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Identification and Characterization of Bovine Mammary Peptide Transporters in Response to Tripeptide and Lactogenic Hormone Treatment

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ABSTRACT

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Oligopeptide transportation is mediated by the peptide transporter (PepT), which consists of two isoforms, PepT1 and PepT2. Because PepT play essential roles in amino acid metabolism and cell growth, the aim of the present study was to identify these transporters in bovine mammary glands and to analyze the potential functions of these transporters in mammary epithelial cells. Abundance of *PepT1* and *PepT2* mRNA was successfully measured in both mammary glands and cultured mammary epithelial cells. In addition, the two proteins were examined using immunohistochemistry, immunocytochemistry, and Western blots. The response of mammary epithelial cells to tripeptide and lactogenic hormone treatment was assayed. The *PepT* mRNA abundance of cultured epithelial cells and secreted protein in the culture medium were increased after tripeptide substitution and addition of hormones such as insulin, hydrocortisone, and prolactin. The response of mammary epithelial cells to tripeptide and hormone treatments suggests that PepT affects the mammary gland function and increases bovine milk production.

Keywords: bovine mammary gland; PepT1; RT-PCR; lactogen

The transportation of oligopeptides is mediated by the peptide transporter (PepT) in a variety of cells. *PepT1* (*SLC15A1*; solute carrier, SLC) is a proton-dependent peptide transporter belonging to the SLC15 family of membrane transporters, which also includes the isoform *PepT2* (*SLC15A2*) and two histidine/peptide transporters, *PhT1* and *PhT2* (Daniel and Kottra 2004). The roles of these transport proteins in mediating the absorption and bioavailability of drugs suggest their considerable importance for pharmacology (Spanier 2014). *PepT1* is primarily expressed in intestinal cells and exhibits unusually broad substrate specificity. There are at least 400 different dipeptides and 8000 different tripeptides that serve as substrates, including all possible mono- or polyvalent species (Kottra et al. 2002). In contrast, *PepT2* exhibits high substrate affinity and selectivity and is expressed in the kid-

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neys, lungs, brain, and mammary glands (Shen et al. 1999, 2004; Groneberg et al. 2001, 2002).

Peptide transporters have 12 transmembrane domains with a large extracellular loop between transmembrane domains 9 and 10. Both the amino and the carboxy terminals are on the cytoplasmic side of the membrane. These proteins also contain potential sites in the intracellular loop for protein kinase-dependent phosphorylation (Chen et al. 2002; Lyons et al. 2014). The crystal structures of di- and tripeptide-bound complexes of a bacterial homolog of PepT1 revealed at least two mechanisms for peptide recognition. The dipeptide was laterally oriented in the binding site, whereas the tripeptide revealed an alternative vertical binding mode (Lyons et al. 2014). Molecular studies on the regulation of *PepT* gene expression in mammals indicates that the promoter of *PepT1* has both an amino acid responsive element (Fei et al. 2000) and binding sites for transcription factors, such as Cdx2, PPARα and Sp1 (Shiraga et al. 1999). Moreover, the transcriptional activation of *PepT1* is also regulated by clock-controlled genes, albumin D site-binding protein, and the insulin/growth factor-TOR signaling pathway (Meissner et al. 2004). These regulatory mechanisms are conserved from worms and birds to mammals.

Although PepT have been thoroughly characterized in terms of function and cellular regulation in both human and animal models, including mice and rats, there is limited knowledge concerning PepT in the dairy cow. Bovine *PepT1* mRNA has been measured in omasum, rumen, duodenum, jejunum, and ileum tissues, but not in the kidneys, colon, and mammary glands (Chen et al. 1999). Presence of *PepT2* mRNA and protein has been detected in bovine mammary epithelial cells. Lactogenic hormones and oligopeptides significantly increased the mRNA levels of *PepT2* in cultured cells (Zhou et al. 2011). Mammary gland *PepT2* in response to epidermal growth factor and thyroid hormone has been investigated in rats and humans (Bravo et al. 2004; Lu and Klaassen 2006). However, there is a relative lack of data regarding the regulation of *PepT* by lactogenic hormones in bovine. In the present study, *PepT1* and *PepT2* expression was detected in both bovine mammary tissue slices and cultured epithelial cells, and the effects of tripeptide and lactogenic hormones on *PepT1* and *PepT2* mRNA abundance were also analyzed in cultured mammary epithelial cells.

