Transcript abundance of amino acid transporters, β -casein, and α -lactalbumin in mammary tissue of periparturient, lactating, and postweaned sows

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ABSTRACT

The objective of these experiments was to test the hypothesis that transcript abundance of cationic AA transporter- and milk protein-encoding genes increase in the porcine mammary gland in response to higher lactation demand. Genes of interest included those encoding for the milk proteins α -lactalbumin (α -LA) and β -casein (β -CN; *LALBA* and *CSN2*, respectively), and AA transporter $b^{0,+}$ AT, y^{+} LAT1, y^{+} LAT2, $ATB^{0,+}$, CAT-1, and CAT-2b (*SLC7A9*, *SLC7A7*, *SLC7A6*, *SLC6A14*, *SLC7A1*, and *SLC7A2*, respectively). Mammary tissue was biopsied from 4 sows on d 110 of gestation (prepartum), on d 2 (early postpartum), on d 5 (early), and d 17 (peak) of lactation, and on d 5 after weaning (postweaning), and mRNA of target genes quantified by reverse transcription quantitative PCR. Compared with prepartum, CAT-1, $ATB^{0,+}$, y^{+} LAT2, β -CN, and α -LA mRNA abundance was higher at early lactation, whereas compared with early lactation, only CAT-1 and α -LA mRNA abundance was higher at peak lactation. The CAT-2b, y^{+} LAT1, and $b^{0,+}$ AT mRNA abundance did not differ when comparing either prepartum or peak lactation to early lactation. Compared with peak lactation, postweaning mRNA abundance of CAT-1, $ATB^{0,+}$, α -LA, and β -CN decreased, y^{+} LAT2, CAT-2b, and $b^{0,+}$ AT remained unchanged, and y^{+} LAT1 increased. The mRNA abundance of y^{+} LAT2 increased from early postpartum to early lactation, and remained unchanged for CAT-1, $ATB^{0,+}$, α -LA, and β -CN. From prepartum to peak lactation, the mRNA abundance of CAT-1, y^{+} LAT2, and $ATB^{0,+}$ was positively correlated with that of β -CN and α -LA. In conclusion, the expression of genes encoding for y^{+} LAT1, CAT-2b, and $b^{0,+}$ AT remained unchanged in porcine mammary glands over

sion of genes encoding for CAT-1, $ATB^{0,+}$, and y^{+} LAT2 was upregulated and positively correlated to expression of genes encoding for the mammary synthesized milk proteins β -CN and α -LA.

Key words: amino acid, lactation, mammary gland, sow

INTRODUCTION

During the second week of lactation an increase in milk demand imposed by the growing piglet exceeds the ability of the sow to produce milk, and cationic and neutral AA including Lys, Thr, and Val may become limiting for milk protein synthesis (NRC, 1998). Therefore, numerous studies have focused on optimizing dietary Lys (NRC, 1998; Dourmad et al., 1998; Touchette et al., 1998), Val (NRC, 1998; Gaines et al., 2006), and Thr (Cooper et al., 2001) intake by the sow to maximize litter growth. One critical step of AA utilization involves cellular AA transport, a process mediated by transporter proteins located in cellular membranes (Shennan and Peaker, 2000; Bröer, 2008). However, little to nothing is known of the AA transporters, their regulation, and the potential that they are coordinately regulated along with milk proteins. Proteins known to facilitate cationic and neutral AA transport into mammalian epithelial cells (Palacín et al., 1998) include those of the y^{+} system of the AA transporter family specific for cationic AA, 2 of which have been identified in lactating porcine mammary tissue, namely CAT-1 and CAT-2b (Pérez Laspiur et al., 2004, 2009). Cationic and neutral AA may also be transported using shared systems with other AA, such as system $B^{0,+}$, of which protein $ATB^{0,+}$ was also shown to be abundantly expressed in porcine mammary tissue (Pérez Laspiur et al., 2004, 2009). However, their expression and that of more novel cationic and neutral AA transporters $b^{0,+}$ and y^{+} LAT in relation to genes encoding for the milk proteins β -CN and α -LA, has not been determined. Furthermore, it is unknown when and if these genes

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change in expression relative to the well-characterized milk production curve in sows (Duncan and Garton, 1966; Nielsen et al., 2002). This information would provide novel insight in the regulation of intracellular AA availability for milk protein synthesis. We hypothesized that cationic AA transporter and milk protein genes increase in expression with higher lactation demand. To test this hypothesis, our objectives were to (1) profile mRNA abundance of genes encoding for cationic AA transporter proteins and for mammary synthesized milk proteins at distinct stages of mammary physiological activity, and (2) determine the relationship between mRNA abundance of each respective candidate AA transporter and mammary synthesized milk protein genes.

