Effects of Vitamin A on Expression of Genes Related to Milk Fat and Milk Protein Synthesis in Dairy Cow Mammary Epithelial Cells (Postprint)

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Abstract

The present study aimed to investigate the effects of vitamin A on the expression of genes related to milk fat and milk protein synthesis in bovine mammary epithelial cells (BMECs). A single-factor completely randomized design was employed, wherein third-passage BMECs were randomly divided into 6 treatments with 6 replicates per treatment. After 24 h of starvation in serum-free medium, the cells were cultured for 24 h in medium containing vitamin A at concentrations of 0 (control), 0.05, 0.10, 0.20, 1.00, and 2.00 g/mL. The results showed that: compared with the control group, 1.00 and 2.00 g/mL vitamin A significantly increased the relative proliferation rate and triglyceride (TG) content ($P<0.05$); vitamin A significantly increased the expression of milk fat synthesis-related genes peroxisome proliferator-activated receptor gamma (PPARG, at 0.05, 0.10, 0.20, 1.00, and 2.00 g/mL), sterol regulatory element-binding protein 1 (SREBP1, at 0.05 and 0.10 μg/mL), and stearoyl-CoA desaturase (SCD, at 0.05 and 0.10 μg/mL) ($P<0.05$); vitamin A also significantly increased the expression of milk protein synthesis-related genes signal transducer and activator of transcription 5 (STAT5, at 0.20 g/mL), s1-casein (CSN1S1, at 0.05 and 0.10 g/mL), and -casein (CSN3, at 0.10 g/mL) ($P<0.05$); vitamin A also significantly increased the activity of the milk fat synthesis-related enzyme acetyl-CoA carboxylase (ACACA, at 0.05, 0.10, 0.20, and 1.00 g/mL) ($P<0.05$). These results suggest that vitamin A promotes the expression of genes related to milk fat and milk protein synthesis in BMECs in a concentration-dependent manner, with 0.10 g/mL vitamin A showing relatively good efficacy.

Full Text

Effects of Vitamin A on Gene Expression Related to Milk Fat and Protein Synthesis in Bovine Mammary Epithelial Cells

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Abstract

This experiment was conducted to investigate the effects of vitamin A on the expression of genes related to milk fat and protein synthesis in bovine mammary epithelial cells (BMECs). Using a single-factor completely randomized design, third-generation BMECs were randomly divided into six treatments with six replicates each. After serum starvation for 24 hours using serum-free medium, cells were cultured for 24 hours in medium supplemented with vitamin A at concentrations of 0 (control), 0.05, 0.10, 0.20, 1.00, and 2.00 g/mL. The results showed that compared with the control group, 1.00 and 2.00 g/mL vitamin A significantly increased the relative growth rate and triglyceride (TG) content ($P<0.05$). Vitamin A significantly enhanced the expression of milk fat synthesis-related genes including peroxisome proliferator-activated receptor (PPARG) at 0.05, 0.10, 0.20, 1.00, and 2.00 g/mL, sterol regulatory element-binding protein 1 (SREBP1) at 0.05 and 0.10 μg/mL, and stearoyl-CoA desaturase (SCD) at 0.05 and 0.10 μg/mL ($P<0.05$). Vitamin A also significantly increased the expression of milk protein synthesis-related genes including signal transducer and activator of transcription 5 (STAT5) at 0.20 g/mL, s1-casein (CSN1S1) at 0.05 and 0.10 μg/mL, and -casein (CSN3) at 0.10 g/mL ($P<0.05$). Additionally, vitamin A significantly elevated the activity of acetyl-CoA carboxylase (ACACA), a key enzyme in milk fat synthesis, at concentrations of 0.05, 0.10, 0.20, and 1.00 g/mL ($P<0.05$). These results suggest that vitamin A exerts concentration-dependent effects on promoting the expression of genes related to milk fat and protein synthesis in BMECs, with 0.10 g/mL vitamin A showing the most favorable effects.

