Differential expression and localization of lipid transporters in the bovine mammary gland during the pregnancy-lactation cycle

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ABSTRACT

The transport of lipids across mammary gland epithelial cells (MEC) determines milk lipid content and composition. We investigated the expression of lipid transporters and their regulators in comparison to blood metabolites during lactation and dry period (DP) in dairy cows. Repeated mammary gland biopsies and blood samples were taken from 10 animals at 7 stages of the pregnancy-lactation cycle. Expression levels of the specific mRNAs were determined by quantitative reverse transcription-PCR, whereas ABCA1 was localized by immunohistochemistry. Blood serum metabolites were determined by common enzymatic chemistries. Elevated mRNA profiles of *ABCA1* and *ABCA7* were found during DP as compared with lactation and were inversely associated with blood cholesterol levels. Elevated levels of *ABCG2*, *NPC1*, *SREBP1*, *SREBP2*, *LXRα*, and *PPARγ* were found postpartum, whereas *ABCG1* did not differ between the functional stages of the mammary gland. The ABCA1 protein was localized in MEC and showed differential activity between DP and lactation suggesting a role of ABCA1 in the removal of excess cellular cholesterol from MEC during the DP. The expression profiles of *ABCA7* and *NPC1* may reflect a role of these transporters in the clearance of apoptotic cells and the intracellular redistribution of cholesterol, respectively. Regulation of lipid transporters in the mammary gland is partially associated with transcription factors that control lipid homeostasis.

Key words: ATP-binding cassette transporter, cholesterol, lipid homeostasis, mammary gland

INTRODUCTION

The development of the mammary gland during pregnancy, lactation, and involution comprises complex physiological processes. The bovine mammary gland

epithelium is established during the first pregnancy and during the dry periods (DP) between the subsequent lactations for the next cycle of milk production to synthesize, transport, and secrete milk constituents such as lipids, proteins, and solutes during the lactation phase. Several mechanisms for milk lipid secretion were proposed. One possible mechanism is that milk fat is secreted after synthesis in the smooth endoplasmic reticulum of mammary gland epithelial cells (MEC) by a unique mechanism in which cytoplasmic lipid droplets are formed. These lipid droplets move to the apical membrane to be secreted as membrane-bound milk fat globules. A second possible mechanism is the transcellular movement of substances across the basal and apical membranes of alveolar cells by membrane transport proteins that actively transfer compounds across cellular membranes (Anderson et al., 2007).

Candidate transporters for the active transport of milk constituents are members of the ATP-binding cassette (ABC) transporter superfamily, one of the most ancient transporter families. The ABC transporters facilitate the efflux of various substrates including metabolites, lipids, and drugs across the cellular membranes by the hydrolysis of ATP. Prokaryotic antibiotic resistance and eukaryotic drug resistance is associated with ABC transporters; for example, ABCB1 and ABCG2, also known as P-glycoprotein, and breast cancer resistance protein (BCRP), respectively. Interestingly, the drug transporter ABCG2 was reported to be strongly induced postpartum in the mammary gland of humans, cows, and mice, to be localized at the apical membrane of MEC, and to participate in the accumulation of drugs and xenotoxins in mouse milk (Jonker et al., 2005). Cohen-Zinder et al. (2005) localized a QTL for milk production genes in dairy cows within the ABCG2 region.

Some members of the ABC superfamily have been functionally involved in cellular lipid transport. A prominent member is ABCA1, which mediates the transport of cholesterol, phospholipids, and other lipophilic molecules across cellular membranes to remove excess cellular cholesterol by export into the high-den-

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sity lipoprotein (**HDL**) pathway (Ikonen, 2008). The *ABCA1* gene is associated with cardiovascular disease and hereditary diseases such as Tangier disease or HDL deficiency (Albrecht et al., 2004a). In this context, it has been shown that missense mutations in the *ABCA1* gene resulted in altered protein trafficking to the plasma membrane and impaired efflux of cellular cholesterol to apolipoprotein A1 (**apoA1**; Albrecht et al., 2004a). Recently, *ABCA1* was demonstrated to transfer cholesterol not only by efflux mechanisms but also by transcytosis through aortic endothelial cells (Cavelier et al., 2006). The half transporter ABCG1 supports *ABCA1* in its function by transferring excess cholesterol from the cell onto mature HDL and thereby reduces cellular cholesterol accumulation (Ikonen, 2008).

The full-size ABC transporter *ABCA7* has the highest known homology to *ABCA1*, has a distinct expression profile from that of *ABCA1*, and is believed to bind lipoproteins to promote the efflux of cellular phospholipids without cholesterol. The gene *ABCA7* mimics *ABCA1* to mediate the production of HDL from cellular lipid when transfected in vitro and may be involved in lipid metabolism in kidney and adipose tissue (Abe-Dohmae et al., 2006). Recently it was shown that *ABCA7*, rather than *ABCA1*, is involved in the engulfment of apoptotic bodies by macrophages (Jehle et al., 2006).

The Niemann-Pick disease related protein 1 (*NPC1*) mediates subcellular cholesterol transport of low-density lipoprotein (**LDL**)-derived cholesterol from late endosomes to other cellular compartments to maintain cholesterol homeostasis. Mutations in the Niemann-Pick disease genes cause lysosomal cholesterol accumulation and impaired LDL cholesterol esterification (Ory, 2004).