MATERIALS AND METHODS

Animals and Tissue Collection

Studies were performed with the approval of the Institutional Animal Care and Use Committee at Michigan State University (AUF#10/08-162-00). All sows were fed a corn-soybean meal-based diet meeting all nutrient requirements for lactating sows nursing piglets with a predicted average daily gain of 250 g/d (NRC, 1998; Table 1). Sows selected on d 107 of pregnancy were moved to farrowing crates in a room maintained at 20°C and fed 3 kg/d divided into 2 meals. The day after farrowing was considered d 1 of lactation and sows were fed 1 kg twice per day (0800 and 1600 h). On d 2 and 3 of lactation, sows were fed 3 and 4 kg, respectively, in 2 equal meals. For the remainder of the study, sows were fed a maximum of 5.5 kg/d to ensure equal DMI among all sows. Sow feed intake was recorded daily throughout lactation. Fresh water was freely available at all times.

In experiment 1, 4 sows (all parity 5, Landrace × Yorkshire) were used. Litters were equalized to 10 piglets within 48 h after farrowing and litter weight was recorded on d 2 and 17 of lactation. Piglets were weaned on d 21. Mammary parenchymal tissue was biopsied from the first and second thoracic glands (Kirkwood et al., 2007) in the morning following a 12-h overnight fast on d 110 of gestation (prepartum), d 5 (early), and d 17 (peak) of lactation, and d 5 after weaning (post-weaning). For mammary tissue sampling, piglets were isolated in an adjacent pen equipped with a heat lamp. Immediately following the biopsy, mammary tissue was flash-frozen and kept in liquid N₂ until later stored at -80°C. Three hours after biopsy, piglets were returned to sows and allowed to nurse.

In experiment 2, 4 sows [parity 2 (n = 2), parity 4 (n = 1), and parity 5 (n = 1); Landrace × Yorkshire] were selected and managed as above, except that litter size

was equalized to 8 and mammary tissue was biopsied on d 2 and 5 of lactation only.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from mammary tissue using the PerfectPure RNA Cell and Tissue Kit according to the manufacturer's instructions (5 PRIME, Inc., Gaithersburg, MD). Isolated RNA was tested for quality and quantity using the Agilent Bioanalyzer 2100 with the RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). The RNA integrity number (RIN) values ranged from 8.7 to 10. Complementary DNA was synthesized using 2 µg of total RNA from each sample as template in reverse transcription reactions using Superscript III reverse transcriptase and oligo(dT)₁₅₋₁₈

Table 1. Ingredient and nutrient composition of lactation diet (as-fed basis)

Item	%
Ingredient	
Yellow corn	61.16
Soybean meal	25.38
Dried beet pulp	5.0
Choice white grease	4.0
Calcium phosphate	1.48
Limestone	0.77
Vitamin premix ¹	0.60
Trace mineral premix ²	0.50
Sow pack ³	0.30
MTB-100 Alltech ⁴	0.10
Natuphos 600 ⁵	0.08
Vitamin E	0.01
Salt	0.50
Calculated analysis	
DM	89.71
ME (Mcal/kg)	3,374
CP	17.86
Lysine, apparent digestible	0.85
Threonine, apparent digestible	0.57
Fat	6.49
NDF	10.25
ADF	4.30
Calcium	0.80
Available phosphorus	0.40
Sodium	0.31
Chloride	0.35

¹Provided the following per kilogram of diet: 4,500 IU of vitamin A, 458 IU of vitamin D₃, 55 IU of vitamin E, 11 mg of vitamin K, 3.66 mg of menadione, 0.0275 mg of vitamin B₁₂, 3.66 mg of riboflavin, 14.67 mg of D-pantothenic acid, 22 mg of niacin, 0.913 mg of thiamine, 0.825 mg of pyridoxine.

²Provided the following per kilogram of diet: 335 g of Ca, 5 g of Fe, 5 g of Zn, 5 g of Cu, 150 µg of Se and 75 µg of I.

³Provided the following per kilogram of diet: 2,755.7 IU of vitamin A, 220.5 µg of biotin, 385.8 mg of choline, 1.65 mg of folic acid.

⁴Non-viable dried yeast of *Saccharomyces cerevisiae* used as an anti-caking agent. Each kilogram contains 22% CP, 0.05 crude fat, and 11% crude fiber (Alltech, Nicholasville, KY).

⁵Provided 500 phytase units of activity per kilogram of diet (500 FTU/kg; BASF Corp., Florham Park, NJ).

primer (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. The concentration of cDNA was quantified by spectrophotometry (NanoDrop 1000), diluted to a working stock containing 10 ng/ μ L and stored at -20°C . Our preliminary studies showed that genes encoding for β -CN and α -LA are highly expressed in mammary cells and, therefore, their cDNA was further diluted (1:100).

