milk yield and milk composition of the right-half udder, and on mammary blood flow and the metabolism of glucose in the right-half udder on d 7 of treatments in dairy cows

Item	98%		70%		SEM	Effect		
	TDM	ODM	TDM	ODM		MF	FL	MF × FL
DMI, kg/d	20.4	19.5	15.2	15.3	0.67	0.50	0.006	0.47
Net energy intake, Mcal/d	30.4	29.2	22.8	22.9	0.99	0.51	0.006	0.47
PDI ³ intake, g/d	1,946	1,879	1,459	1,467	64.6	0.58	0.007	0.49
Milk yield, kg/d	13.3	10.6	11.4	9.3	0.37	0.0001	0.04	0.27
Milk protein								
g/d	439	357	349	297	12.3	0.0002	0.02	0.09
%	3.33	3.38	3.07	3.22	0.036	0.02	0.008	0.2
Milk fat								
g/d	533	484	456	378	33.2	0.05	0.09	0.58
%	4.05	4.66	3.99	4.06	0.268	0.23	0.27	0.32
Lactose								
g/d	652	508	556	429	15.8	0.0001	0.01	0.52
%	4.88	4.77	4.85	4.62	0.077	0.06	0.31	0.42
Mammary blood flow, L/min	5.76	5.11	4.91	4.28	0.203	0.007	0.03	0.97
Arterial glucose, mM	4.02	4.21	3.95	4.05	0.066	0.01	0.24	0.28
Glucose extraction rate, %	21.5	18.8	21	18.5	0.72	0.006	0.63	0.85
Mammary glucose uptake, mmol/min	3.73	2.92	3	2.25	0.122	0.0009	0.008	0.81
Lactose output:glucose uptake	73.7	72.2	79.3	79	2.9	0.75	0.1	0.84

¹TDM = twice-daily milking; ODM = once-daily milking.

²Feeding level: 98 and 70% of requirements determined the week before the trial.

³Protein truly digested in the small intestine (Jarrige, 1989).

liter of milk as expected (Feng et al., 2007). Purified MEC represented 2% of the total milk cells prepared. Only 2.7×10^6 purified MEC were prepared from 1.75 L of milk, which shows that very few MEC are present in bovine milk (Lee et al., 1980). Despite the small number of purified MEC collected, the immunomagnetic MEC purification method demonstrated excellent efficiency for isolating this population from total milk cells.

Because MEC are in the minority in the bovine milk cell population (Feng et al., 2007), the quantification of mammary mRNA in total milk cell samples was dependent on the proportion of MEC. As previously

reported in the goat (Boutinaud et al., 2002), we observed marked variations in the percentage of purified MEC obtained. Hence, the quantification of mammary transcripts in total milk cell samples by using a housekeeping gene expressed in leukocytes was not suitable. Variations in the levels of mammary transcripts in total milk cells, expressed as a ratio of housekeeping gene expression, would mainly depend on variations in the proportion of MEC. This method described for MEC purification prior to RNA extraction enables quantification without contamination from leukocyte RNA and suppresses variations in the proportion of MEC.

Table 4. Effect of milking frequency (MF)¹ and feeding level (FL)² on RNA levels of galactosyltransferase [GAT (1,4)], α -LA, κ -CN, type 1 glucose transporter (GLUT-1), type 1 sodium glucose cotransporter (SGLT-1), bcl2, and bax in purified mammary epithelial cells on d 7 of treatments in dairy cows³

Item	98%		70%		SEM	Effect ⁴		
	TDM	ODM	TDM	ODM		MF	FL	MF × FL
GLUT-1	567.8	362.1	185.9	225.0	56.5	0.64	0.034	0.27
SGLT-1	13.4	5.0	3.9	4.1	1.9	0.39	0.07	0.08
α -LA	63.3	24.5	82.3	14.7	12.1	0.002	0.60	0.25
GAT-(1,4)	94.6	104.8	75.5	95.1	16.0	0.21	0.52	0.42
κ -CN	75.3	15.1	55.2	3.2	12.1	0.0001	0.94	0.06
bcl2	2.5	58.9	11.3	84.1	34.37	0.01	0.33	0.58
bax	2.2	65.4	10.7	45.8	40.35	0.04	0.51	0.81
bcl2:bax	3.6	1.6	5.9	3.2	2.21	0.69	0.31	0.62

¹TDM = twice-daily milking; ODM = once-daily milking.

²Feeding level: 98 and 70% of needs determined the week before the trial.

³Data are expressed in arbitrary units as a ratio of cyclophilin mRNA levels.

⁴Statistical analyses were performed on log₁₀-transformed values.