Keywords: vitamin A; milk fat; milk protein; gene expression

Introduction

Milk fat and protein, as the main components of milk, are important nutritional indicators for dairy products. Vitamin A is an essential nutrient required for maintaining normal physiological metabolism in animals. It can influence animal growth, immunity, and metabolism by regulating the expression of various genes, and also affects lipid metabolism in multiple animal species. Wang et

al. [1] found that reducing dietary vitamin A levels increased beef marbling scores, while Arnett et al. [2] reported that supplementing lambs with high levels of vitamin A for 112 days increased their total intramuscular fat content. Ding [3] observed that adding 2,000–4,000 IU/kg vitamin A to grouper feed significantly enhanced lipid catabolism, which was markedly higher than in the 0 and 2,000 IU/kg groups. These findings demonstrate that vitamin A significantly influences lipid metabolism in animals. Oldham et al. [4] showed that dairy cows fed 170,000 IU/d vitamin A from 60 days prepartum to 42 days postpartum exhibited significantly increased milk yield and enhanced synthesis of milk components including milk fat and protein compared to cows fed 50,000 IU/d. Our research group previously found that adding high-dose rumen-protected vitamin A (220 IU/kg BW) to dairy cow diets tended to increase milk yield, milk fat percentage, milk protein percentage, and daily yields of milk fat and protein compared to low-dose supplementation (110 IU/kg BW) [5]. However, few reports have addressed whether vitamin A directly affects milk fat and protein synthesis in dairy cows. Bovine mammary epithelial cells (BMECs) are the primary site for milk fat and protein synthesis and secretion, and their biosynthetic capacity determines the lactation performance of the mammary gland. Therefore, this study used BMECs as a model to investigate the effects of vitamin A on milk fat and protein synthesis by examining the activity of related enzymes and gene expression levels, providing a basis for rational vitamin A supplementation in dairy production and improvement of milk composition.

1.1 Main Reagents

Collagenase II (17101-015), DMEM/F12 basal medium (12400-024), insulin-transferrin-selenium (51500-056), fetal bovine serum (FBS, 10099-141), trypsin/ethylenediaminetetraacetic acid (EDTA, 25300054), and penicillin-streptomycin (15140-122) were purchased from Gibco. Amphotericin B (AE437), prolactin (PRL, L6520), retinoic acid (RA, R2625), epidermal growth factor (EGF, E4127), and hydrocortisone (IJ0135) were obtained from Sigma. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Amresco. Phosphate-buffered saline (PBS) was from HyClone. PrimeScript™ RT Master Mix (DRR036A), SYBR Premix Ex Taq™ II (DRR820A), and RNAiso Plus (D9109B) were obtained from Takara Bio (Dalian, China). Chloroform, absolute ethanol, and isopropanol were purchased from Tianjin Fengchuan Chemical Reagent Technology Co., Ltd.

1.2 Reagent Preparation

Growth medium was prepared by adding 10 mL FBS, 2 mL penicillin-streptomycin, 0.5 mL insulin-transferrin-selenium, 100 L each of PRL, amphotericin B, and hydrocortisone, and 10 L EGF to 100 mL DMEM/F12 basal medium.

Vitamin A stock solution was prepared by dissolving 100 mg RA (the main

active metabolite of vitamin A) in 5 mL DMSO to create a 20.00 mg/mL stock, which was then diluted to concentrations of 0, 0.05, 0.10, 0.20, 1.00, and 2.00 mg/mL. The solutions were filtered through a 0.22 mol/L filter.

1.3 BMEC Culture

BMECs were cultured using the collagenase digestion method. Healthy Holstein cow mammary tissue was obtained from a slaughterhouse and transported to the laboratory under low temperature. The tissue was cut into 1 cm × 1 cm × 1 cm pieces and washed three times with 3× antibiotic PBS, then cleaned with 75% ethanol for 30 seconds, followed by three washes with 1× antibiotic PBS. Alveolar-rich regions were selected and placed in 5 mL enzyme-free sterile centrifuge tubes. After mincing to a paste-like consistency, an equal volume of 0.5% collagenase II was added, and the mixture was digested at 37 °C for 1 hour with inversion every 20 minutes to ensure complete digestion. The digest was filtered through an 80-mesh screen and centrifuged at 172×g for 5 minutes. After washing with PBS and centrifuging again for 3 minutes, cells were resuspended in growth medium and seeded into 25 cm^2 ventilated cell culture flasks. Cells were incubated at 37 °C in a 5% CO incubator until they reached 90% confluence, after which they were purified and passaged using 0.05% trypsin/EDTA.

1.4 Experimental Design

The experiment employed a single-factor completely randomized design. Third-generation BMECs were randomly divided into six treatments with six replicates each. After serum starvation with serum-free medium for 24 hours, cells were treated with medium containing vitamin A at concentrations of 0, 0.05, 0.10, 0.20, 1.00, and 2.00 μg/mL, where the 0 μg/mL group served as the control. After 24 hours of culture, cells were collected according to the required methods for subsequent analyses.