Primer Design and Optimization

Primer sequences for reference and target genes are presented in Table 2. To facilitate the readership throughout the remainder of the manuscript, target genes will be referred as the common name of the AA transporter and milk proteins that they encode for. Hence *SLC7A9* will be referred to as $\text{b}^{0,+}\text{AT}$, *SLC7A7* as $\text{y}^{+}\text{LAT1}$, *SLC7A6* as $\text{y}^{+}\text{LAT2}$, *SLC6A14* as $\text{ATB}^{0,+}$, *SLC7A1* as CAT-1, *SLC7A2* as CAT-2b, *CSN2* as β -CN, and *LALBA* as α -LA. Reference genes *API5*, *VAPB*, and *MRPL39* were selected based on a previous study that support their stability in porcine mammary gland during lactation (Tramontana et al., 2008; supplementary material in the Appendix), although primers used were different from those published to optimize the efficiency of the reverse transcription quantitative PCR (qPCR) reaction in our samples. Primers were designed based on publicly available swine cDNA and on expressed sequence tag (EST) sequences deposited in the National Center for Biotechnology database using Primer Express software (v. 3.0, Applied Biosystems, Foster City, Ca) with default settings. Designed primers were blasted against published swine (*Sus scrofa*), human (*Homo sapiens*), bovine (*Bos taurus*), and rat (*Rattus norvegicus*) genome sequences; pairs that showed significant alignment (i.e., high query coverage) with nucleotide sequences other than the protein of interest in any of the species mentioned were discarded. Primer pairs were optimized for concentration using a primer optimization matrix (Mikeska and Dobrovic, 2009) and a relative standard curve was used to determine the efficiency (Yuan et al., 2006). The standard curve was constructed using cDNA synthesized from an RNA pool made of all samples using the following amounts of cDNA (in duplicate): 40, 4, 0.4, 0.04, and 0.004 ng for β -CN and α -LA, and 40, 20, 10, 5, and 2.5 ng for all other genes. Efficiency of the qPCR reaction for each gene was calculated from the slope of the standard curve using the formula $(10^{-1/\text{slope}} - 1) \times 100$, as described by Yuan et al. (2006). Specific hybridization of the primers was validated by the presence of a unique peak in the dissociation curve at the end of the qPCR amplification. Non-template controls were

included in all qPCR plates to validate that primers were not amplifying contaminating DNA.

Reverse Transcription qPCR Assay

Reverse transcription qPCR reactions were performed in MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems). To each well were added 3 μ L (30 ng) of template cDNA, 12.5 μ L of SYBR Green master mix (Applied Biosystems), 6 μ L each of 10 μ M forward and reverse primers, and 3.5 μ L of diethylpyrocarbonate (DEPC)-treated and nuclease-free water (Fisher Scientific, Fair Lawn, NJ). Plates were sealed, centrifuged at $380 \times g$ for 1 min, and loaded into the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The amplification program included 2 initial steps (50°C for 2 min and 95°C for 10 min), followed for 40 cycles (step 3; 95°C for 15 s and 60°C for 1 min), and a dissociation curve (step 4; 95°C for 15 s, 60°C for 1 min, 95°C for 15 s). Data were analyzed with the 7000 RQ Sequence Detection Systems Software (version 2.2.1; Applied Biosystems).

Normalization and Statistical Analysis

Analysis of target gene expression was made by subtracting from each of the target gene cycle thresholds (Ct) the arithmetic mean of the 3 selected reference gene Ct (Equation 1; see description of the statistical analysis in the Appendix).

$$\Delta\text{Ct} = \text{Ct}_C - \left[\frac{1}{3} (\text{Ct}_{\text{R1}} + \text{Ct}_{\text{R2}} + \text{Ct}_{\text{R3}}) \right]. \quad [1]$$

Changes in ΔCt for each target gene in response to physiological phases of mammary activity were assessed using a linear mixed model procedure (PROC MIXED) of SAS (SAS Institute, Inc., Cary, NC) that included the fixed effect of time and the random effect of sow (Equation 2):

$$Y_{ij} = \mu + \alpha_i + b_j + e_{ij}, \quad [2]$$

where Y_{ij} is ΔCt of target gene for the j th sow within the i th stage of lactation, μ is the overall ΔCt mean, α_i is the fixed effect of the i th stage of lactation, b_j is the random effect of the j th sow, and e_{ij} is the experimental error. In experiment 1, ΔCt for each target gene were compared between early lactation and prepartum, peak lactation, and early lactation and postweaning and peak lactation. In experiment 2, ΔCt of target genes were compared between d 2 and 5 of lactation. Mul-