Lipid homeostasis in peripheral cells is regulated by several transcription factors including nuclear receptors and sterol responsive element binding proteins (**SREBP**). The liver X receptor (**LXR**) α together with the retinoid X receptor (**RXR**) acts as a heterodimer and activates the transcription of genes involved in cholesterol homeostasis such as *ABCA1*; *LXR* α is activated by oxysterols that are produced by oxidation when intracellular cholesterol levels are high (Schmitz and Langmann, 2005). Other regulators of lipid homeostasis genes are the peroxisome proliferator-activated receptors (**PPAR**). These proteins act as nutritional sensors that regulate a variety of homeostatic functions including metabolism, inflammation, and development. Peroxisome proliferator-activated receptor α , is the main metabolic regulator for catabolism, whereas *PPAR* γ regulates anabolism or storage and activates the transcription of *RXR* and *LXR* genes in macrophages (Schmitz and Langmann, 2005). The **SREBP**

are important regulators of lipogenesis and cholesterol synthesis; **SREBP-1c** activates lipogenic genes when cholesterol levels are high, whereas **SREBP2** induces among other target genes the expression of cholesterol synthetic genes (Desvergne et al., 2006).

The ABC transporters play a substantial role in hereditary human diseases. However, only scarce information exists about the expression and localization of ABC transporters in the mammary gland (Cohen-Zinder et al., 2005; Jonker et al., 2005; Farke et al., 2006, 2008; Viturro et al., 2006; Bionaz and Loo, 2008). In addition, their function in the mammary gland remains mostly elusive. As the lipid transporters described previously are crucial for maintaining lipid homeostasis in peripheral tissues, we hypothesized that 1) the milk lipid composition depends on the differential expression and regulation of lipid transporters in mammary gland tissue; and 2) expressional and functional differences of these lipid transporters are associated with the remodeling processes and the metabolic changes occurring in the mammary gland during the cycle of lactation. Therefore, the objectives of this work were 1) to study expression levels of genes involved in lipid transport and homeostasis in the bovine mammary gland during various functional stages; 2) to investigate potential relationships between mRNA expression profiles and relevant metabolite concentrations in the blood; and 3) to determine in parallel mRNA expression and the cellular localization of selected transporters in mammary tissue at different stages of the pregnancy-lactation cycle.

MATERIALS AND METHODS

The animal experiment was conducted according to protocols approved by the Danish Animal Experiments Inspectorate and complied with the guidelines of the Danish Ministry of Justice concerning animal experimentation and care of animals under study.

Animals, Diets, and Sampling

The experimental setup, animal characteristics, and sampling procedures were described previously (Sorensen et al., 2006). Briefly, 10 Holstein-Friesian dairy cows of the first to fourth parity were housed in tie-stalls, milked twice daily, and fed a ration based on grass silage and concentrate. The feeding regimen was ad libitum throughout lactation and restricted during the DP according to current recommendations. The cows were artificially inseminated according to herd procedure at approximately d 60 (between 45 and 76) after parturition. Two cows were reinseminated at d 122 and 169. The cows were dried off 52 d before expected parturition. Seven mammary gland biopsies were ob-

tained from each cow. All biopsies were obtained from front quarters at 7 lactational stages (end of lactation at d 77 before next parturition; during the DP at d 48 and d 16 before parturition; during lactation at d 14, 42, 88, and 172). The cows were healthy, although 4 were treated once for mastitis within 5 d of biopsy sampling (Sorensen et al., 2006). Eight cows received a prophylactic antibiotic treatment at dry off. Milk yield was within the expected range and not affected by biopsy sampling, and the cows can be regarded as a representative sample of the dairy cow population. The majority of biopsy tissue was snap frozen in liquid nitrogen and kept at -80°C until analysis of mRNA abundance. A small portion of tissue was used for immunohistochemical analysis, and was fixed overnight in 4% neutral buffered formalin, dehydrated, and embedded in paraffin according to standard techniques. Blood samples were obtained from the jugular vein; serum was harvested and kept at -80°C until analyzed for metabolites.

Isolation of RNA, Reverse Transcription, and PCR

Cow mammary gland sample preparation for analysis of mRNA abundance was carried out as described by Sorensen et al. (2006). Briefly, mammary tissue was homogenized and total RNA was purified using the RNeasy Mini kit (Qiagen, Crawley, UK). Purified RNA was reverse-transcribed using Superscript II RNaseH reverse transcription kit (Invitrogen, Taastrup, Denmark). Complementary DNA was stored at -20°C . Specific primers for the analysis of candidate genes were designed to span exon-exon boundaries (Table 1). Primers were tested by conventional PCR and were analyzed by gel electrophoresis on 1.5% agarose gels. The bands were cut, frozen, and sequenced for confirmation of the specificity of each primer pair. With the same primers, quantification by quantitative reverse transcription PCR was performed using Power SYBRGreen Master Mix (Applied Biosystems, Foster City, CA) in a final volume of 25 μL , a final primer concentration of 150 nM, and 2 μL of template. Quantitative reverse transcription PCR analyses were performed on an ABI Prism 7500 real-time PCR detection system (Applied Biosystems). Prior to annealing, a polymerase activation step at 95°C was performed for 10 min. For each primer pair, annealing was performed at 60°C for 1 min. In every PCR run, a nontemplate control consisting of primers and water was included for each primer pair. In addition, standard curves were generated from dilution series of pooled samples to determine detection limits and efficiencies for each primer pair. Amplified products underwent melting curve analysis to specify

the integrity of amplification. To remove nonspecific signals, a higher fluorescence acquisition temperature was chosen (Table 1), according to the melting curve that was recorded for each reaction.

Immunohistochemistry

Serial sections of paraffin-embedded tissue (4 μm) were prepared and picked up on SuperFrost (SuperFrost, Braunschweig, Germany) slides. After drying, sections were dewaxed in xylol and hydrated through graded alcohols. Endogenous peroxidase activity was blocked by dipping the slides in 3% H_2O_2 solution for 10 min. An antigen recovery step in sodium citrate buffer (10 mM, pH 5.5) was performed in a microwave oven at 98°C for 5 min. After blocking with 4% BSA, slides were incubated in a humid chamber at 4°C overnight with or without (negative controls) ABCA1-specific polyclonal antibody supplied by Abcam (ab 7360, Cambridge, UK). After washing the slides, affinity-purified, biotin-conjugated goat anti-rabbit antibody (Dako, Glostrup, Denmark) was applied for 60 min at room temperature in a humid chamber. After washing, slides were exposed for 45 min to avidin-biotin-horseradish peroxidase complex prepared from reagents supplied by Dako. Bound peroxidase was detected in a 7- to 12-min incubation at room temperature with 3,3'-diaminobenzidine substrate. Sections were finally briefly counterstained with hematoxylin and mounted in Aquatex (Merck, Darmstadt, Germany).