Table 5. Pearson correlation coefficients between data on mRNA levels [galactosyltransferase (GAT (1,4), α -LA, κ -CN, type 1 glucose transporter (GLUT-1), type 1 sodium glucose cotransporter (SGLT-1), bcl2, and bax], and milk, lactose, and CN yields and net mammary balance data

Item	κ -CN	α -LA	GAT-(1,4)	GLUT-1	SGLT-1	bcl2	bax
Milk yield, kg/d	0.450	0.527	NS	0.592	0.452	NS	NS
Lactose, g/d	0.434	0.544	NS	0.567	NS	NS	NS
CN, g/d	0.466	0.481	NS	0.682	0.458	NS	NS
Mammary blood flow, L/min	0.495	0.493	NS	0.579	NS	NS	NS
Glucose arterial supply, mmol/min	0.469	0.465	NS	0.579	NS	NS	NS
Glucose extraction rate, %	0.596	0.680	NS	NS	NS	NS	NS
Mammary glucose uptake, mmol/min	0.593	0.614	NS	0.556	NS	NS	NS
κ -CN		0.832	NS	NS	NS	NS	NS
α -LA			NS	NS	NS	NS	NS
GAT-(1,4)				NS	NS	NS	NS
GLUT-1					0.578	NS	NS
SGLT-1						NS	NS
bcl2							0.829

The purification of MEC before RNA extraction was useful because it was possible to analyze non-mammary-specific genes such as bax and bcl2. Indeed, these 2 genes are expressed in all types of cells; for instance, they are involved in regulating apoptosis in milk neutrophils (Boutet et al., 2004). Moreover, leukocytes in milk undergo spontaneous apoptosis (Paape et al., 2003). Hence, study of the gene expression of bax and bcl2 in total milk cell samples is not appropriate for analyzing apoptosis in the mammary epithelium, unlike the analysis of such genes in purified MEC.

The quality of RNA was better in purified MEC samples than in total milk cell samples. The method used to purify cells, which requires that cells be in contact with beads, may allow the selection of cells that retain their membrane integrity, and eliminates cells containing degraded RNA. Previously, the quality of total RNA prepared from total milk cells in goats and cows was observed on agarose gel (Boutinaud et al., 2002; Murrieta et al., 2006). The bioanalyzer method was more accurate and reflected a concrete measurement of RNA degradation. The improved quality of total RNA from purified MEC samples provides further evidence that purified MEC are more appropriate than total milk cells for analysis of mRNA levels. Because of the sensitivity of reverse transcription PCR, it was possible to quantify mRNA levels with only the small amount of total RNA provided by the small number of purified MEC from milk.

Several analyses were performed to test the validity of the data obtained from purified MEC. Cytokeratin 8 mRNA levels were compared in cell samples before and after purification to confirm the epithelial state of the purified cell population. As expected, expression of cytokeratin 8 was greater in purified MEC samples than in total milk cell samples. The mRNA levels of the other transcripts analyzed in purified MEC exhibited significant correlations. The consistency of the results

obtained with purified MEC was confirmed by comparisons with the other measurements performed during this study. Messenger RNA levels in the 2 milk protein genes and 2 glucose transporter genes studied revealed significant correlations with milk yields and with mammary balance for glucose. Moreover, the analysis of mammary transcripts in purified MEC provided consistent results concerning the effect of ODM. Indeed, the regulation of α -LA mRNA levels in purified MEC under ODM was in agreement with the regulation of lactose synthase enzyme activity reported in ODM cows (Farr et al., 1995), suggesting that purified MEC constitute a valuable material for analyzing the effects of the treatments on mammary transcripts.

Effects of Feed Restriction

The results presented show that feed restriction induced a significant reduction in GLUT-1 mRNA levels and a trend toward a reduction in SGLT-1 mRNA levels, but no effects on α -LA, GAT-(1,4), κ -CN, bcl2, and bax mRNA levels. To our knowledge, no previous studies have reported on either GLUT-1 or SGLT-1 mRNA level variations in MEC attributable to diet. Starvation induced a down-regulation of α -LA and several CN genes in goat mammary glands (Ollier et al., 2007). The lower level of GLUT-1 mRNA may induce a decrease in the amount of GLUT-1 protein. Further studies are needed to analyze GLUT-1 protein levels and confirm this hypothesis. In addition to mRNA modifications, feed restriction may act on the subcellular localization of GLUT-1, insofar as GLUT-1 translocation from the plasma membrane to the Golgi apparatus was a major control mechanism regarding glucose transport into mammary cells after starvation (Prosser, 1988; Camps et al., 1994).

The correlation between GLUT-1 mRNA levels and arterial supplies of glucose to the mammary gland ($r =$

0.58) raises the question of whether the regulation of GLUT-1 mRNA is responsible for the reduction in glucose uptake or if it is a consequence of cell adaptation to the lower glucose supply to the mammary gland. More generally, GLUT-1 and SGLT-1 mRNA levels were correlated with milk, lactose, and protein yields, suggesting either that the regulation of glucose transporter transcription is a determinant for milk production, or that MEC may adjust the expression of glucose transporters to the level required for milk production. Because GLUT-1 was shown as the main glucose transporter expressed in the mammary gland (Zhao et al., 1996), regulation of the glucose mammary extraction rate may involve the regulation of GLUT-1 transcription. Still, our data suggest that the regulation of glucose transporter transcription was not a factor that affected the ability of MEC to extract arterial glucose. Indeed, glucose extraction rates did not vary under feed restriction, whereas GLUT-1 mRNA levels were reduced.