MATERIAL AND METHODS

Animals, tissue preparation, and cell culture. Three post-peak lactating Holstein cows in the second parity, four years of age, were used under the approval of the Laboratory Animal Administration Committee of Inner Mongolia. Deep gland tissue was collected, cut into pieces, and stored in Dulbecco's Phosphate-Buffered Saline (DPBS). For tissue slices, the gland tissue samples were fixed for 12 h in 4% paraformaldehyde in DPBS. For cell culture, the gland tissue pieces were cut into small pieces and digested with type II collagenase for an hour and subsequently transferred to culture bottles containing DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin-streptomycin, 2500 µg/ml Fungizone, and 5 µg/ml Insulin-Transferrin-Selenium Solution (Gibco). The cells were incubated at 37°C in a water-saturated atmosphere containing 5% CO_2.

Immunohistochemistry and immunocytochemistry. Paraffin-embedded tissue samples were cut into 6 µm sections and mounted on poly-lysine coated slides, followed by deparaffinization and rehydration. Sections were boiled in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) 20 min using a domestic microwave for epitope retrieval. The sections were successively washed in dH_2O, incubated in 4% hydrogen peroxide, blocked in DPBS with 1% bovine serum albumin (BSA) for 2 h at room temperature, and immunohistochemistry performed. The primary antibodies were anti-SLC15A1 (1 : 200, ab203043; Abcam, UK) and anti-SLC15A2 (1 : 200, ab83771; Abcam), and the secondary antibody was goat anti-rabbit IgG H&L (Biotin, 1 : 200, ab6720; Abcam). Streptavidin HRP (1 : 5000, ab7403; Abcam) and DAB (1 : 100, ab103723; Abcam) were used as the detection system. The sections were counterstained with hematoxylin to visualize cell nuclei and mounted with DPX, imaged using a microscope (Olympus, Japan). For immunocytochemistry, cultured mammary epithelial cells at passage 2 were directly plated onto glass coverslips laying in the 6-well plates for 48 h and fixed 10 min in paraformaldehyde. After permeabilization for 10 min in DPBS containing 0.25% Triton X-100, the cells were blocked with 1% BSA in PBS for 30 min at room temperature. The primary antibodies were the same as previously described, while the secondary antibody was either goat anti-rabbit

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IgG H&L (Biotin, 1 : 200, ab6720; Abcam) for immunocytochemistry or goat anti-rabbit IgG H&L (FITC, 1 : 500, ab6717; Abcam) for fluorescent staining. Biotin detection system was as described. The fluorescent samples were counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, USA). The primary antibody was replaced with β-actin antibody and buffer as positive and negative controls, respectively.

Real-time PCR. Total RNA was extracted from both gland tissue and cultured cells using the SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. cDNA was synthesized using the PrimeScript RT Master Mix (TaKaRa Biotechnology, China), followed by quantitative PCR using the ABI Prism^{TM} SYBR Fast qPCR Kit (KAPA Biosystems, USA) on a ViiA^{TM} 7 system (Applied Biosystems, USA). The Ct values for each gene were calculated under default settings using real-time sequence detection software (Applied Biosystems). The fold changes detected at the mRNA level were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used for real-time PCR span at least one intron are shown in Table 1. Template cDNA substituted with dH2O was used as negative control to ensure no contamination of reagents.

Western blot analysis. Proteins were extracted from both gland tissue and cultured cells using a buffer (Beyotime Institute of Biotechnology, China) supplied with 1% phenylmethanesulfonylfluoride (PMSF) (Sigma-Aldrich, USA) and Protease Inhibitor Cocktail (Promega, USA). The protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto nitrocellulose membranes, followed by blocking. The membrane was incubated with primary antibody at a 1 : 1000 dilution overnight at 4°C, and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Promega) at a 1 : 2000 dilution for 1 h at room temperature. The reactions were visualized using ECL Western Blotting Substrate with 1 min exposure. β-Actin was used as the gel loading control.

Genomic DNA extraction and protein collection from culture medium. Genomic DNA was extracted using the EasyPure Genomic DNA Kit (Transgen, China). OD260/280 values and DNA concentrations were calculated using a Synergy^{TM} H4 Hybrid Microplate Reader (Bio-Tek, China). The total protein content in the medium was determined as previously (Zhou et al. 2011). Briefly, the culture medium was precipitated using cold acetone, lyophilized, and redissolved in DPBS containing 1 mmol EDTA. The protein concentration was calculated based on the absorbance measured at 562 nm. Redissolved protein solution was replaced with DPBS containing 1 mmol EDTA as a control, and BSA was used as a standard. The concentration of the tested protein was calculated using a linear equation after adjusting for dilution.