Table 2. Primer information for reverse transcription quantitative PCR (RT-qPCR) assays

Accession number ¹	Gene	Protein	Primer ²	Primer (5'-3')	E ³ (%)
CV872150.1	<i>API5</i>	Apoptosis inhibitor 5	F. 502 R. 568	CTGGAGTGGTGGCAATAATCTCT CCAAGGGAGCTCAGGTTTAGC	99.4
AY610067.1	<i>MRPL39</i>	Mitochondrial ribosomal protein L39	F. 540 R. 601	TCGCTGGAGCTTTCTGCTATG TGTGGCATCCACTCATCAAG	103.5
NM_001123213.1	<i>VAPB</i>	Vesicle-associated membrane protein-associated protein B/C	F. 1012 R. 1072	TGGCGCTGGTGGTTTTG CCTACAAGGCGATCTTCCCTATG	101.9
NM_001110421.1	<i>SLC7A7</i>	y ⁺ LAT1	F. 51 R. 108	TTTGGTTCCCAAGGTTGCA GCAGCTTCCTGGCATTGC	115.6
CX064558.1	<i>SLC7A6</i>	y ⁺ LAT2	F. 114 R. 172	CTGCCGCTGCATGTGT TGTGCCCCACTTGACATAGG	101.9
NM_001012613.1	<i>SLC7A1</i>	CAT-1	F. 1172 R. 1234	GGGCTGCTGTTTAAGTTTTTGG CGTGGCGATTATTGGTGTGTTT	100.4
NM_001110420.1	<i>SLC7A2</i>	CAT-2b	F. 1723 R. 1071	GCCCCAGAATCAGCAAAAAGTA GATGCTGAAGGCTGGCAAAA	110.7
NM_001166042.1	<i>SLC6A14</i>	ATB ^{0,+}	F. 162 R. 226	CCGTGGTAACTGGTCCAAAAA CCAATCCCACTGCATATCCAA	101
NM_001110171.1	<i>SLC7A9</i>	b ^{0,+} AT	F. 138 R. 205	CCAGAGCACTGAACCCAAGAC TGATGCAGATGCCACTGAACA	97.1
NM_214434.1	<i>CSN2</i>	β-Casein	F. 333 R. 398	CTGTGGTGGTGCCTCTTCTTC GAACAATGGTCTCCTTAGCTTTGG	96.4
NM_214360.1	<i>LALBA</i>	α-Lactalbumin	F. 433 R. 495	CCCGCTGTCTTGCTGCTT AGGTAGCCTTAGGGAAGAGGAGTT	94.5

¹Accession number corresponds to the cDNA or the expressed sequence tag (EST) sequence deposited in the National Center for Biotechnology Information (NCBI; Bethesda, MD) database from which the primers were designed.

²Direction (F. = forward; R. = reverse) and hybridization position for each primer (5'-3') within the nucleotide sequence from which the primers were designed.

³The reverse transcription quantitative PCR (RT-qPCR) efficiency (E) was calculated as follow: $E = -1 + 10^{(-1/\text{slope})} \times 100$. The R² values for all standard curves for reference and target genes were >0.98, indicating excellent linear relationships between quantities of serially diluted cDNA and cycles to threshold (Ct) when RT-qPCR was performed.

multiple comparisons in experiment 1 were accounted for with Bonferroni adjustment. Gene expression results reported as ΔCt values may often be confusing, as increasing ΔCt reflect decreasing mRNA abundance. Thus, subtracting ΔCt values from a constant value before or after statistical analysis allows to for an easier interpretation of the data (i.e., increasing ΔCt reflects increasing mRNA abundance, without altering the P -value or the standard error). To further simplify the interpretation of results, this constant was selected to be an entire number higher than any ΔCt values among all genes, so that the values presented in the figures are all positive. Thus, mRNA abundance of individual

genes is reported as $13 - \Delta\text{Ct} \pm \text{SEM}$. Differences between physiological stages of mammary activity were considered at $P \leq 0.05$.

To investigate the relationship between mRNA abundance of each respective AA transporter and mammary synthesized milk protein genes, correlations between mRNA abundance (ΔCt) of AA transporter genes and that of milk protein genes were determined using the PROC CORR of SAS. Pearson correlation coefficient (r) was considered significant at $P \leq 0.05$.

