The effect of G protein-coupled receptor kinase 2 (GRK2) on lactation and on proliferation of mammary epithelial cells from dairy cows

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ABSTRACT

Milk protein is an important component of milk and a nutritional source for human consumption. To better understand the molecular events underlying synthesis of milk proteins, the global gene expression patterns in mammary glands of dairy cow with high-quality milk (>3% milk protein; >3.5% milk fat) and low-quality milk (<3% milk protein; <3.5% milk fat) were examined via digital gene expression study. A total of 139 upregulated and 66 downregulated genes were detected in the mammary tissues of lactating cows with high-quality milk compared with the tissues of cows with low-quality milk. A pathway enrichment study of these genes revealed that the top 5 pathways that were differentially affected in the tissues of cows with high-versus low-quality milk involved metabolic pathways, cancer, cytokine-cytokine receptor interactions, regulation of the actin cytoskeleton, and insulin signaling. We also found that the G protein-coupled receptor kinase 2 (GRK2) was one of the most highly upregulated genes in lactating mammary tissue with low-quality milk compared with tissue with high-quality milk. The knockdown of GRK2 in cultured bovine mammary epithelial cells enhanced CSN2 expression and activated signaling molecules related to translation, including protein kinase B, mammalian target of rapamycin, and p70 ribosomal protein S6 kinase 1 (S6K1), whereas overexpression of GRK2 had the opposite effects. However, expression of genes involved in the mitogen-activated protein kinase pathway was positively regulated by GRK2. Therefore, GRK2 seems to act as a negative mediator of milk-protein synthesis via the protein kinase B-mammalian target of rapamycin signaling axis. Furthermore, GRK2 may negatively control milk-protein synthesis by activating the mitogen-activated protein kinase pathway in dairy cow mammary epithelial cells.

Key words: mammary gland, G protein-coupled receptor kinase 2, lactation, proliferation

INTRODUCTION

Milk protein, which is composed primarily of casein and whey protein, is an important nutritional source for human consumption (Haug et al., 2007). Casein accounts for ~80% of total milk protein (Cerbulis and Farrell, 1975). The bovine casein consists of approximately 40% αS1-CN, 10% αS2-CN, 37% β-CN, and 13% κ-CN (Stewart et al., 1987).

Milk-protein synthesis is tightly regulated by specific genes (Bionaz and Loor, 2011). High-throughput transcription profiling has been used to evaluate global gene expression in mammary tissues in response to development and lactation. This information can provide insights into mechanisms contributing to milk synthesis. Microarray analyses used to study biologically relevant patterns of gene expression in mouse mammary gland development and lactation have been reported (Master et al., 2002; Rudolph et al., 2003). Similarly, for bovine mammary tissue, microarray analysis has been used to characterize changes in gene expression that may be related to development (Suchyta et al., 2003), the onset of lactation (Finnucane et al., 2008), or milking frequency (Connor et al., 2008). Recently, a digital gene expression (DGE) study was used to profile gene expression changes in the bovine mammary gland before and after parturition (Gao et al., 2013). However, no study has directly compared the changes in gene expression in lactating dairy cow mammary glands associated with different lactation capacities.

Several genes have been identified that are involved in lactation, but the roles of some of these genes have not been characterized. The 7 known G protein-coupled receptor kinase isoforms (GRK; Pitcher et al., 1998) are members of the serine or threonine protein kinase family, and these proteins have important and various roles in regulating essential cellular processes (Jiang et al., 2009; Kahsai et al., 2010; Penela et al.,...
Among them, GRK2 is ubiquitously expressed throughout the body (Watari et al., 2014). The first identified function for GRK2 involved desensitization of GRK-receptor signaling by phosphorylating agonist-activated 7-transmembrane receptors (Benovic et al., 1986). Recently, evidence has accumulated documenting GRK2 interactions with cytosolic proteins involved in signaling pathways, which are relevant to essential cellular processes (e.g., proliferation, migration, trafficking, cell cycle, and development; Penela et al., 2010). However, reports are currently not available concerning the roles of GRK2 in normal mammary epithelial cell proliferation and differentiation. The mitogen-activated protein kinase (MAPK)-signaling pathway has been associated with cell proliferation in response to growth factor stimulation in the mouse mammary gland (Fata et al., 2007). In vascular smooth muscle cell proliferation, GRK2 appears to serve as a RhoA-activated scaffold protein for the ERK-MAPK cascade (Robinson and Pitcher, 2013). Whether GRK2 affects mammary epithelial cell proliferation by activating MAPK in the dairy cow mammary gland is not clear. Furthermore, the relationship between GRK2 and lactation has not been described.