Treatment of mammary epithelial cells with tripeptide. Cells at passage 2 were seeded onto 6-well plates at a density of 1×10^5 cells per well for 24 h prior to tripeptide addition. Final concentration of phenylalanine (Phe) in treating medium was 117 mg/l (Zhou et al. 2011). Free Phe was replaced with threonine (Thr)-Phe-Phe (synthesized at Sangon, China) at ratios of 0, 5, 10, 15, and 20% of the total Phe. Free Thr was also adjusted accordingly to maintain the same final concentration of total Thr in all treatment media. After a 48-hour treatment, the culture medium was collected for secreted protein determination, and the cells were trypsinized for total DNA and RNA extraction.

Treatment of mammary epithelial cells with lactogenic hormones. Lactogenic hormone-con-

Table 1. Primer sequences used in this study

Primer name	Primer sequence (5'- 3')	Product length (bp)	T_m (°C)	GenBank Acc. No.
PepT1	TGGTCAATGAGTTCTGCGAAAG CGAGGATGGGCGTTAGGTAG	150	58	NM_001099378
PepT2	ATGGCAATGCCCAATGAAG CACCAACACAGCAACAAACAAA	105	61.4	NM_001079582
GAPDH	GCCAAGAGGGTCATCATCTC GGTCATAAGTCCCTCCACGA	197	60	AJ_000039
α*s1 casein*	CCTAAACATCCTATCAAGCACCAA ATTGACCTTCTCCTTTCCAAACAC	111	59	NM_181029

T_m = annealing temperature

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Table 2. Concentrations of additional lactogenic hormones

Lactogenic hormone	Concentration					
Prolactin (IU/ml)	0	0.0002	0.002	0.02	0.2	2
Insulin (ng/ml)	0	5	50	500	5 000	50 000
Hydrocortisone (ng/ml)	0	1	10	100	1 000	10 000

taining medium was added 24 h after the cells had been seeded. As shown in Table 2, different doses of prolactin (L6520), insulin (I6634), and hydrocortisone (H0135) (all Sigma-Aldrich) were added to medium containing 10% Thr-Phe-Phe. After treatment for 24 h, the culture medium was collected for milk protein determination, and the cells were trypsinized for total DNA and RNA extractions.

Statistical analysis. The treatment involved three cows so the study presents results referring to these three individuals. The data are presented as means ± standard deviations. Comparisons between groups were evaluated using the *t*-test, and *P*-values < 0.05 were considered significant.

RESULTS

Identification of PepT1 and PepT2 in bovine mammary glands. Gene specific amplification yielded the expected band sizes (Figure 1A). The specific gene amplification of *PepT1* and *PepT2* from the mammary tissue sample extracts was confirmed by sequencing. Immunohistochemistry revealed that both PepT1 (Figures 1C, D) and PepT2 (Figures 1E, F) were expressed in the bovine mammary glands. The PepT1 and PepT2 expression was also confirmed using Western blot analysis (Figure 1G).

PepT1 and PepT2 were expressed in cultured mammary epithelial cells. Gene specific amplification from primary mammary epithelial cell extracts showed expected band sizes (Figure 2A). Sequencing showed that the specific gene amplification of *PepT1* and *PepT2* from the mammary epithelial cells was confirmed. Immunocytochemistry showed staining for PepT1 (Figures 2D, E) and PepT2 (Figures 2F, G) in the bovine mammary epithelial cells. Moreover, mammary epithelial cells were fluorescence stained. Figures 2I, J indicated