RESULTS

Experiment 1

Lactation performance between d 2 and 17 of lactation was uniform among sows, as evidenced by a piglet ADG of 257.8 ± 9 g and a daily sow feed intake of 4.7 ± 0.3 kg. Compared with prepartum, CAT-1 mRNA abundance increased ($P = 0.02$) at early lactation and increased further ($P = 0.02$) from early to peak lactation. Compared with peak lactation, CAT-1 mRNA abundance was lower postweaning ($P = 0.001$; Figure 1a). Compared with prepartum, $\text{ATB}^{0,+}$ and $\text{y}^+\text{LAT}2$ mRNA abundance increased ($P < 0.0001$ and $P = 0.001$, respectively) at early lactation, but did not differ ($P > 0.1$) at peak compared with early lactation (Figure 1b). Compared with peak lactation, $\text{ATB}^{0,+}$ and $\text{y}^+\text{LAT}2$ mRNA abundance decreased postweaning ($P < 0.0001$; Figure 1b). The mRNA abundance of CAT-2b, $\text{y}^+\text{LAT}1$, and $\text{b}^{0,+}\text{AT}$ remained unchanged over prepartum to peak lactation periods ($P > 0.1$; Figures 1a and b). Compared with peak lactation, postweaning CAT-2b and $\text{b}^{0,+}\text{AT}$ mRNA abundance did not differ and that of $\text{y}^+\text{LAT}1$ increased ($P < 0.0001$; Figures 1a and b). Both β -CN and α -LA mRNA abundance were higher ($P = 0.003$ and $P < 0.0001$, respectively) in early lactation compared with prepartum but did not differ at peak compared with early lactation. Compared with peak lactation, β -CN and α -LA mRNA abundance decreased ($P < 0.0001$) postweaning (Figure 1c). Over the prepartum to peak lactation periods, mRNA abundance of $\text{ATB}^{0,+}$, CAT-1, and $\text{y}^+\text{LAT}2$ was positively correlated to β -CN mRNA abundance ($r = 0.76$ and $P = 0.0034$; $r = 0.78$ and $P = 0.0023$; $r = 0.71$ and $P = 0.008$, respectively) and to α -LA mRNA abundance ($r = 0.87$ and $P < 0.001$; $r = 0.71$ and $P = 0.008$; $r = 0.86$ and $P < 0.001$, respectively). The mRNA abundance of CAT-2b, $\text{y}^+\text{LAT}1$, and $\text{b}^{0,+}\text{AT}$ was not correlated with that of either β -CN or α -LA ($P > 0.1$).

Experiment 2

In experiment 2, piglet ADG and daily sow feed intake between d 2 and 5 of lactation was 357.2 ± 41.4 g and

Figure 1. Messenger RNA abundance of AA transporters $\text{b}^{0,+}\text{AT}$, $\text{y}^+\text{LAT}1$, $\text{y}^+\text{LAT}2$, $\text{ATB}^{0,+}$, CAT-1, CAT-2b, and milk protein genes β -casein and α -lactalbumin in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represents $13 - \Delta\text{Ct} \pm \text{SE}$ ($n = 4$). Data were compared between: d 5 of lactation (early) and d 110 of gestation (prepartum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (postweaning) and peak lactation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

4.0 kg, respectively. Compared with d 2 of lactation, mRNA abundance of y^+ LAT2 was higher ($P = 0.005$; Figure 2a) on d 5. No difference in CAT-1, $ATB^{0,+}$, α -LA, and β -CN mRNA abundance between d 2 and 5 of lactation was observed ($P > 0.1$; Figures 2a and b). Between d 2 and 5, mRNA abundance of CAT-1 and y^+ LAT2 was correlated to that of β -CN ($r = 0.76$ and $P = 0.003$; $r = 0.67$ and $P = 0.01$, respectively) and of α -LA ($r = 0.82$ and $P = 0.04$; $r = 0.83$ and $P = 0.03$, respectively). The mRNA abundance of $ATB^{0,+}$ was not correlated with that of β -casein or α -lactalbumin ($P > 0.1$).

DISCUSSION

Previous studies performed in lactating rats using mammary tissue explants have indicated that Lys is transported into mammary cells by cationic AA transporters of the y^+ system (Shennan et al., 1997; Shennan and Peaker, 2000). In the current study, genes encoding for the cationic-specific AA transporters CAT-1 and CAT-2b, and for the shared cationic-neutral AA transporters $ATB^{0,+}$, $b^{0,+}$ AT, y^+ LAT1, and y^+ LAT2, were