Despite the decrease in GLUT-1 mRNA levels, MEC retained their ability to produce lactose, because α -LA, GAT-(1,4) mRNA levels did not vary. It appears that MEC can adapt their metabolism to compensate for loss in the glucose available for lactose synthesis.

Considering the effect of feeding level, the reductions in glucose mammary uptake and milk yield involved a clear down-regulation of glucose transporter gene expression at a cellular level without affecting mRNA indicators of lactose synthesis and cell death, in agreement with the absence of a low-energy diet effect on apoptosis in the bovine mammary gland (Norgaard et al., 2005).

Effects of ODM

The milking frequency treatment produced modifications to gene expression that were supported with the decrease in mammary glucose uptake. Still, these modifications triggered mechanisms that differed from those arising under feed restriction. Once daily milking decreased the α -LA and κ -CN mRNA levels, increased the bax and bcl2 mRNA levels, but had no effects on GAT-(1,4), GLUT-1, and SGLT-1.

Once daily milking induced significant reductions in both mammary glucose uptake and the mammary glucose extraction rate (Table 3; Guinard-Flament et al., 2007). A key effect of ODM on glucose uptake was probably due to regulation at a level other than that regulating glucose transporter transcription because GLUT-1 and SGLT-1 were unaffected by milking frequency. A degree of this regulation could be due to prolactin, because it was required to maintain GLUT-

1 protein levels in the rat mammary gland through posttranscriptional regulation (Baldwin et al., 1994). Hence, ODM may induce a decrease in GLUT-1 protein levels as a result of the reduced prolactin release into plasma induced by milking.

One of the main ODM effects on mammary transcripts was a marked reduction in α -LA mRNA levels, but no effect on GAT-(1,4) mRNA levels. α -Lactalbumin is known to alter the enzyme specificity of GAT-(1,4) and regulate its activity for lactose synthesis. Therefore, a drop in α -LA mRNA levels could result in a concomitant reduction in lactose synthesis (Table 3). Similarly to α -LA, ODM decreased κ -CN mRNA levels. The effect of ODM on CN expression was reported in cows, where 2 d of ODM induced a -20% decrease in β -CN mRNA levels (Yang et al., 2005). Our results showed a correlation between κ -CN and α -LA mRNA levels ($r = 0.83$). A decline in milking-induced prolactin release into plasma under ODM may be responsible for the coordinated down-regulation of the 2 genes' expression.

Significant correlations were observed between α -LA and κ -CN mRNA levels and milk, protein, and lactose yields (Table 5). These results suggest that the decrease in milk synthesis under ODM probably involves the regulation of milk protein transcription. As expected, a high correlation was obtained between α -LA mRNA levels and lactose yield ($r = 0.54$). The mRNA levels of α -LA and κ -CN were correlated with data on the mammary balance for glucose (Table 5). The mRNA level of α -LA was particularly correlated with the mammary glucose extraction rate ($r = 0.68$). Thus, reductions in mammary glucose uptake and glucose extraction rate during ODM may be due to a reduced glucose demand by MEC to achieve lactose synthesis.

Once daily milking induced a modulation of genes involved in apoptosis by increasing proapoptotic bax and antiapoptotic bcl2 mRNA levels. Nonetheless, the bcl2:bax ratio did not vary. Insofar as cell death was demonstrated after 3 wk of ODM (Li et al., 1999), ODM may initiate cell death after 7 d of treatment by activating the transcription of genes involved in regulating cell death in the mitochondria. The effect of milk accumulation on the up-regulation of bax was reported in the bovine mammary gland (Singh et al., 2005). The elevation of bcl-2 expression could be the consequence of a compensatory effect with respect to cell death. Conversely, not all the mechanisms that lead to cell death in the mammary gland are yet understood. More genes involved in cell death and indicators other than mRNA need to be analyzed to confirm the onset of cell death after 7 d of ODM.

The reduction in glucose mammary uptake and milk yield under ODM was associated at the cellular level with a clear down-regulation of α -LA and κ -CN expression without affecting glucose transporter transcription. Moreover, the modulation of cell death may be involved in regulating the milk yield after 7 d of ODM.

CONCLUSIONS

The immunomagnetic method used to purify MEC from milk cells can improve the quantification of mammary transcripts. This method enabled investigation of the mammary nutrient balance and the level of mammary transcripts, but without damaging mammary tissue.

The results indicate that feed restriction and ODM exert opposite effects on MEC in terms of modulating glucose uptake and milk synthesis. The effects of feed restriction on milk yield and mammary glucose uptake were associated with a down-regulation of glucose transporter gene expression, associated with a reduction in the supply of glucose to the mammary gland. Thus, during feed restriction, the entry of glucose into MEC may be more limited than the capacity of these cells to synthesize lactose. The regulation of cell death was probably not involved after 7 d. In contrast, ODM induced a decrease in the levels of α -LA and κ -CN transcripts associated with decreases in milk and lactose synthesis. Limitation for the use of glucose for lactose synthesis by MEC may be responsible for lower glucose extraction rates under ODM. The induction of cell death under ODM may be involved in reducing milk yield.

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