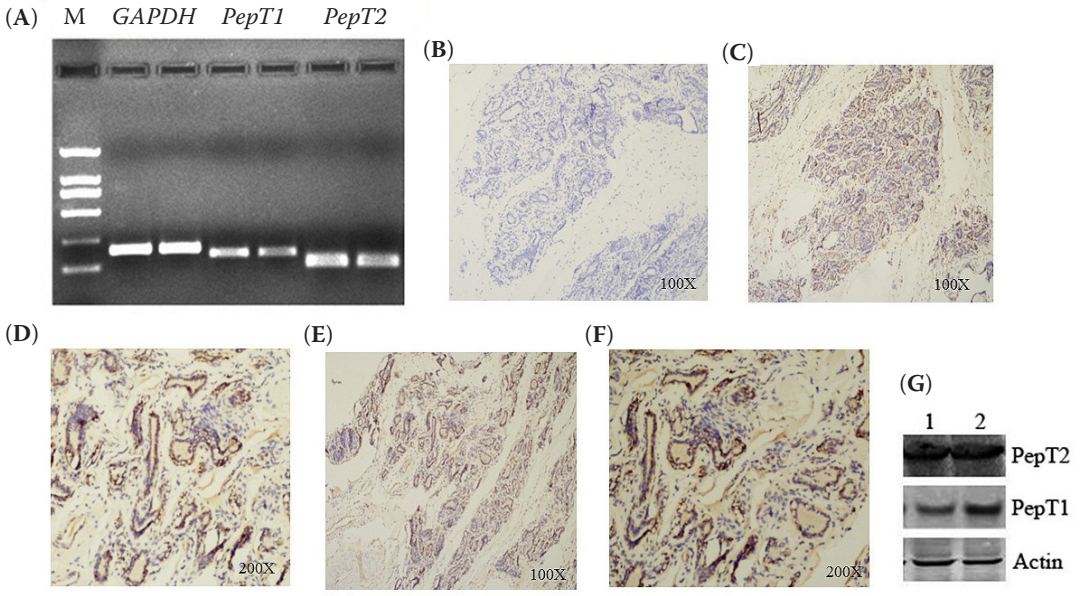

Figure 1. *PepT* expression in bovine mammary glands
Real-time PCR was conducted to amplify *PepT1* and *PepT2* from bovine mammary glands using specific primers (**A**). Bovine mammary gland sections were stained using immunohistochemistry with antibodies to detect the presence of PepT1 (**C**, **D**) and PepT2 (**E**, **F**). Control stained in the absence of primary antibody did not show specific staining (**B**). Tissue lysate was also probed with PepT1 and PepT2 antibodies (**G**)

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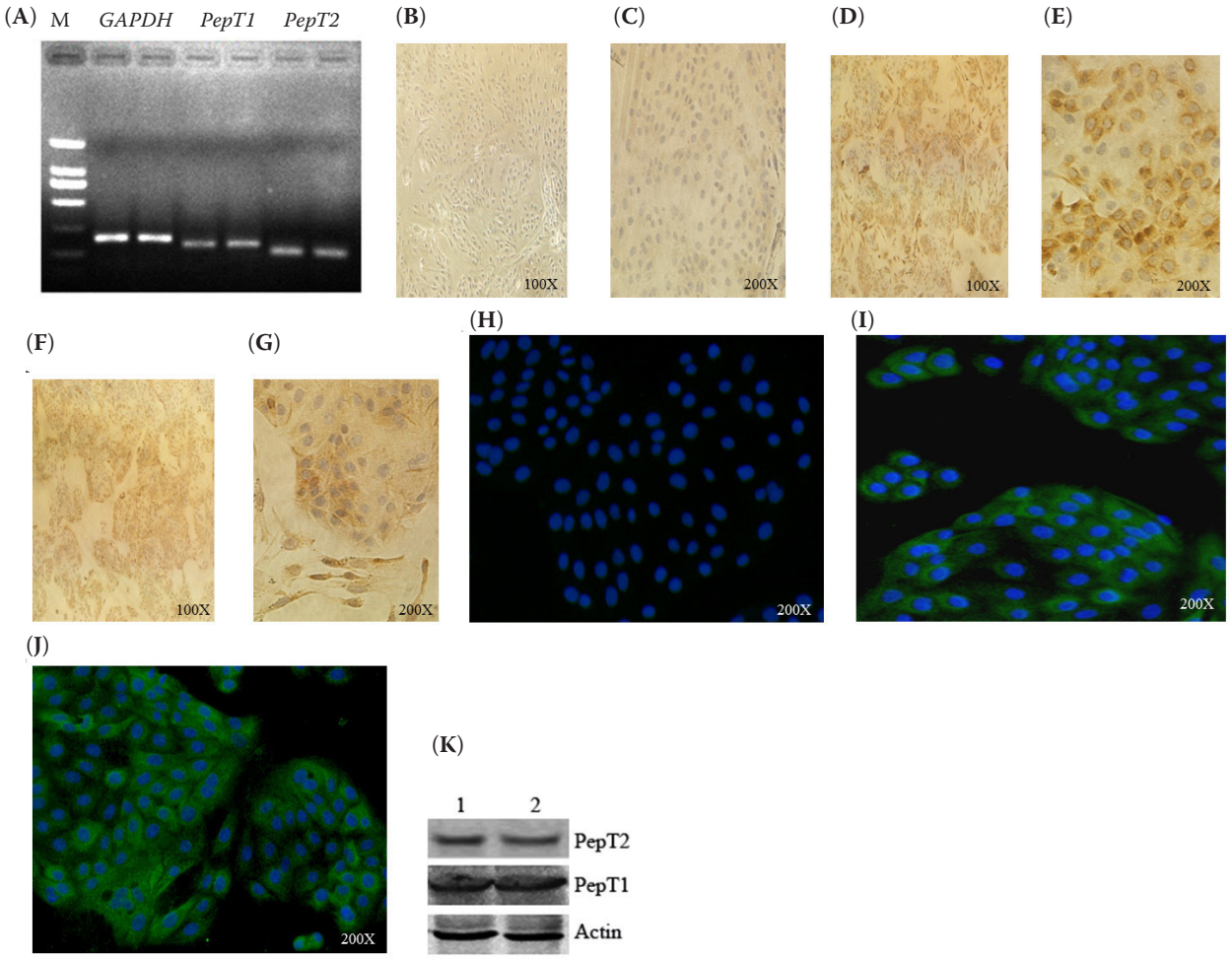