expressed at the transcription level in porcine mammary tissue. Amino acid transporters CAT-1 and CAT-2b (system y^+) are Na^+ independent and highly specific for Lys and Arg (Devés and Boyd, 1998), whereas $ATB^{0,+}$ (system $B^{0,+}$) is Na^+ dependent and mediates intracellular transport of both cationic and neutral AA in intestinal epithelial cells (Bröer, 2008). In this study, mRNA of CAT-1 and of $ATB^{0,+}$ was highly abundant in porcine mammary tissue collected during lactation relative to that of other AA transporters. Furthermore, CAT-1 and $ATB^{0,+}$ mRNA abundance increased with the expected increase in piglet growth and was highly related to changes in β -CN and α -LA expression at the mRNA abundance level. Both transporters were highly abundant and upregulated before or at the onset of lactation, because no increase in mRNA occurred as early as between d 2 and 5 of lactation. In contrast, AA transporter CAT-2b was low in abundance and remained unchanged between prepartum and postweaning. Pérez Laspiur et al. (2009) reported that both CAT-1 and CAT-2b were unaffected by increase in milk demand between d 7 and 18 of lactation, and suggested that these transporters may be upregulated earlier in lactation. In the present study, mammary biopsies were taken no later than d 5, and CAT-1 increased from d 5 to 17 of lactation. In humans, CAT-1 and CAT-2b isoforms demonstrate clear differences in their functionality with CAT-1, having a 3-fold higher substrate affinity and being more sensitive to *trans* stimulation than CAT-2b (Closs et al., 1997). The higher dependence of CAT-1 on substrate concentration at the *trans* side of the membrane may facilitate cationic AA uptake into mammary epithelial cells.

Similarly to CAT-2b, AA transporter $b^{0,+}$ AT (system $b^{0,+}$) was relatively low in abundance and demonstrated no change in mRNA abundance between the prepartum and postweaning periods. In contrast to mammary tissue, $b^{0,+}$ AT is now recognized as the major protein mediating cationic AA transport across small intestinal and kidney tubular cells. In those organs, $b^{0,+}$ AT-mediated transport is an Na^+ -independent process localized to the apical rather than basolateral membrane; in those cells, $b^{0,+}$ AT is highly abundant and functions to reabsorb and absorb, in the renal tubules and intestinal epithelial cells, respectively, cationic AA in exchange for neutral AA (Chillarón et al., 1996; Bröer, 2008; Sperandio et al., 2008). Thus, $b^{0,+}$ AT may also be localized to the apical aspect of mammary cells rather than to the basolateral side. Its role in porcine mammary cells during lactation is unclear; it may function to recover free cationic AA from the alveolar lumen where free milk AA concentration constitutes a very small fraction of total milk nitrogen (Wu and Knabe, 1994). With these notions in mind, $b^{0,+}$ AT mRNA abundance would

Figure 2. Messenger RNA abundance of AA transporters $ATB^{0,+}$, y^+ LAT2, CAT1, and milk protein genes β -casein and α -lactalbumin in sow mammary tissue quantified by reverse transcription quantitative PCR (RT-qPCR). Each bar represent $13 - \Delta Ct \pm SE$ ($n = 4$). Data at d 2 of lactation were compared with that of d 5 of lactation (early). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

not be expected to change in relation to that of β -CN and α -LA.

Amino acid transporters y^+LAT1 and y^+LAT2 (system y^+L) have similar properties (Pfeiffer et al., 1999; Bröer et al., 2000). Both transporters are Na^+ independent and shown to be localized to the basolateral membrane of kidney and intestinal epithelial cells, where they mediate the uptake of circulating neutral AA in exchange for intracellular cationic AA (Closs et al., 2004; Bröer, 2008). Thus, both transporters are likely to be expressed on the basolateral aspect of mammary epithelial cells. However, mRNA abundance of y^+LAT1 was unrelated to that of the milk protein genes, as it remained unchanged from prepartum to peak lactation, whereas that of y^+LAT2 was upregulated with the onset of lactation and highly correlated to β -CN and α -LA mRNA. Therefore, it may be possible that both transporters contribute similarly to neutral AA uptake during late gestation in the porcine mammary gland, with only y^+LAT2 subjected to further upregulation with the onset of lactation to ensure adequate leucine, valine, and isoleucine uptake for milk protein synthesis.

Increasing milk protein synthetic demand with the advancement of lactation decreases the arterial concentration of Lys (Trottier et al., 1997), the most limiting AA in the diet of lactating sows (NRC, 1998). Consequently, Lys transporters such as CAT-1, $ATB^{0,+}$, and y^+LAT2 may play a critical role in adapting to fluctuating plasma Lys levels to ensure intracellular Lys concentrations are maintained at high levels to keep tRNA fully charged, as suggested by the high correlation found between their mRNA abundance and that of β -CN and α -LA. Based on the marked increase in expression of CAT-1, $ATB^{0,+}$, and y^+LAT2 from the prepartum to early lactation periods, we further quantified their transcript abundance, along with α -LA and β -CN, during the very early stage of lactation (i.e., between d 2 and 5). However during that short time period, only expression levels of CAT-1 and y^+LAT2 were correlated to those of α -LA and β -CN, but only mRNA abundance of y^+LAT2 increased on d 5. It is possible that increase in mRNA abundance of y^+LAT2 occurs after farrowing, whereas CAT-1 and $ATB^{0,+}$ increase before farrowing in preparation for galactopoiesis.