For our study, lactating mammary glands from dairy cows with high- or low-quality milk were selected for the analysis of global gene expression profiles in dairy cow using the DGE approach. Among the DGE, we found that GRK2 was significantly expressed at a high level in the mammary glands of cows with low-quality milk compared with those of cows with high-quality milk. To address the role of GRK2 in mammary epithelial cell growth and lactation in the dairy cow, in vitro experiments with cultured mammary epithelial cells were carried out.

MATERIALS AND METHODS

Animals and Tissue Collection

All experiments involving animals were performed in strict accordance with the guidelines for the care and use of experimental animals at Northeast Agricultural University (Harbin, China).

For DGE profiling, 6 multiparous, lactating Holstein dairy cows were used. The cows were in the third parity, in the same lactation stage (90 DIM), and were separated into high-quality milk (33.9 ± 0.01 kg/d of milk yield; >3% milk protein; >3.5% milk fat) and low-quality milk (33.7 ± 0.5 kg/d of milk yield; <3% milk protein; <3.5% milk fat) groups (3 cows per group) according to their milk protein and fat contents (Table 1). All cows were slaughtered at 90 DIM, and mammary parenchyma tissues were excised from the upper third of the posterior area of one of their udders. These tissue samples were trimmed of connective tissue, cut into small blocks (~200 mg), and frozen in liquid nitrogen until use for RNA isolation. For the culture of mammary epithelial cells, lactating mammary tissues were collected, rinsed in 0.9% (wt/vol) sterile aqueous saline solution, and brought to the laboratory immediately.

Reagents and Antibodies

A short interfering RNA (siRNA) specific for dairy cow GRK2 and a scrambled siRNA (SCR) were purchased from Shanghai GenePharma Co., Ltd. (China). Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, 12400–024), fetal bovine serum (FBS, 10082–147), TRIZol Reagent (15596–026), Opti-MEM I Reduced Serum Medium (31985–047), Lipofectamine 2000 Transfection Reagent (11668–019), and Hanks’ balanced salt solution (HBSS, 14175) were purchased from Life Technologies (Carlsbad, CA). PrimeScript RT reagents (RR037A), SYBR Premix Ex Taq (RR420A), EcoRI (1040A), and SalI (1080A) were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Anti-GRK2 (ab137666), anti-phospho-protein kinase B (PKB, also known as AKT1, ab8932), and anti-phospho-mammalian target of rapamycin (mTOR, Ser2448, ab84400) were purchased from Cell Signaling Technology (Beverly, MA). Normal goat serum (ZLI-9021), normal rabbit serum (ZLI-9025), goat anti-rabbit IgG-horseradish peroxidase (HRP, ZB-2301), goat anti-mouse IgG-HRP (ZB-2305), and rabbit anti-goat IgG-HRP (ZB-2306) were purchased from ZSGB-BIO (Beijing, China). Nitrocellulose membranes (P-66485) were purchased from Pall Corporation (Port Washington, NY). Insulin, prolactin, hydrocortisone, and a scrambled siRNA (SCR) were purchased from Shanghai GenePharma Co., Ltd. (China). Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, 12400–024), fetal bovine serum (FBS, 10082–147), TRIZol Reagent (15596–026), Opti-MEM I Reduced Serum Medium (31985–047), Lipofectamine 2000 Transfection Reagent (11668–019), and Hanks’ balanced salt solution (HBSS, 14175) were purchased from Life Technologies (Carlsbad, CA). PrimeScript RT reagents (RR037A), SYBR Premix Ex Taq (RR420A), EcoRI (1040A), and SalI (1080A) were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Anti-GRK2 (ab137666), anti-phospho-protein kinase B (PKB, also known as AKT1, ab8932), and anti-phospho-mammalian target of rapamycin (mTOR, Ser2448, ab84400), were purchased from Cell Signaling Technology (Beverly, MA). Normal goat serum (ZLI-9021), normal rabbit serum (ZLI-9025), goat anti-rabbit IgG-horseradish peroxidase (HRP, ZB-2301), goat anti-mouse IgG-HRP (ZB-2305), and rabbit anti-goat IgG-HRP (ZB-2306) were purchased from ZSGB-BIO (Beijing, China). Nitrocellulose membranes (P-66485) were purchased from Pall Corporation (Port Washington, NY). Insulin, prolactin, hydrocortisone,
and other reagents were purchased from Sigma-Aldrich (St Louis, MO), unless otherwise stated.