Determination of Blood Serum Metabolites

The plasma concentrations of glucose, cholesterol, and triglycerides were measured by using enzymatic kits purchased from Biomérieux (#61270, #61219, and #61236, respectively; Marcy l'Etoile, France). The concentrations of NEFA in plasma were measured with a kit from Wako Chemicals (#994-75409, Neuss, Germany).

Quantification and Statistics

To evaluate mRNA quantities, data were obtained as cycle threshold values (C_T ; the cycle number at which logarithmic plots cross calculated threshold lines). Samples were measured in duplicates. The C_T values were used to determine ΔC_T values (ΔC_T = arithmetic mean C_T value of the 3 housekeeping genes β -actin, ubiquitin, and GAPDH minus the C_T value of the target gene). Variation of the housekeeping genes between days of the pregnancy-lactation cycle was determined by one-way ANOVA using the statistical software Kaleida-

Table 1. Nucleotide and PCR product specifications

Gene ¹	Nucleotide sequence [5' to 3']	Accession number	Acquisition temperature (°C) ²	Fragment length (bp)
ABCG2	GCTCCTGAAGAGGATGTC CAGCGGAAACCTATGGCTC	NM_001037478.2	81	174
ABCA1	GGACATGTGCAACTACGTGG TGATGGACCACCCATACAGC	NM_001024693	78	133
ABCA7	CACAATGTCCATCCTGAGTG CATCCACAGTCAGTAGGTCA	XM_589159.3	77	152
ABCG1	GACTCGGTCTCCTCACGCAC CGGAGAAACACGCTCATCTC	XM_587930	81	203
NPC1	GCTTCTGGTGGATTGCGAAA GTTTCATCTATCTGGTAGGTC	NM_174758.2	77	199
LXR α	CTGCGATTGAGGTGATGCTC CGGTCTGCAGAGAAGATGC	NM_001014861.1	78	229
PPAR γ	CTCCAAGAGTACCAAAGTGCAATC CCGGAAGAAACCCCTTGCATC	NM_181024.2	78	198
SREBP1	GACGGCCAGGTGAATCCAGA CAGGACCATCTCTGCCCTCA	NM_001113302	78	207
SREBP2	GACTGATGCCAAGATGCACA CCCTTCAGGAGTTTGCTCTT	XM_583656.3	78	140
GAPDH	GGACTTATGACCACTGTCCA ATGCCAGTGAGCTTCCCCTT	NM_001034034	81	175
Ubiquitin	TTCACAGGTCAAAATGCAGA ATCTGCATACCACCCCTCAG	NM_174133.2	81	237
β -Actin	AACTCCATCATGAAGTGTGACG GATCCACATCTGCTGGAAGG	NM_173979.3	78	214

¹ABCG2, ABCA1, ABCA7, ABCG1 = different ATP-binding cassette transporter genes; NPC1 = Niemann-Pick disease related protein 1; LXR α = liver X receptor α ; PPAR γ = peroxisome proliferator-activated receptor γ ; SREBP1 and SREBP2 = sterol responsive element binding proteins.

²After annealing at 60°C, the fluorescence acquisition step was performed at the indicated temperature for 32 s to avoid measurement of nonspecific products.

Graph (SynergySoftware, Reading, PA). Least squares mean ΔC_T values with standard errors of the mean were calculated for each time point using SAS statistical software (SAS Institute Inc., Cary, NC). Fold difference values were calculated using the $2^{(-\Delta\Delta C_T)}$ method described in the ABI user bulletin number 2 (Livak and Schmittgen, 2001). All statistics were performed at the ΔC_T level to avoid bias by a transformation of normally distributed logarithmic values in not normally distributed fold difference values. For the determination of metabolite levels, the mean concentrations $\pm$ standard errors of the means were calculated for each time point. To test whether mRNA abundance and metabolite concentrations were significantly different between the stages, the Mixed Procedure in SAS was applied using ΔC_T values (Littell et al., 1998). In Figures 1, 2, and 3, a–d superscripts indicate significantly different mean values ($P < 0.05$). Correlations are expressed as Pearson correlation coefficients on raw data of relative mRNA profiles and metabolite profiles, entering ΔC_T values and metabolite concentrations using the Corr Procedure in SAS. A positive or negative correlation was considered as statistically significant when $P < 0.05$ (Table 2).

RESULTS

Differential mRNA Expression of Lipid Transporters and Their Regulators

To determine whether lactation changes the gene expression of transporters and/or their regulators, relative mRNA abundance in mammary gland tissues at different days of the pregnancy-lactation cycle was compared. Variance analysis of the C_T values originating from the arithmetic means of the housekeeping genes β -actin, ubiquitin, and GAPDH by one-way ANOVA revealed no significant changes between the functional stages of the mammary gland ($P = 0.2885$, data not shown). In Figures 1A and 2, the mRNA abundances (ΔC_T values as described in the Quantification and Statistics section) and statistical analyses of all candidate genes are summarized. Relative mRNA abundance of ABCG2 was significantly down-regulated by a factor of 11.6 after drying off [$2^{(-\Delta\Delta C_T)}$; d –48 vs. –77; $P < 0.0001$], and then significantly increased 51.3-fold postpartum (d –16 vs. 14; $P < 0.0001$) to constant levels during lactation (Figure 2). The lipid transporters ABCA1 (Figure 1A) and ABCA7 (Figure 2) were significantly increased