Figure 2. *PepT* expression in cultured mammary epithelial cells

Real-time PCR was conducted to amplify *PepT1* and *PepT2* from mammary epithelial cells using specific primers (**A**). Mammary epithelial cells were stained using immunocytochemistry with antibodies to detect the expression levels of PepT1 (**D**, **E**) and PepT2 (**F**, **G**). Fluorescent staining showed results similar to those obtained by immunocytochemistry (**I**, **J**). Control stained in the absence of primary antibody did not show specific staining (**B**, **C**, **H**). Cell lysate was also probed with PepT1 and PepT2 antibodies (**K**)

that PepT1 and PepT2 were located in the cell membrane and cytoplasm, consistent with the functions of these proteins. Western blot analyses of the total protein extracted from the cultured cells also showed the presence of both PepT1 and PepT2 (Figure 2K).

Effects of tripeptide on PepT. The mRNA abundance of *PepT* and protein secretion into the medium were quantified using real-time PCR and absorbance calculation methods, respectively. As shown in Figures 3A–C, *PepT* mRNA abundance was significantly ($P < 0.05$) enhanced when 5%, 10% or 15% of free Phe was replaced with Thr-Phe-Phe. The highest mRNA abundance was observed when mammary epithelial cells were incubated with 10% Thr-Phe-Phe. Tripeptide substitution also increased the level of proteins in the culture medium. Based on a curve fit, the best ratio of Thr-Phe-Phe was 8.2% (Figure 3D). Specifically, the increased abundance of *PepT*, α_{s1}*casein*, and secreted protein showed a dose-dependent effect in response to the tripeptide.

Effects of lactogenic hormones on PepT. To examine the influence of lactogenic hormones on mammary epithelial cells, concentration gradients of the three hormones (insulin, hydrocortisone, and prolactin) were added to medium containing 10% tripeptide replacement. All three hormones up-regulated *PepT* mRNA abundance (Figures 4–6). As shown in Figures 4 and 5, 50 ng/ml insulin and