**RNA Isolation**

Total RNA was isolated from mammary tissue using TRIzol reagent as described (Wang et al., 2014a), and RNA quality was assessed according to its UV spectral characteristics (NanoDrop 2000c; Thermo Scientific, Wilmington, DE). As a control, RNA integrity was analyzed by agarose gel electrophoresis. The intensity of the 28S rRNA band was approximately twice that of the 18S rRNA band.

**DGE Profiling**

BGI Tech (Shenzhen, China) performed the DGE analyses. A false discovery rate of ≤0.001 and an absolute value of log2 ratio ≥1 were used as cutoffs to judge the significance of DGE. The functions of the DGE were assigned using the gene ontology (GO) functional enrichment module online (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi). A corrected P-value of ≤0.05 was selected as the threshold for significant enrichment of the gene sets. Pathway enrichment analysis for the DGE used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). Pathways with P ≤ 0.05 were considered strongly enriched in DGE.

**Quantitative Real-Time PCR**

Selected gene expression data from the DGE study were confirmed by quantitative real-time (q)PCR. Each RNA sample was reverse transcribed with PrimeScript RT reagents to cDNA. Each gene was PCR amplified in a separate reaction, and each reaction was performed in triplicate using SYBR Premix Ex Taq regents and a 7300 Real-time PCR system (Applied Biosystems, Grand Island, NY; Liu et al., 2015). Primers were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA) and those used are shown in Table 2. The relative amount of an expressed gene pair was calculated using the comparative cycle threshold (Ct) method (Livak and Schmittgen, 2001). The expression of β-actin was not significantly different between lactating mammary tissues with high- and low-quality milk and, therefore, was selected as the housekeeping gene. Thus, the expression values obtained were normalized against that of β-actin.

**Mammary Epithelial Cell Isolation and Culture**

Mammary epithelial cells were isolated from lactating mammary tissues with high-quality milk as described in Jedrzejczak and Szatkowska (2014) with minor modifications. Briefly, mammary tissues were minced with surgical scissors. Minced samples were washed with HBSS and digested with collagenase III for 2 h at 37°C. Each digest was filtered through a nylon mesh, and the filtrate was centrifuged for 10 min at 150 × g. Each pellet was washed twice with HBSS, and the isolated cells were cultured in DMEM/F-12 medium supplemented with 10% (vol/vol) FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 5 μg/mL of insulin, 1 μg/mL of prolactin, and 1 μg/mL of hydrocortisone at 37°C in a humidified atmosphere containing 5% CO2. The primary cell cultures were trypsinized at ~80% confluency with 0.25% (wt/vol) trypsin and 0.02% (wt/vol) EDTA and passaged to remove fibroblasts. Immunofluorescence was used to detect cytokeratin18 expression in the cultured cells to confirm that the isolated cells were purified mammary epithelial cells [Supplemental Figure S1 (http://dx.doi.org/10.3168/jds.2015-10560); Wang et al., 2014a].
Plasmid Construction

Full-length GRK2 was PCR amplified from mammary tissue cDNA and inserted into the EcoRI/SalI sites of the vector pGCMV-IRES-EGFP (Tiandz, Beijing, China). The forward primer was 5'-AAGATGGCG-GACCTGGA-3', and the reverse primer was 5'-TTG-GCGGGAACAAATAATA-3'. Plasmid integrity was verified by DNA sequencing.