2.9 and 3.2-fold, respectively, during the dry-off phase compared with lactation (d 42 vs. -16; $P = 0.0324$ and $P < 0.0001$, respectively). Moreover, ABCA7 was upregulated 3.5-fold after drying off (d -77 vs. -16; $P < 0.0001$). At this stage, ABCA1 levels showed the same trend but did not reach statistical significance (Figure 1A). Mean values of ABCG1 showed no significant differences between the different stages (Figure 2). The mRNA abundance of the intracellular cholesterol transporter NPC1 was significantly down-regulated by a factor of 2.4 after drying off (d -16 vs. -77; $P = 0.0067$) and then increased 3.5-fold after parturition (d -16 vs. 42; $P = 0.0004$). The mRNA abundance of LXR α significantly increased 2.2-fold after parturition (d -16 vs. 14; $P = 0.0134$). The mRNA expression of PPAR γ was significantly down-regulated by a factor of

2 four days after drying off (d -48 vs. -77; $P = 0.0326$) and then was elevated 2.6-fold throughout lactation (d -48 vs. 172; $P = 0.0015$). The protein SREBP1 was significantly down-regulated by a factor of 2.5 after the end of the previous lactation (d -77 vs. 48; $P = 0.0233$); it was enhanced 6.8-fold after parturition and throughout lactation (d -16 vs. 172; $P < 0.0001$), reaching the highest value at d 172 postpartum; a similar expression pattern was observed for SREBP2 where a 2.3-fold increase was found after drying off until d 172 (d -48 vs. d 172; $P < 0.0056$).

Blood Serum Metabolite Profiles

The results of metabolite profiles at the different functional stages of the mammary gland are shown in

Figure 1. Relative mRNA abundance and immunohistochemical localization of ATP-binding cassette transporter A1 (ABCA1) in mammary gland tissues during the dry period and lactation. A) ABCA1 mRNA abundance was determined by quantitative reverse transcription PCR. Cycle threshold (C_T) values were normalized to the arithmetic mean of 3 housekeeping genes (β -actin, ubiquitin, and GAPDH) to obtain ΔC_T values and are presented as box whisker plots ($n = 7$ to 10). The box contains 50% of the data, the median (line), the mean (filled circles), and the upper (75%) and lower (25%) quartiles. The whiskers indicate maximal and minimal values without outliers. Outliers (empty circles) are defined as values being either higher than [upper quartile + $1.5 \times$ interquartile distance] or smaller than [lower quartile - $1.5 \times$ interquartile distance]. Significantly different means ($P < 0.05$) are marked by the letters a and b. B) The left and middle panels show immunohistochemical staining of ABCA1 protein (dark coloring) in mammary gland biopsies taken from a representative animal at d 16 before expected parturition and at d 42 of lactation. For the negative control (right panel), only secondary antibody was applied. Magnification = $40\times$. Color version available online at <http://jds.fass.org/content/vol92/issue8/>.

Figure 3. Concentrations of NEFA in the blood serum were significantly increased after parturition (d -16 vs. 14; $P < 0.0001$) and decreased to baseline levels at d 88 (d 14 vs. 88; $P < 0.0001$). In contrast, cholesterol levels decreased from high levels during the dry-off phase to lowest levels at the onset of lactation (d -48 vs. 14; $P < 0.0001$) and increased during lactation to maximum levels at d 172 (d 14 vs. 172; $P < 0.0001$). Glucose levels decreased by 20% after parturition and increased by 30% until peak lactation at d 88 (d -16 vs. 14; $P < 0.0001$; d 14 vs. 88; $P < 0.0001$, respectively). Triglyceride concentrations were elevated after drying off by 40% (d -77 vs. -48; $P = 0.0004$). In early lactation, at d 14, the lowest triglycerides concentrations were observed; triglyceride levels increased during lactation by 70% until d 88 (d -14 vs. -88; $P < 0.0001$) and then decreased again by 20% until d 172 (d 88 vs. 172; $P < 0.0127$).

Correlations Between Transporters, Regulators, and Metabolites

To investigate potential associations between mRNA expression patterns and metabolic parameters, Pearson correlation analysis was performed. In Table 2, Pearson coefficients of significant ($P < 0.05$) correlations are listed. Comparison of the expression patterns between lipid transporters revealed positive associations between the cholesterol transporters ABCA1, ABCA7, and ABCG1 (Figure 4A). Comparisons between transporters and regulators revealed positive correlations between ABCA7 and LXR α , and between ABCG1, LXR α , and PPAR γ (Table 2). Interestingly, NPC1 showed a strong positive correlation with PPAR γ , SREBP1 (Figure 4B), LXR α , and SREBP2 (Table 2). A similar pattern was found for ABCG2 (Table 2).

Correlation analysis between transporter mRNA profiles and blood serum metabolites revealed negative correlations between ABCA1, ABCA7, ABCG1, LXR α , and cholesterol levels (Table 2). The strikingly opposite trend of ABCA1 mRNA abundance and cholesterol serum concentrations is illustrated in Figure 4C. Non-esterified fatty acids positively correlated with ABCG2, LXR α , and NPC1; glucose negatively correlated with ABCG2. Triglycerides were inversely correlated with ABCG2, PPAR γ , and SREBP2 mRNA profiles.