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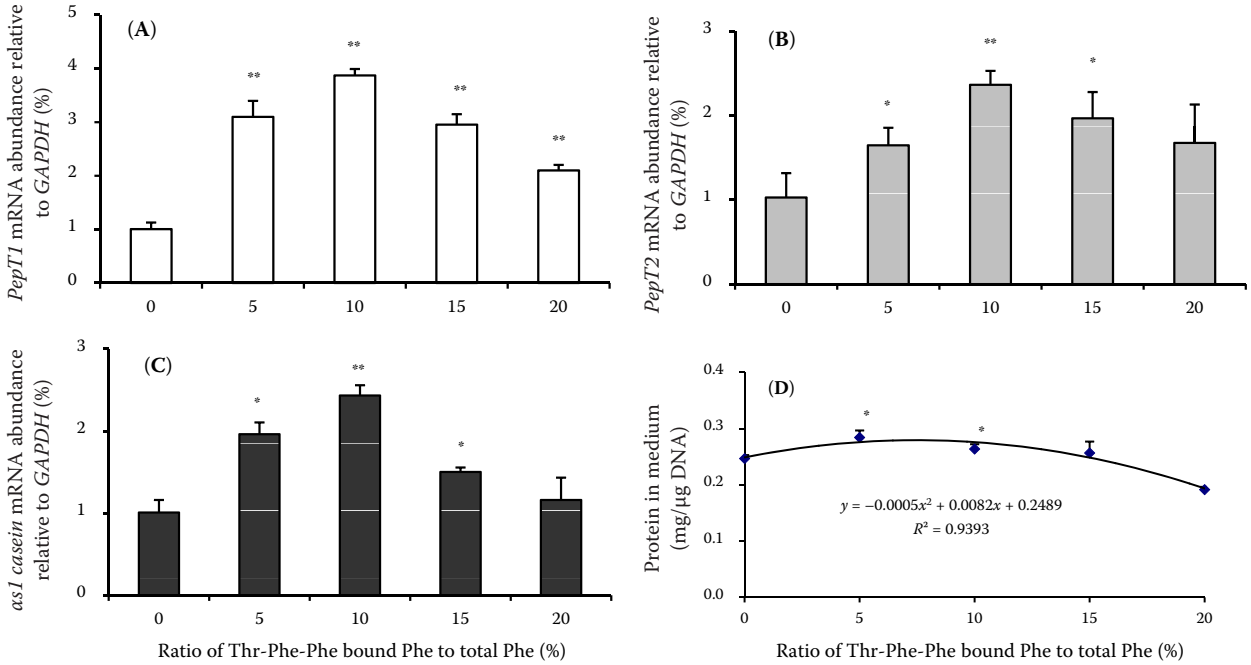

Figure 3. Effects of tripeptide on mammary epithelial cells

Free phenylalanine (Phe) was substituted with the tripeptide threonine (Thr)-Phe-Phe at ratios of 5, 10, 15, and 20%. Expression levels of *PepT1* (**A**), *PepT2* (**B**), and α_{s1}*casein* (**C**) increased ($P < 0.05$), and these proteins were secreted into medium (**D**); *significant difference, **extremely significant difference

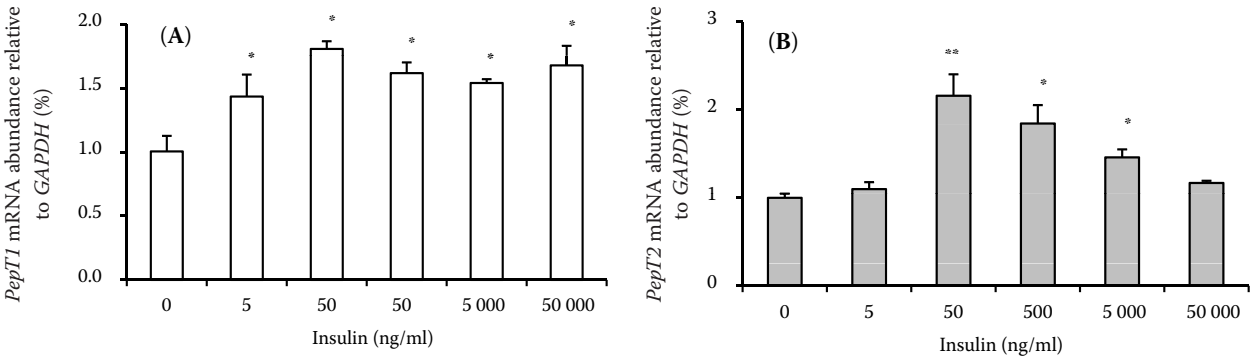

Figure 4. Effects of insulin on *PepT* gene expression

Cultured bovine mammary epithelial cells were incubated in medium containing 10% threonine (Thr)-phenylalanine (Phe)-Phe and different concentrations of insulin for 24 h. The addition of insulin significantly increased *PepT* expression, and 50 ng/ml insulin showed the highest abundance ($P < 0.05$). Fold changes in *PepT1* and *PepT2* mRNA abundance levels relative to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) were determined using real-time PCR. Values are means ± SD from three independent analyses; *significant difference, **extremely significant difference

100 ng/ml hydrocortisone generated the highest increases in *PepT* mRNA abundance. However, as the prolactin concentration increased, the *PepT* gene expression decreased (Figure 6). These results indicated that lactogenic hormones increased the *PepT* mRNA abundance *in vitro*.