Transfection

Transient transfection was performed using Lipofectamine 2000 (Wang et al., 2014a). Briefly, mammary epithelial cells were plated into the wells of 6-well culture plates at 10⁶ cells/well. When the cells reached 80% confluency, transfection was performed. For GRK2 knockdown, cells were transfected with GRK2 siRNA or with SCR as the negative control. For GRK2 overexpression, cells were transfected with the GRK2-containing pGCMV-IRES-EGFP-GRK2 plasmid or a pGCMV-IRES-EGFP empty plasmid as the negative control (Supplemental Figure S2; http://dx.doi.org/10.3168/jds.2015-10560). The transfection efficiency was assessed by specific mRNA and protein levels in cell lysates isolated 24 h post-transfection. After 24 h of transfection, cells in triplicate wells were harvested for Western blotting.

Western Blotting

Abundance of proteins involved in cell proliferation (MAPK, phospho-MAPK, and cyclin D1) and milk-protein synthesis (β-CN, AKT1, phospho-AKT1, mTOR, phospho-mTOR, S6K1, and phospho-S6K1) were assessed by Western blotting as described (Wang et al., 2014b). Briefly, cells were lysed in ice-cold RIPA buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% vol/vol NP-40, 1% wt/vol sodium deoxycholate, 0.1% wt/vol SDS]. Proteins were separated via SDS-PAGE (10% wt/vol acrylamide gel), and then transferred to a nitrocellulose membranes that was then blocked for 1 h at 37°C in 5% (wt/vol) BSA in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST). Membranes were incubated overnight at 4°C with an antibody against GRK2 (1:500 dilution), MAPK (1:1,000 dilution), phospho-MAPK (1:2,000 dilution), cyclin D1 (1:1,000 dilution), β-CN (1:1,000 dilution), AKT1 (1:200 dilution), phospho-AKT1 (1:200 dilution), mTOR (1:200 dilution), phospho-mTOR (1:400 dilution), S6K1 (1:250 dilution), or phospho-S6K1 (1:1,000 dilution). After washing 3 times with TBST, each membrane was incubated for 1 h at 37°C with HRP-conjugated goat anti-rabbit IgG (1:500 dilution), goat anti-mouse IgG (1:500 dilution), or rabbit anti-goat IgG (1:500 dilution). Each membrane was washed 3 times with TBST, and antibody-bound proteins were detected using the ECL detection reagent.

Statistics

Data from at least 3 independent experiments were subjected to statistical analysis and plotted using Prism 6.0 (Graph Pad Software, La Jolla, CA). Results are presented as the mean ± SEM. To analyze the effect of GRK2 on expression of β-CN and of the signaling molecules involved in milk-protein synthesis and cell proliferation, the 2-tailed unpaired t-test was used to compare means between the GRK2 knockdown or overexpression group and the control group. Statistical significance was set at P < 0.05.

RESULTS

Identification of DGE in the Lactating Mammary Glands of Dairy Cow

The DGE analysis showed that 205 genes were significantly differentially expressed in the lactating mammary tissues of cows with high-quality milk compared with the tissues of cows with low-quality milk. Among them, 139 genes were upregulated, and 66 genes were downregulated (Supplemental Table S1; http://dx.doi.org/10.3168/jds.2015-10560). To understand their functions, all 205 DGE were mapped to GO database terms and their numbers compared with terms for the whole genome background. Figure 1A shows the top 10 GO terms in the molecular function, cellular component, and biological process categories. The majority of these DGE are associated with cellular processes in the biological process ontology category, with cell and cell part in the cellular component ontology category, and with binding activities in the molecular function ontology category. Pathway enrichment analysis revealed that the top 5 pathways associated with the DGE involved metabolic pathways, cancer, cytokine-cytokine receptor interactions, regulation of the actin cytoskeleton, and insulin signaling (Figure 1B).