1.5 Measurement Indicators and Methods

Cell viability was measured using the MTT colorimetric assay [6] and expressed as relative growth rate (RGR). Four hours before the end of culture, 20 L MTT was added to each well. After 4 hours, the supernatant was removed, 100 L DMSO was added to each well, and after shaking for 10 minutes, the absorbance (OD) at 490 nm was measured using a microplate reader. RGR (%) = (OD nm of treatment group / OD nm of control group) × 100.

Intracellular triglyceride (TG) content was determined according to the kit instructions (E1013, Beijing Pulilai Gene Technology Co., Ltd.). After culture, the supernatant was removed, cells were washed twice with PBS, and 200 L lysis buffer was added per well for 10 minutes. The lysate was collected in 1.5 mL centrifuge tubes and centrifuged at 1,200×g for 5 minutes. The supernatant was heated at 70 °C for 10 minutes, then centrifuged at 424×g for 5 minutes, and the clear supernatant was used for measurement.

Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene, real-time quantitative PCR (ABI-7500, ABI) was used to determine the expression of genes related to milk fat and protein synthesis. The target genes included milk fat synthesis-related genes: fatty acid synthase (FASN), acetyl-CoA carboxylase (ACACA), stearoyl-CoA desaturase (SCD), lipoprotein lipase (LPL), fatty acid binding protein 3 (FABP3), sterol regulatory element-binding protein 1 (SREBP1), and peroxisome proliferator-activated receptor (PPARG); and milk protein synthesis-related genes: s1-casein (CSN1S1), -casein (CSN2), -casein (CSN3), eukaryotic initiation factor 4E (eIF4E), eukaryotic initiation factor 4E-binding protein 1 (4EBP1), mechanistic target of rapamycin (mTOR), p70 ribosomal protein S6 kinase 1 (S6K1), and signal transducer and activator of transcription 5 (STAT5). Primer sequences and parameters are shown in . The reaction program was: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s for 40 cycles; final extension at 72 °C for 7 minutes; melting curve analysis from 70–95 °C with 0.5 °C increments every 6 s for 51 cycles. Gene expression was calculated using the 2 ΔΔCt method.

The activity and protein content of enzymes related to milk fat and protein synthesis were determined according to enzyme-linked immunosorbent assay (ELISA) kit instructions, including the activities of milk fat synthesis-related enzymes FASN, ACACA, LPL, and SCD, as well as the activity of milk protein synthesis-related enzyme S6K1 and mTOR content in BMECs.

1.6 Statistical Analysis

Experimental data were analyzed using the ANOVA procedure in SAS 9.0 software, with Duncan' s multiple comparison test used for post-hoc analysis. $P<0.05$ was considered statistically significant, and $0.05 P<0.10$ was considered a trend toward significance.

Results

2.1 RGR, TG Content, and Expression of Milk Fat Synthesis-Related Genes

As shown in , RGR and TG content in the 1.00 and 2.00 μg/mL vitamin A groups were significantly higher than in the control group ($P<0.05$). For FASN gene expression, the 0.10 μg/mL vitamin A group showed the highest value, significantly higher than the control group ($P<0.05$) but not significantly different from the 0.05 μg/mL group ($P>0.05$). For ACACA gene expression, all vitamin A groups except the 2.00 μg/mL group were significantly higher than the control group ($P<0.05$), with the 0.10 μg/mL group showing the highest expression. All vitamin A groups exhibited significantly higher PPARG expression compared to the control group ($P<0.05$), with the 1.00 μg/mL group showing the highest level. For SREBP1 and SCD gene expression, the 0.05 and 0.10 μg/mL vitamin A groups were significantly higher than the control group ($P<0.05$), while the 0.20, 1.00, and 2.00 μg/mL groups showed no significant difference from the

control (P>0.05).

2.2 Expression of Milk Protein Synthesis-Related Genes

As shown in , the 0.05 and 0.10 g/mL vitamin A groups showed higher CSN1S1 gene expression, significantly greater than the control and other vitamin A groups (P<0.05), while the 0.20 and 1.00 g/mL groups were also significantly higher than the control. For CSN3 gene expression, the 0.10 g/mL vitamin A group was significantly higher than the control and the 0.20, 1.00, and 2.00 g/mL groups (P<0.05), with the latter three groups showing no significant difference from the control (P>0.05). The 0.10 g/mL vitamin A group showed significantly higher mTOR gene expression compared to the control (P<0.05), while other groups did not differ significantly (P>0.05). For STAT5 gene expression, the 0.05, 0.10, and 0.20 g/mL vitamin A groups showed higher values, particularly the 0.20 g/mL group, which was significantly higher than the control (P<0.05), while other vitamin A groups showed no significant difference (P>0.05). The 0.10 and 1.00 g/mL vitamin A groups showed a trend toward increased eIF4E gene expression compared to the control (P=0.07).