DISCUSSION

In the present study, we identified *PepT1* and *PepT2* in both bovine mammary glands and cultured mammary epithelial cells. To our knowledge, this study is the first to demonstrate the

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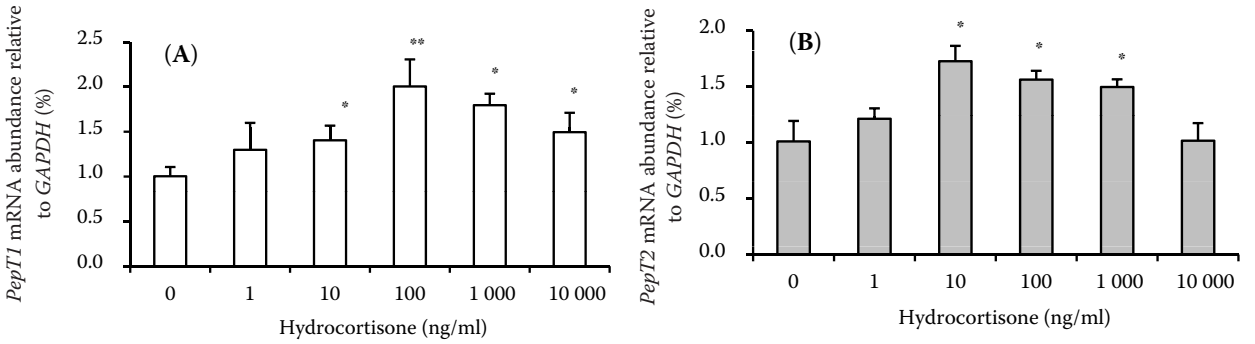

Figure 5. Effects of hydrocortisone on *PepT* gene expression

Cultured bovine mammary epithelial cells were incubated in medium containing 10% threonine (Thr)-phenylalanine (Phe)-Phe and different concentrations of hydrocortisone for 24 h. Addition of hydrocortisone significantly increased *PepT* expression, and 100 ng/ml hydrocortisone showed the highest abundance ($P < 0.05$). Values are means ± SD from three independent analyses; *significant difference, **extremely significant difference

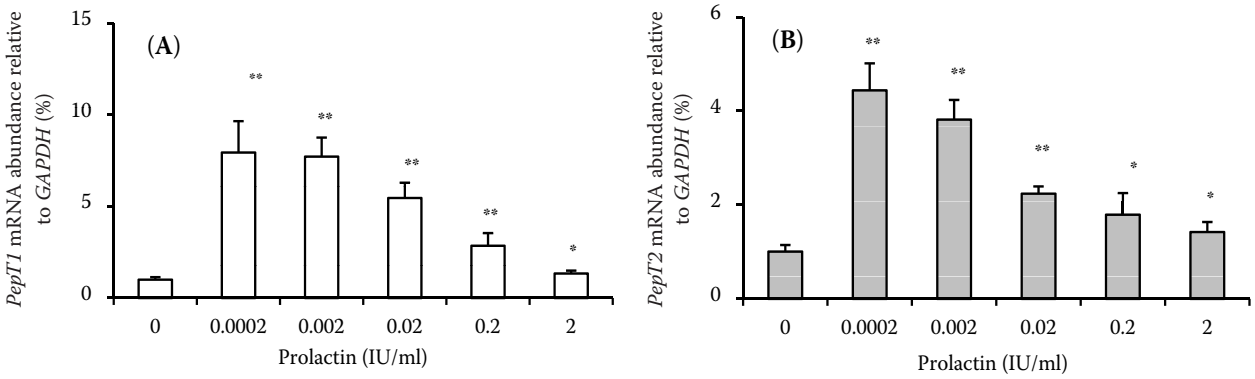

Figure 6. Effects of prolactin on *PepT* gene expression

Cultured bovine mammary epithelial cells were incubated in medium containing 10% threonine (Thr)-phenylalanine (Phe)-Phe and different concentrations of prolactin for 24 h. Addition of prolactin enhanced *PepT1* and *PepT2* mRNA abundance ($P < 0.05$), and the *PepT* mRNA level decreased with increasing prolactin concentration. Values are means ± SD from three independent analyses; *significant difference, **extremely significant difference

expression of *PepT1* in bovine where it is mainly expressed in the intestine and in the mammary gland. Additionally, the replacement of free Phe with a tripeptide in the medium and lactogenic hormone supplementation increased *PepT* mRNA abundance in mammary epithelial cells cultured *in vitro*.