Validation of the DGE Using qPCR

To confirm the DGE results, 5 genes which correlated with lactation (Sigl et al., 2014) were selected for qPCR analysis. Among these genes, the expression of CSN2, AKT1, the gene encoding signal transducer and activators of transcription 5 (STAT5), and the gene encoding E74-like factor 5 (ELF5), an enhancer of STAT signal-
ing) was upregulated in the lactating mammary tissues of cows with high-quality milk compared with the tissues of cows with low-quality milk. The expression of \textit{GRK2} was significantly lowered in the tissues of cows with high-quality milk than in those with low-quality milk (Figure 1C). The qPCR results were consistent with the DGE analysis, indicating that the DGE profiling was valid.

**GRK2 Negatively Regulates Lactation in Mammary Epithelial Cells of Dairy Cows**

To analyze the potential effect of GRK2 on lactation, we performed RNA interference experiments in cultured mammary epithelial cells. Our results showed that compared with control, \textit{GRK2} siRNA significantly decreased the mRNA expression and protein abundance of GRK2 24 h after transfection ($P < 0.01$, Figure 2). The knockdown of GRK2 in the mammary epithelial cells resulted in higher expression of $\beta$-CN ($P < 0.05$, Figures 2B and C).

The effect of overexpression of \textit{GRK2} on $\beta$-CN in mammary epithelial cells following transient transfection with pGCMV-IRES-EGFP-GRK2 was investigated. Mammary epithelial cells transiently transfected with empty pGCMV-IRES-EGFP vector were used as the negative control. Compared with the pGCMV-IRES-EGFP-transfected cells, levels of both \textit{GRK2}
mRNA and protein significantly increased 24 h after pGCMV-IRES-EGFP-GRK2 transfection (P < 0.01; Figure 3). As expected, transient transfection with pGCMV-IRES-EGFP-GRK2 resulted in a lower expression of β-CN (P < 0.05, Figures 3B, and C).

To explore the signaling pathway by which GRK2 negatively regulates β-CN expression, the abundance of AKT1, mTOR, and S6K1 proteins and their phosphorylated forms, which mediate milk-protein synthesis (Bionaz and Loor, 2011), was examined by Western blotting. The levels of phospho-AKT1, phospho-mTOR, and phospho-S6K1 in GRK2-knockdown cells were significantly increased (P < 0.01; Figures 4A and B). In contrast, overexpression of GRK2 in the cells significantly decreased AKT1, phospho-AKT1, mTOR, phospho-mTOR, S6K1, and phospho-S6K1 levels (P < 0.05; P < 0.01; Figures 4C and D). Overall, these results suggested that GRK2 negatively regulates β-CN synthesis via the AKT1-mTOR signaling axis.

GRK2 Affects the Proliferation of Mammary Epithelial Cells of Dairy Cows

To investigate if GRK2 affects mammary epithelial cell proliferation, the protein abundance levels of signaling molecules involved in proliferation were analyzed in cells transfected with GRK2 siRNA by Western blotting. The knockdown of GRK2 significantly decreased MAPK, phospho-MAPK, and cyclin D1 abundance in the cells (P < 0.01; P < 0.05; Figures 5A and B). In contrast, the overexpression of GRK2 correlated with increased of MAPK, phospho-MAPK, and cyclin D1 (P < 0.01; P < 0.05; Figure 5C and D). Taken together, these results suggest that GRK2 participates in the regulation of cell proliferation in the mammary gland of the dairy cow.