Earlier studies have shown that as lactation progresses in the sow, arterial extraction rate increases for the majority of indispensable AA, likely to support the increasing need for milk protein synthesis (Trottier et al., 1997; Nielsen et al., 2002). In the present study, however, only CAT-1 mRNA abundance increased between early and peak lactation. It is well known that an increase in mRNA abundance does not always translate into an increase in protein level. However, despite CAT-1 protein levels not being measured, *in vitro* (Aulak et

al., 1999) and *in vivo* (Pérez Laspiur et al., 2009) studies showed parallel changes in CAT-1 protein and transcript abundance in porcine mammary tissue. Therefore, it is possible that an increase in extraction efficiency by CAT-1 is supported by an increase of mRNA and protein abundance [maximum reaction rate (V_{max}) effect], whereas uptake regulation of neutral AA by y^+LAT2 and $ATB^{0,+}$ is mediated by a change in binding affinity of the transporters [Michaelis constant (K_m) effect]. Alternatively, it is possible that CAT-1 carries out more functions in the mammary gland than merely providing AA to the cells. Increased CAT-1 mRNA abundance during lactation may play a key role in porcine mammary cell growth and turnover by sensing changes in extracellular Arg and Lys concentrations; for instance, in the fat body of mosquitoes, active uptake of cationic AA via specific transporters activated vitellogenesis through the target of rapamycin (TOR) pathway (Attardo et al., 2006). In addition, CAT-1 may offer a unique port of entry for Arg in endothelial cells. Arginine plays a pivotal role in enhancing blood flow in the mammary gland during lactation, as it is the substrate for synthesis of the vasodilator nitric oxide (Mann et al., 2003). Indeed, arterial Arg extraction rate by the mammary gland increases with lactation (R. Manjarin, J. P. Steibel, R. N. Kirkwood, N. P. Taylor, and N. L. Trottier, unpublished data; Trottier et al., 1997), and others have shown that CAT-1 is located on the basolateral membrane of endothelial cells (Woodard et al., 1994; Mann et al., 2003).

In conclusion, changes in mRNA abundance indicate that expression of selected AA transporters is regulated, in part, at a pre-translational level. Messenger RNA abundance of CAT-1, $ATB^{0,+}$, and y^+LAT2 was upregulated between prepartum and peak lactation, and positively correlated to expression of genes encoding for the mammary synthesized milk proteins β -CN and α -LA. As such, CAT-1, $ATB^{0,+}$, and y^+LAT2 may be involved in the transport of limiting AA in cells of the mammary parenchyma tissue, constituting molecular targets for improving sow milk production during lactation. Further studies are critically needed to determine the regulatory mechanisms that control the expression of these genes.

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APPENDIX

Selection of Reference Genes for Reverse Transcription qPCR Analysis

Recent studies have shown that upregulation of genes involved in milk synthesis causes a large increase in transcript abundance of those genes, thus diluting the mRNA abundance of reference genes that actually maintain a stable amount of mRNA per cell during lactation (Bionaz and Loor, 2007; Tramontana et al., 2008). In support of these authors' work, results from the present study indicated a significant increase ($P < 0.05$; Figure 3a in Appendix) in RNA per milligram of mammary tissue over prepartum to peak lactation, whereas relative mRNA abundance of the 3 selected reference genes decreased during over the same time period ($P < 0.05$; Figure 3b in Appendix; statistical analysis of RNA concentration and reference gene mRNA raw data has been added as supplementary material to the Appendix). Accordingly, relative mRNA

Figure 3. (a) RNA concentration (mg/g of tissue) in sow mammary tissue isolated with PerfectPure RNA Cell and Tissue Kit (5 PRIME, Inc., Gaithersburg, MD) and quantified using the Agilent Bioanalyzer 2100 with the RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). Data was compared between d 5 of lactation (early) and d 110 of gestation (prepartum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (postweaning) and peak lactation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (b) Log-transformed relative mRNA abundance [efficiency-corrected cycle threshold (Ct)] of reference genes *API5*, *VAPB*, and *MRPL39* in sow mammary tissue measured by reverse transcription quantitative PCR (RT-qPCR). Data was compared between d 5 of lactation (early) and d 110 of gestation (prepartum), d 17 of lactation (peak) and early lactation, and d 5 after weaning (postweaning) and peak lactation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

abundances of *API5*, *VAPB*, and *MRPL39* were positively correlated to each other ($P < 0.001$; Figure 4 in Appendix) and negatively correlated to total RNA per milligram of mammary tissue ($P < 0.05$; Figure 4 in Appendix) over the prepartum to postweaning periods. These findings are suggestive of an artifactual change in gene expression caused by variation in total cellular RNA (an external factor to the qPCR reaction directly related to transcript abundance of all genes in the cell). Although such artifactual dilution causes an apparent decrease in mRNA abundance of the 3 reference genes, the work by Bionaz and Loor (2007) and Tramontana et al. (2008) demonstrated that the same dilution rate applied to all genes. Accordingly, normalizing the target genes with suitable reference genes cancels out the dilution caused by RNA.