2.3 Activity of Enzymes and Protein Content Related to Milk Fat and Protein Synthesis

As shown in , for ACACA activity, all vitamin A groups except the 2.00 g/mL group were significantly higher than the control group (P<0.05), with the 0.10 g/mL group showing the highest activity. Vitamin A had no significant effect on the activities of milk fat synthesis-related enzymes FASN and LPL (P>0.05), although numerically, the 0.05 and 0.10 g/mL groups showed the highest FASN activity. SCD activity tended to be higher in the 0.05 and 0.10 g/mL vitamin A groups compared to the control (P=0.08). The 1.00 g/mL vitamin A group increased mTOR content compared to the control, though not significantly (P>0.05), but was significantly higher than the 0.05, 0.20, and 2.00 g/mL groups (P<0.05). The 0.10 and 1.00 g/mL vitamin A groups showed slightly higher S6K1 activity than other groups, but the difference was not significant (P>0.05).

Discussion

3.1 Effects of Vitamin A on Expression of Milk Fat Synthesis-Related Genes in BMECs

Milk fat is primarily composed of TG, which accounts for approximately 98% of all milk fat components. Volatile fatty acids synthesized de novo in the mammary gland and absorbed from blood are synthesized into TG on the endoplasmic reticulum of BMECs and subsequently secreted as lipid droplets of varying sizes [10]. In this study, 1.00 and 2.00 μg/mL vitamin A increased RGR and TG content in BMECs, indicating that vitamin A can promote milk fat synthesis in these cells. ACACA and FASN act on acetate and -hydroxybutyrate in BMECs

to synthesize short- and medium-chain fatty acids de novo; approximately half of C16:0 and nearly all C4:0–C14:0 in milk depend on de novo synthesis in the mammary gland. ACACA is the rate-limiting enzyme in the first stage of fatty acid synthesis, while medium-chain fatty acid synthesis is primarily mediated by FASN, which participates throughout the carbon chain elongation process. Our results demonstrate that vitamin A supplementation increased the expression of ACACA and FASN genes in BMECs, suggesting that vitamin A may promote de novo fatty acid synthesis in BMECs, leading to increased TG content. Zhang et al. [11] analyzed the expression of milk fat synthesis-related genes in mammary glands of dairy cows with different milk quality and found that high-quality cows had lower FASN expression than low-quality cows, with no significant difference in ACACA activity between groups, indicating that while ACACA is an important rate-limiting enzyme for de novo fatty acid synthesis, its activity may not be the primary factor affecting milk fat content. In our experiment, although vitamin A increased TG content in BMECs, the promoting concentrations differed from those for ACACA and FASN gene expression, suggesting that the increase in TG content may not be achieved entirely through promoting de novo fatty acid synthesis, and the specific mechanisms require further investigation.

PPARG and SREBP1 belong to the nuclear transcription factor family and nuclear hormone receptor family of ligand-activated receptors, respectively. Both serve as important transcriptional regulators in the milk fat synthesis gene network and can directly regulate target genes including ACACA, FASN, SCD, and LPL, thereby modulating fatty acid transport, synthesis, and desaturation. The expression levels and enzyme activities of milk fat synthesis-related genes are important factors regulating milk fat secretion in ruminants [12], and long-chain fatty acids entering BMECs require desaturation [13]. SCD is the key rate-limiting enzyme catalyzing the formation of monounsaturated fatty acids from saturated fatty acids, and its catalytic products are essential components of TG synthesis. Studies have shown that activated PPAR can form heterodimers with retinoid X receptor (RXR) and interact with target genes to regulate transcription and expression, while 9-cis RA, a vitamin A metabolite, is an effective ligand for RXR [14]. Our results demonstrated that vitamin A upregulated PPARG, SREBP1, and SCD gene expression while also promoting ACACA and FASN gene expression, with ACACA, FASN, and SCD activities showing similar trends to their target gene expression levels. This suggests that the increased TG content in BMECs following vitamin A supplementation may be related to enhanced PPARG and SREBP1 expression, which subsequently upregulates the transcription of downstream target genes encoding fatty acid synthesis enzymes such as FASN, ACACA, and SCD. However, few studies have reported on these specific mechanisms, necessitating further investigation.