DISCUSSION

To better understand the relationship between gene expression in the lactating mammary glands of dairy cows and their production of high- or low-quality milk,
Our DGE study suggests that the lactation capacity of dairy cows can be affected by GRK2 expression, which negatively correlates with β-CN expression in the lactating mammary glands of dairy cows. This reveals a previously unrecognized role of GRK2. We also showed that GRK2 knockdown enhances β-CN expression in mammary epithelial cells which correlated with increased amount of phospho-AKT1. In lactating mice, AKT1 is required for the synthesis of sufficient quantities of milk to support their offspring (Boxer et al., 2006). Transgenic expression of the AKTI in mouse mammary gland enhances β-CN expression and results in more differentiated cells surviving in the tissue during lactation at a time when other receptor tyrosine kinases are nearly absent (Schwertfeger et al., 2001). In a mouse myeloid progenitor cell line, mTOR was found to be phosphorylated in vitro and in vivo by AKT1 (Sekulić et al., 2000). In the present study, activation of AKT1 probably increased the expression of mTOR and its active form, phospho-mTOR, in the dairy cow cells. Numerous components involved in protein synthesis are regulated by mTOR, including initiation and elongation factors and the ribosomes biogenesis (Wang and Proud, 2006). Research in ruminants has highlighted a role for mTOR in regulating milk-protein synthesis (Prizant and Barash, 2008). For the core proteins involved in mTOR signaling, the expression of FRAP1 (the gene encoding mTOR) was found to be significantly increased during lactation, and the expression pattern of FRAP1 was very similar to that of milk-protein expression (Bionaz and Loor, 2011). Ribosomal protein S6 kinase 1 is a known target of mTOR (Toerien et al., 2010) and its activation has been shown to be dependent on AKT1 and mTOR in mouse HC11 cells (Galbaugh et al., 2006). In the present study, activated mTOR probably phosphorylated S6K1, which in turn probably phosphorylates the ribosomal protein S6 and several other proteins to accelerate mRNA translation. Overall, our results reveal that GRK2 negatively regulates β-CN expression via the AKT1-mTOR-signaling pathway in mammary glands of dairy cows.

Our results also showed that GRK2 could activate the MAPK pathway and cyclin D1 expression in the mammary epithelial cells. The MAPK-signaling pathway activation is a common feature of many GRK2-receptor substrates (Lappano and Maggiolini, 2011). The MAPK-signaling pathway is also associated with cell proliferation in response to growth-factor stimulation in the mammary gland (Fata et al., 2007). Cyclin D1 is a central cell-cycle regulator (Arnold and Proud, 2006). Research in ruminants has highlighted a role for GRK2 on mammary epithelial cell proliferation.

During the lactation period, the increase in milk yield until it peaks has been correlated with an increase in cellular synthetic capacity rather than increased cell proliferation (Capuco et al., 2001). In the present study, we showed that GRK2 expression is lower in the lactating mammary glands of cows with high-quality...
milk, suggesting that GRK2 upregulates milk-protein synthesis rather than cell proliferation. In mouse HC11 cells, inhibition of MAPK signaling seems to increase STAT5 expression, indicating enhanced mammary epithelial cell differentiation and milk synthesis (Faulds et al., 2004). Our in vitro experiments also demonstrated that GRK2 knockdown decreased the expression and activity of MAPK in mammary epithelial cells of dairy cows, whereas it increased milk-protein synthesis. Conversely, members of epidermal growth factor family, such as the Neu differentiation factor and epidermal growth factor, have been reported to inhibit mammary epithelial cell differentiation, concomitant with stimulation of the Ras/Mek/Erk pathway in HC11 cells (Marte et al., 1995; Merlo et al., 1996; Cerrito et al., 2004). In the present study, GRK2 expression was greater in lactating mammary glands of cows with low-quality milk, suggesting that GRK2 may block the lactogenic differentiation of mammary epithelial cells, concomitant with the activation of the MAPK pathway. Overall, these results are consistent with the report that, in the bovine mammary gland at the onset of lactation, upregulation of genes involved in milk-protein synthesis is concomitant with inhibition of genes related to cell proliferation (Finnucane et al., 2008).

CONCLUSIONS

Mammary epithelial proliferation and lactation are regulated by specific gene expression. In the lactating mammary gland of the dairy cow, GRK2 expression negatively correlates with milk-protein synthesis and occurs in concert with stimulation of mammary epithelial cell proliferation.

ACKNOWLEDGMENTS

This work was supported by grants from National Basic Research Program (2011CB100804) from the Ministry of Science and Technology of China and the National Natural Science Foundation of China (31200984 to X. Hou, and 31401109 to Y. Lin).