Immunohistochemical Localization of ABCA1

Biopsy sections from the different stages in the pregnancy-lactation cycle ($n = 6$ animals per stage) were stained with a polyclonal antibody against ABCA1 and were evaluated by qualitative analysis. To exclude non-specific staining, negative controls were treated with

Figure 2. Relative mRNA abundances in the bovine mammary gland during the cycle of lactation. Relative mRNA abundance was determined by quantitative reverse transcription PCR. Delta cycle threshold (ΔC_T) values are presented as box whisker plots ($n = 7$ to 10). For definition of the box whisker plots, see Figure 1. The ATP-binding cassette transporter A1 (ABCA1) mRNA profile is shown in Figure 1A. Significantly different means ($P < 0.05$) are marked by the letters a-c. ABCG2, ABCA7, ABCG1 = different ATP-binding cassette transporter genes; NPC1 = Niemann-Pick disease related protein 1; LXR α = liver X receptor α ; PPAR γ = peroxisome proliferator-activated receptor γ ; SREBP1 and SREBP2 = sterol responsive element binding proteins.

secondary antibody alone and showed no staining and no background (Figure 1B, right panel). The ABCA1 protein showed differential localization in epithelial cells depending on the stage of the pregnancy-lactation cycle. At the end of lactation at d -77 relative to parturition, ABCA1 was predominantly localized at the apical plasma membrane of alveolar cells (data not shown). In late pregnancy, at day -16 relative to parturition, almost all alveolar cells were highly positive for ABCA1 and showed predominantly cytoplasmic and membrane staining (Figure 1B, left panel). Some myoepithelial cells and vessels were also positive. During lactation, at d 42 after parturition, ABCA1 was localized in only few epithelial cells, where it was basally distributed (Figure 1B, middle panel); some animals showed cells with perinuclear ABCA1 distribution. Throughout lactation, only few epithelial cells were positively stained with low intensity. In contrast to differential ABCA1 protein expression between the dry-off phase and lactation in epithelial cells, stromal cells were stained with similar intensity and localization throughout all stages (Figure 1B). These preliminary results indicate that in the mammary gland ABCA1 protein expression follows a pattern similar to the ABCA1 mRNA abundance (Figure 1A).

DISCUSSION

The mRNA Expression of Transporters and Regulators and Their Relationship to Metabolite Levels

This work describes differential expression patterns of lipid transporters during lactation and DP in the bovine mammary gland. The mRNA profiles of candidate transporters and their regulatory genes were seen in the context of the metabolic state reflected by metabolic parameters measured in the blood serum of the same animals. The mRNA abundance of ABCA1 was elevated during the DP and early lactation (Figure 1). It is well established that ABCA1 prevents cholesterol accumulation by mediating cholesterol efflux onto lipid-poor apoA1 in peripheral cells (Ikonen, 2008). Therefore, as lipids accumulate in the mammary gland during involution caused by cessation of milking and tissue remodeling processes, it is tempting to speculate that ABCA1 plays a role in removing excess cholesterol from epithelial cells during the DP. In concordance with the established role of ABCA1 in reverse cholesterol transport in peripheral cells, it is possible that this transporter is implicated in maintaining cholesterol homeostasis in the mammary gland by effluxing cholesterol accumulating in MEC into the blood circulation. Recently, apolipoprotein E (apoE) and apoA1, key

Figure 3. Blood serum metabolite levels during the pregnancy-lactation cycle. Blood serum metabolite concentrations at 7 stages of the pregnancy-lactation cycle are represented as box whisker plots. The means of serum concentrations are indicated as black circles ($n = 8$ to 10 per group) and outliers as empty circles. For definition of the box whisker plots, see Figure 1. Significantly different means ($P < 0.05$) are marked by the letters a–d.

acceptors of cholesterol effluxed by ABCA1 (Ikonen, 2008), were identified in bovine milk fat globule (MFG) membranes of colostral and normal milk (Fong et al., 2007). These findings suggest that cholesterol might also be removed from MEC by MFG secretion into the alveolus. Recently it was demonstrated that ABCA1 is able to transfer cholesterol by transcytosis through aortic endothelial cells (Cavelier et al., 2006). It is currently not clear if this process also occurs in mammary tissue. However, if transcytotic transport processes are identified in MEC, it is likely that ABCA1 could also play a role in the transfer of cholesterol from the blood into the milk.

The transporter ABCG1 was shown to support ABCA1 in its function by transferring cholesterol onto mature HDL particles (Ikonen, 2008). In contrast to ABCA1 and ABCA7 profiles, ABCG1 mRNA abundance showed no significant differences between the stages in the pregnancy-lactation cycle. This may indicate either that basal ABCG1 levels are sufficient to support ABCA1 in its function or that ABCG1 could have another, as yet unidentified, role in mammary tissue. However, correlation analysis between the expression levels (Figure 4A) revealed that the profiles of

Table 2. Summary of Pearson correlation analysis¹ between relative mRNA profiles of transporters, regulators, and blood serum metabolite profiles during the pregnancy-lactation cycle²

Transporters vs. transporters	ABCG2	ABCA1	ABCA7	ABCG1	NPC1
ABCG2	1.00				
ABCA1	−0.31	1.00			
ABCA7	−0.26	0.73	1.00		
ABCG1	NS	0.68	0.52	1.00	
NPC1	0.67	NS	NS	0.25	1.00
Transporters vs. regulators	LXR α	PPAR γ	SREBP1	SREBP2	
ABCG2	0.37	0.60	0.62	0.52	
ABCA1	NS	NS	NS	NS	
ABCA7	0.29	NS	NS	NS	
ABCG1	0.27	0.37	NS	0.39	
NPC1	0.49	0.57	0.62	0.55	
Regulators vs. regulators	LXR α	PPAR γ	SREBP1	SREBP2	
LXR α	1.00				
PPAR γ	0.48	1.00			
SREBP1	NS	0.62	1.00		
SREBP2	0.35	0.64	0.40	1.00	
mRNA vs. metabolites	NEFA	Glucose	Cholesterol	Triglycerides	
ABCG2	0.32	−0.43	NS	−0.32	
ABCA1	NS	NS	−0.39	NS	
ABCA7	NS	NS	−0.51	NS	
ABCG1	NS	NS	−0.29	NS	
NPC1	0.29	NS	NS	NS	
LXR α	0.38	NS	−0.26	NS	
PPAR γ	NS	NS	NS	−0.30	
SREBP1	NS	NS	NS	NS	
SREBP2	NS	NS	NS	−0.27	

¹Pearson correlation coefficients from correlation analysis between relative mRNA profiles (ΔC_T values) and metabolite concentrations ($n = 61$) were calculated by SAS statistical software (SAS Institute Inc., Cary, NC) using the Corr Procedure. Only correlation coefficients with $P < 0.05$ are listed; NS = nonsignificant ($P > 0.05$).