Reverse Transcription qPCR and Statistical Analysis of Reference Genes

A relative standard curve (Larionov et al., 2005) was used as the reverse transcription qPCR (RT-qPCR) method to measure relative mRNA abundance of *API5*, *MRPL39* and *VAPB* over the prepartum to postweaning periods. The use of a relative standard curve corrected for differences in total cDNA used on each RT-qPCR reaction and for differences in primer amplification efficiency between genes (Larionov et al., 2005), consequently increasing accuracy of further statistical analysis based on raw data of reference genes. For each sample, the relative standard curve quantification software generated 2 expression values. These values were log-transformed and averaged, and referred as efficiency-corrected Ct values for further analyses. Normality of the residuals was tested using the Shapiro-Wilk test under the UNIVARIATE procedure of SAS.

A test for invariance expression of reference genes and RNA concentration over prepartum to postweaning was performed using a linear mixed model in SAS. The model included stage of lactation as a fixed effect and sow as random effect. The statistical model (Equation 3) is as follows:

$$Y_{ij} = \mu + \alpha_i + b_j + e_{ij}, \quad [3]$$

where Y_{ij} is the efficiency-corrected Ct of potential reference gene or RNA concentration for each sow (j) at each stage of lactation (i), μ is the overall efficiency-corrected Ct or RNA concentration mean, α_i is the fixed effect of the i th level of stage of lactation, b_j is the random effect of the sow, and e_{ij} is the experimental error. Results are reported as least squares means $\pm$ standard error (Figure 3a and b in the Appendix). $P < 0.05$ was used for determining significance.

To investigate the relationship between efficiency-corrected Ct of 3 reference genes, and between each respective reference gene and mammary RNA concentration, correlations were determined using the PROC CORR procedure of SAS. The Pearson correlation coefficient (r) was considered significant at $P \leq 0.05$. Results are reported in Figure 2 in the Appendix.

Arithmetic Versus Geometric Mean of Reference Genes to Normalize Expression of Target Genes

Analysis of AA transporter and milk protein gene expression was made by subtracting from the target gene Ct, the arithmetic mean of the Ct of 3 selected reference genes (Equation 1). The authors acknowledge that relative amounts of a target gene (Q_C) from a standard curve must be divided by the geometric mean of

Figure 4. Correlation between log-transformed relative mRNA abundance [efficiency-corrected cycle threshold (Ct)] of reference genes *API5*, *VAPB*, and *MRPL39* with each other and with RNA concentration in mammary tissue at d 110 of gestation (prepartum), d 5 (early) and d 17 of lactation (peak), and d 5 after weaning (postweaning).

the relative amounts of 3 or more reference genes (Q_R ; Vandesompele et al., 2002). However, the geometric mean of several reference genes can be converted into an arithmetic mean to normalize the efficiency-corrected Ct instead of amounts, according to the following formula:

$$\begin{aligned}
 Y_{ij} &= \text{Log} \left(\frac{Q_C}{\sqrt[3]{Q_{R1} \times Q_{R2} \times Q_{R3}}} \right) = \text{Log}(Q_C) - \text{Log}(Q_{R1} \times Q_{R2} \times Q_{R3})^{\frac{1}{3}} \\
 &= \text{Log}(Q_C) - \frac{1}{3} (\text{Log } Q_{R1} + \text{Log } Q_{R2} + \text{Log } Q_{R3}) \\
 &= \text{Corrected Ct}_C - \frac{1}{3} \left(\text{Corrected Ct}_{R1} + \text{Corrected Ct}_{R2} + \text{Corrected Ct}_{R3} \right),
 \end{aligned}
 \quad [4]$$

where Y_{ij} are target gene normalized values for the j th sow within the i th stage of lactation, QC_{ij} and QR_{ij} are target and reference gene quantities from the thermocycler software corrected by the standard curve, respectively. If the efficiency of target and reference genes is close to 100%, efficiency-corrected Ct are not significantly different from Ct, and, therefore, the formula described above can be used to normalize the expression of target genes using the arithmetic mean of 3 reference genes, without using a standard curve on each plate to obtain relative quantities of both target and reference genes.