In this study, the effects of vitamin A on milk fat synthesis in BMECs were concentration-dependent. Vitamin A at 1.00 and 2.00 g/mL showed good efficacy in promoting intracellular TG content. All vitamin A concentrations promoted the expression of FASN, ACACA, PPARG, and SCD genes, with FASN,

SREBP1, and SCD expression being highest in the 0.05 and 0.10 g/mL groups. ACACA expression was most markedly increased in the 0.10 g/mL group, while PPARG expression was most significantly upregulated at 1.00 g/mL. Although 2.00 g/mL vitamin A promoted TG content, it did not significantly affect milk fat synthesis-related gene expression compared to the control. Overall, 0.10 g/mL vitamin A demonstrated the strongest promoting effect on milk fat synthesis in BMECs.

3.2 Effects of Vitamin A on Expression of Milk Protein Synthesis-Related Genes in BMECs

Milk protein is primarily composed of caseins (approximately 80%), including CSN1S1, CSN1S2, CSN2, and CSN3, among which CSN1S1 and CSN3 are the main proteins reflecting milk protein synthesis, and their gene expression levels can indicate the capacity for milk protein synthesis in BMECs [15]. Milk protein synthesis is mainly regulated by two signaling pathways: the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, which primarily regulates milk protein synthesis at the transcriptional level under the action of prolactin (PRL) [16], and the mTOR signaling pathway, which plays a major role in regulating milk protein synthesis at the translational level [17]. mTOR belongs to the phosphatidylinositol kinase-related kinase superfamily and is a serine/threonine kinase. In bovine mammary tissue, the mTOR signaling pathway controls the translation of milk protein-related genes, and S6K1, a downstream kinase of mTOR, promotes milk protein synthesis in BMECs when both are phosphorylated and activated. Our results showed that supplementation with certain concentrations of vitamin A significantly increased CSN1S1 and CSN3 gene expression while also significantly enhancing STAT5 gene expression. Additionally, mTOR and eIF4E gene expression were higher in the 0.10 and 1.00 g/mL vitamin A groups compared to the control, with similar trends observed for mTOR content and S6K1 activity. This suggests that vitamin A may promote milk protein synthesis by activating mTOR and S6K1 through the mTOR signaling pathway. These findings indicate that the enhancing effect of vitamin A on CSN1S1 and CSN3 gene expression may be related to the mTOR and STAT5 signaling pathways, though relevant studies are scarce and require further investigation.

The insulin-like growth factor (IGF) system comprises polypeptide growth factors highly homologous to insulin. RA can induce changes in the expression of IGF-I, IGF-II, and IGF-binding proteins (IGFBP) in different cell lines [18]. He et al. [19] found that adding different concentrations of IGF-I to cultured BMECs for 24 hours significantly promoted CSN2 and ACACA gene expression in a concentration-dependent manner, indicating that IGF-I can regulate mammary gland lactation function as a signaling molecule. Wang et al. [20] reported that treating BMECs with insulin for 24 hours significantly upregulated the expression of CSN2, CSN3, STAT5, ACACA, FASN, and SREBP1 genes. Therefore, the effects of vitamin A on milk fat and protein synthesis-

related genes observed in this study may also be mediated through increased expression of IGF and IGFBP, which requires further research.

Our results also demonstrated that the effects of vitamin A on milk protein synthesis-related gene expression in BMECs were concentration-dependent, with low concentrations showing better efficacy. The 0.05 and 0.10 g/mL vitamin A groups significantly upregulated CSN1S1 and mTOR gene expression, while the 0.10 and 0.20 g/mL groups showed better promotion of CSN3 and STAT5 gene expression. In contrast, the high concentration of 2.00 g/mL vitamin A showed no significant difference from the control for any of these genes. Overall, 0.10 g/mL vitamin A demonstrated favorable effects on promoting milk protein synthesis gene expression in BMECs.

Conclusion

1. Vitamin A can enhance BMEC viability and TG content.
2. Vitamin A can upregulate the expression of milk fat synthesis-related genes (FASN, ACACA, PPARG, SREBP1, SCD) and milk protein synthesis-related genes (CSN1S1, CSN3, STAT5, mTOR) in BMECs, though its effects are concentration-dependent.
3. Vitamin A at 0.10 g/mL shows optimal promoting effects on the expression of genes related to milk fat and protein synthesis.

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Note: Figure translations are in progress. See original paper for figures.