²Genes: ABCG2, ABCA1, ABCA7, ABCG1 = different ATP-binding cassette transporter genes; NPC-1 = Niemann-Pick disease related protein 1; LXR α = liver X receptor α ; PPAR γ = peroxisome proliferator-activated receptor γ ; SREBP1 and SREBP2 = sterol responsive element binding proteins.

ABCA1, ABCA7, and ABCG1 were highly associated, suggesting that these genes might be involved in similar physiological processes.

The transporter ABCA7 was elevated during the DP and declined during lactation in a similar pattern as ABCA1; ABCA7 shows high sequence similarity to ABCA1 and was implicated in the clearance of apoptotic cells by phagocytosis (Jehle et al., 2006). Apoptosis of MEC mainly occurs during the DP and at the onset of lactation to remove the excess of newly synthesized cells in the bovine mammary gland (Sorensen et al., 2006). During involution, macrophages expressing ABCA7 invade the mammary tissue to clear debris of apoptotic MEC (Monks et al., 2002). Therefore, the elevated ABCA7 mRNA abundance observed in our study during involution and early lactation may occur because of the high apoptosis rate of MEC during these stages and subsequent invasion of macrophages that express ABCA7 (Abe-Dohmae et al., 2006). Previous investigations in our laboratories (Farke et al., 2008) showed a trend toward an enhanced CD14 expression

in the mammary gland 2 wk after drying off, but this tendency did not reach statistical significance because of high interindividual variations. In those preliminary studies, only 4 cows were included and only expression patterns in lactation versus DP were investigated. Nevertheless, for ABCA1, ABCG1, ABCG2, and SREBP1, the mRNA expression profiles were similar to results obtained in the present study. With regard to ABCA7, however, significant upregulation of ABCA7 was found after parturition (Farke et al., 2008). This discrepancy may be due to different and less frequent sampling schedules as well as to the low number of animals included. Other studies have implicated ABCA7 in mediating the transfer of phospholipids to apolipoproteins (Abe-Dohmae et al., 2006). Whether this could be a potential function of ABCA7 in the mammary gland should be elucidated by localization studies and functional assays.

Studies of Niemann-Pick disease type C have shown that NPC1 is required for the transport of LDL-derived cholesterol from late endosomes and lysosomes to other

Figure 4. Correlations between mRNA profiles of selected transporters, regulators, and blood serum metabolites; mRNA abundance and blood serum concentrations with significant Pearson correlations ($P < 0.05$) and high association (see Table 2) are compared. A) Correlations between relative mRNA abundance [ΔC_T values] of the ATP-binding cassette (ABC) transporter A1, A7, and G1. Data points with linear regression line, equation, and Pearson correlation coefficients (r) are indicated; B) Niemann-Pick disease related protein 1 (NPC1) mRNA abundance is plotted against the relative mRNA abundance of regulatory genes involved in lipid homeostasis; C) relative ABCA1 mRNA profile and blood serum cholesterol concentrations are compared (Pearson correlation coefficient $r = -0.39$, $P = 0.0017$). Means $\pm$ SEM per group are shown.

compartments and to the plasma membrane caveolae (Ory, 2004). In many cell types, these efflux mechanisms keep the cholesterol content in late endosome membranes low. Although it has been demonstrated that cholesterol in milk is derived from blood serum cholesterol, the transfer mechanism across MEC remains elusive. In mice, it has been previously shown

that approximately 15% of the cholesterol found in milk is transferred across MEC by the uptake from LDL involving a non-LDL receptor-mediated process (Monks et al., 2001). In our study, NPC1 mRNA abundance was elevated after parturition and remained constant during lactation, suggesting that in bovine mammary tissue NPC1 is required to redistribute LDL-derived cholesterol from the lysosomal system to various other intracellular compartments.

In addition to the previously mentioned lipid transporters, we also measured ABCG2 expression in our mammary gland samples. Previously, ABCG2 was shown to be involved in the transport of drugs and xenobiotics into milk and was strongly induced in the mammary gland of mice, cows, and women during lactation (Jonker et al., 2005). In agreement with these data and with previous investigations of our laboratories (Farke et al., 2008), ABCG2 mRNA abundance was significantly increased after parturition and declined from the lactating to the nonlactating state in the bovine mammary gland. It is currently unclear why and to what extent ABCG2 is functionally active in the mammary gland. Therefore, it is essential to identify physiological substrates for ABCG2 and to investigate which of them may account for the high expression during lactation. Interestingly, a missense mutation in the ABCG2 gene was found to affect milk yield, milk fat, and protein concentration in cattle (Cohen-Zinder et al., 2005), suggesting a functional role for ABCG2 in milk secretion.

The lipid transporters investigated in our studies are involved in lipid homeostasis and are regulated by transcription factors such as nuclear receptors and SREBP. To gain some insight into regulatory pathways in the mammary gland at the different functional stages, we analyzed the relationship between mRNA profiles of lipid transporters and their regulatory genes. Transcription of ABCA1 is activated by LXR α that is a sensor for cellular cholesterol accumulation (Ikonen, 2008). However, in the present study LXR α and ABCA1 mRNA profiles showed no significant associations. As it has been proposed that LXR α is also a sensor for high cellular glucose levels (Mitro et al., 2007), it may be upregulated because of the increased glucose influx into MEC from the blood for the synthesis of lactose at the onset of lactation (Anderson et al., 2007). It was also shown that ABCA1 is upregulated by PPAR agonists inducing the transcription of LXR α (Ikonen, 2008). However, in our study PPAR γ expression levels showed no correlations to ABCA1. The increase of PPAR γ mRNA abundance at the onset of lactation more likely may be explained by the fact that PPAR γ is involved in lipid anabolism and activates lactogenic genes (Schmitz and Langmann, 2005). In contrast to ABCA1, we found

a positive correlation between ABCG1 and LXR α , suggesting that ABCG1 is regulated by LXR α in the mammary gland as it has been previously shown in other cell types (Ikonen, 2008).