The cloning, characterization, and distribution of *PepT1* and *PepT2* have provided valuable information about peptide transport in mammalian species, including worms, fish, chickens, mice, rabbits, domestic animals, and humans (Liang et al. 1995; Boll et al. 1996; Fei et al. 1998, 2000; Chen et al. 1999; Rubio-Aliaga et al. 2000; Ronnestad et al. 2007). PepT1 is a low-affinity and high-capacity peptide transporter, whereas PepT2 is a high-affinity and low-capacity peptide transporter. *PepT1* is primarily expressed in the intestinal tract and is weakly expressed in the kidneys, while *PepT2* is primarily expressed in the kidneys (Inui et al. 2000). The expression and distribution of *PepT2* has been investigated in the lactating mammary glands of rats and mice and in human epithelial cells derived from breast milk (Groneberg et al. 2002). *PepT2* has also been detected in bovine mammary glands (Zhou et al. 2011). Previous studies using Northern blot analysis demonstrated that no hybridization of *Pept1* was observed in the mammary glands of dairy cows (Chen et al. 1999). However, using reverse transcription-polymerase chain reaction (RT-PCR), *PepT1* and *PepT2* mRNAs were detected in bovine mammary gland extracts

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and mammary epithelial cells. Additionally, immunohistochemistry and immunocytochemistry showed PepT in bovine mammary gland extracts and mammary epithelial cells.

The uptake of amino acids is mediated by a number of integral cell membrane carriers that transport amino acids either in free or peptide-bound forms. PepT1 and PepT2 transport short-chain peptides and a variety of pharmacologically important compounds, including select β-lactam antibiotics, angiotensin-converting enzyme inhibitors, and antiviral and antineoplastic drugs (Rubio-Aliaga et al. 2000; Meissner et al. 2004). Studies on the regulation of *PepT1* by dietary factors in mammals have been conducted for several years (Spanier 2014). The results suggest that *PepT1* expression is modulated by amino acid response elements (AAREs) in promoters and transcription factors, such as Cdx2 and Sp1 (Shimakura et al. 2005, 2006). Consistently, synthesized Thr-Phe-Phe tripeptide treatment significantly increased *PepT* and *casein* abundance in cultured mammary epithelial cells.

The expression and function of peptide transporters are regulated by a variety of factors, including health, substrates, hormones, and pH (Fei et al. 1994; Alcorn et al. 2002; Sala-Rabanal et al. 2008). Although growth hormones and epidermal growth factors affect the abundance of *PepT1* and *PepT2*, the underlying molecular mechanism has not been elucidated (Avissar et al. 2001). The tyrosine kinases Janus kinase (JAK)2 and JAK3 also influence *PepT1* and *PepT2* (Hosseinzadeh et al. 2013; Warsi et al. 2013). Lactogenic hormones (prolactin, hydrocortisone, and insulin) play major roles in pregnancy, milk protein synthesis, postpartum development of the mammary gland, and lactation (Brennan et al. 2008; Lee et al. 2009). Prolactin enhances the production of total protein and casein in the mammary glands and also affects mammary differentiation and milk production after birth (Oakes et al. 2008). Insulin is an effective lactogenic factor that increases cell growth and maintains the number and function of mammary cells (Akers 2006). Hydrocortisone is a glucocorticoid that functions as a part of adrenal cortex hormone pathways. This hormone enhances the mRNA levels of casein (Kabotyanski et al. 2009). The lactogenic hormone-induced increasing of PepT and secreted protein suggests that peptide transporters play roles in lactation of dairy cows.

In conclusion, this study demonstrates the presence of *PepT1* mRNA and protein in bovine mammary glands and cultured mammary epithelial cells. The effect of tripeptides and lactogenic hormones on PepT and casein was examined using RT-PCR and concentration measurement. While the mechanisms underlying the effects of peptides and hormones on the mammary glands remain elusive, results of the present study suggest that PepT could have an effect on milk production in bovine.

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