The *ABCA7* gene is regulated by sterols through the SRE/SREBP2 pathway (Abe-Dohmae et al., 2006). However, correlation analysis between mRNA abundance of both SREBP2 and *ABCA7* did not reveal clear associations. A positive correlation was found for *ABCA7* and LXR α , suggesting that *ABCA7* might be regulated by LXR α in the mammary gland. However, it is likely that the *ABCA7* transporter in mammary tissue is also regulated at the post-transcriptional level as it was reported for other cell types (Abe-Dohmae et al., 2006).

The PPAR γ , SREBP1, and SREBP2 profiles highly correlated with ABCG2 profiles. The drug transporter ABCG2 affects milk yield and fat composition in cattle (Cohen-Zinder et al., 2005) and is regulated in a PPAR γ /RXR manner in human dendritic cells (Szatmari et al., 2006). We also found an increase in PPAR γ levels at the onset of lactation reflecting an elevated physiological demand of PPAR γ for the activation of ABCG2. The QTL located in ABCG2 might induce the SREBP pathway, activating the transcription of key enzymes for milk fat synthesis in MEC (Anderson et al., 2007). In this context, it has been shown that the activity and mRNA abundance of fatty acid synthase and acetyl coenzyme A carboxylase were highly elevated at the onset of lactation in mammary gland tissue (Sorensen et al., 2006).

It has been demonstrated that NPC1 is regulated on the transcriptional level by the SRE/SREBP pathway in human fibroblasts (Garver et al., 2008). The mRNA profile of NPC1 was highly positively correlated with SREBP1 (Figure 4B), suggesting that this factor is actively involved in the transcriptional regulation of NPC1 in the mammary gland. Levels of PPAR γ also correlated with NPC1 levels. Whether this suggests an alternative regulatory process in the mammary gland, or is rather a consequence of the concomitant induction of other genes, remains to be elucidated. It was reported that NPC1 and NPC2 regulate cellular cholesterol homeostasis through the production of LDL-derived oxysterols (Ory, 2004). It is currently unclear whether these ligands are present in the mammary gland and may bind to LXR α that, in turn, could activate SREBP1, as it was shown in the liver of mice (Repa et al., 2000). In our study, a significant correlation between NPC1 and LXR α was found, suggesting that LXR α may also indirectly upregulate NPC1 via SREBP1.

In general, regulatory networks controlling the expression of lipid transporters during the cycle of lactation are complex. The induction of lactation is triggered by

lactogenic hormones and growth factors and is not completely understood. These signals presumably induce many regulatory and lactogenic genes at the onset of lactation. Expressional changes of lactogenic genes can therefore influence the expression of regulatory genes that are also involved in controlling the expression of lipid transporters. Moreover, the expression of membrane transport proteins is often regulated not only at the transcriptional but also at the posttranscriptional level (Schmitz and Langmann, 2005). To dissect direct and indirect effects and functional relationships between regulators and lipid transporters, it is therefore crucial to investigate regulatory mechanisms of lipid transporters by functional in vitro assays in the presence and absence of lactogenic parameters.

In parallel to mRNA profiles, we also investigated blood serum metabolites profiles to reveal potential relationships to transporter gene expression. A marked negative correlation with blood serum cholesterol levels was found for *ABCA1* (Table 2; Figure 4C). A decrease in cholesterol levels in the blood during the DP compared with lactation has been previously reported (Seifi et al., 2007) and was explained by the requirement of cholesterol for the development of the fetus. Moreover, cholesterol is presumably also required for the development of the lactating mammary gland and for synthesis of the colostrum (Anderson et al., 2007). Several reports exist regarding the origin of the cholesterol fraction found in the milk; although some milk cholesterol is synthesized in the mammary gland (Anderson et al., 2007), it is basically derived from serum cholesterol (Monks et al., 2001). The colostrum contains higher levels of cholesterol than mature milk; the cholesterol/triglyceride ratio of the MFG membranes in the milky secretion found in the alveolus during late pregnancy is higher than during normal lactation (Bitman et al., 1992). In our study, the major changes in mRNA abundance of the ABC transporters were observed during late pregnancy and early lactation. The cholesterol transporters increased during DP and recovered until peak lactation, whereas serum cholesterol profiles showed the opposite pattern. This relationship may suggest that cholesterol accumulates during late pregnancy and early lactation in the mammary gland because of higher influx from the blood, and is secreted into milk by active transport processes presumably involving *ABCA1* as soon as a functional epithelium is established.

The transporter *ABCA7* showed a very similar mRNA profile as *ABCA1* and was highly inversely correlated with cholesterol levels (Table 2). During involution, debris of apoptotic cells have to be removed by macrophages and by MEC (Monks et al., 2002). As *ABCA7* was shown to be involved in phagocytosis (Jehle et al., 2006), it may play a role in the engulfment of

cellular debris of apoptotic MEC. Cholesterol accumulating in phagocytic cells might be removed by ABCA1 and ABCG1 as it has been previously demonstrated in macrophages (Oram and Lawn, 2001). Thus, the accumulation of cholesterol in phagocytic cells could be a further factor underlying increased ABCA1 expression in the mammary gland during late pregnancy. This is in line with the finding that ABCG1 also showed a weak inverse correlation with cholesterol levels, suggesting a role for ABCG1 in supporting ABCA1 in this function as previously reported in macrophages (Oram and Lawn, 2001). Only moderate correlations with serum cholesterol levels were found for NPC1. This is in agreement with recent findings in mice, where NPC1 was not regulated by the amount of cholesterol that flows through the cells (Garver et al., 2005). Nevertheless, the increase in NPC1 mRNA expression after parturition implies that NPC1 may be required for intracellular trafficking of LDL-derived cholesterol from late lysosomes to other compartments during lactation.

The blood serum metabolite profiles measured in the present study are comparable with previous studies in cows (Seifi et al., 2007; Figure 3). High NEFA levels and low triglyceride levels at the onset of lactation indicate the mobilization of fatty acids from peripheral adipocytes and show the negative energy balance of the cow due to requirements for the development of the fetus and milk production. Increased triglyceride levels after drying off suggest that triglycerides presumably are transferred back into the periphery for storage in the adipose tissue. Decreased triglyceride and glucose levels postpartum may indicate their transfer to the mammary tissue because of the requirements for milk fat synthesis (Anderson et al., 2007). Interestingly, ABCG2 mRNA abundance in the mammary gland correlated with NEFA, triglycerides, and glucose serum levels. The transporter ABCG2 is known to transport various substrates into milk and the QTL for milk yield and fat composition is located on the *ABCG2* gene (Cohen-Zinder et al., 2005); therefore, the increase in ABCG2 may partially influence the uptake of glucose, NEFA, and triglycerides from the blood for the synthesis of milk fat. Regarding associations between regulators and metabolites, LXR α showed a weak negative correlation with serum cholesterol levels. Because LXR α is sensitive to cholesterol accumulation (Schmitz and Langmann, 2005), this finding may reflect an accumulation of cholesterol in the mammary gland during lactation and hence underline the need for inducing effective cholesterol efflux and phagocytosis mechanisms as outlined previously. This again may underscore a role for ABCA1 and ABCA7 in these processes.

Localization of ABCA1 Protein in Mammary Gland Tissue

Based predominantly on the ABCA1 mRNA expression profiles, we proposed that ABCA1 might play a role in the removal of cholesterol in the mammary gland during involution. Because discrepancies between ABCA1 mRNA and protein expression have been observed (Wellington et al., 2002; Albrecht et al., 2004b) and localization data for ABCA1 in the mammary gland are not available, we performed ABCA1 localization studies in mammary tissue at the different stages of the pregnancy-lactation cycle using immunohistochemistry. During lactation and involution, ABCA1 was localized in stromal cells (fat depleted adipocytes, fibroblasts, macrophages, smooth muscle cells, and blood vessels) and in MEC. During involution, ABCA1 localized at the apical and the basolateral membranes and in the cytoplasm of MEC with higher intensity compared with the lactating stage (d 42 postpartum). During lactation, it was localized basal and partly perinuclear in vesicular structures. A vesicular intracellular distribution pattern of ABCA1 has previously been demonstrated in ABCA1 overexpressing cells where ABCA1 protein resided on the cell surface and on intracellular vesicles (Neufeld et al., 2001). In general, the number of positive cells, the distribution, the signal to background ratio, and the subcellular localization changed between lactation and DP in the epithelium, whereas the expression in stromal cells was not altered (Figure 1B). Preliminary analysis of the intensity and number of positive MEC suggests that in the mammary gland, in contrast to others tissues (Albrecht et al., 2004b), ABCA1 protein and mRNA expression do correspond. Therefore, the increased ABCA1 mRNA expression occurring during dry-off phase is an effect of changes in the epithelium (Figure 1).

These immunohistochemical data support the notion that ABCA1 could be involved in the removal of cholesterol from MEC during the DP and early lactation. During these stages, cholesterol may accumulate in MEC because of phagocytosis of apoptotic cells and the engulfment of cholesterol-rich debris. Cholesterol might also accumulate in functional epithelial cells because of the increased influx of cholesterol from the blood and may be removed by as yet unclear mechanisms involving the ABCA1 transporter. The basal and apical distribution in MEC during lactation and DP could suggest that ABCA1 may efflux cholesterol into milk by MFG secretion or into the blood by transferring it onto lipid-poor apoA1. Alternatively, it cannot be excluded that during lactation ABCA1 could act as "receptor"

molecule for the uptake and intracellular trafficking of cholesterol-rich lipoproteins and may transfer apolipoproteins such as apoA1 by transcytosis through the epithelium. However, to shed light on the physiological role of ABCA1 in the mammary gland, it is crucial to investigate whether ABCA1 is expressed in MFG and/or other subcellular compartments of MEC.

CONCLUSIONS

In summary, lipid transporters were differentially expressed between lactation and DP in the bovine mammary gland and partially correlated with expression profiles of their regulators. Levels of ABCA1, ABCG1, and ABCA7 showed inverse correlations to serum cholesterol levels. Cellular ABCA1 protein localization in MEC changed at the transition from the DP to lactation. These findings may imply a role of ABCA1 and ABCG1 in the transport of cholesterol in the mammary gland and suggest that ABCA1 is involved in maintaining cholesterol homeostasis in MEC during involution and early lactation. It is possible that ABCA1 might also act as a "receptor" molecule for the uptake of cholesterol-rich lipoproteins from the serum, and that ABCA7 may play a role in phagocytosis of apoptotic cells during involution. Intracellular LDL-derived cholesterol in MEC may be redistributed from late endosomes to other compartments by NPC1 during lactation. Regulatory mechanisms are complex and should be additionally assessed by functional assays. To elucidate the exact roles of these lipid transporters in the mammary gland, detailed localization studies of lipid transporters, lipoproteins, and accessory proteins in mammary tissue, in cultured cells, and in MFG membranes are needed. Such studies, together with functional in vitro models, will shed light on the implication of these transporters in mammary gland physiology.

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