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Effects of Saturated Long-chain Fatty Acid on mRNA Expression of Genes Associated with Milk Fat and Protein Biosynthesis in Bovine Mammary Epithelial Cells

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ABSTRACT: This study was conducted to determine the effects of saturated long-chain fatty acids (LCFA) on cell proliferation and triacylglycerol (TAG) content, as well as mRNA expression of αs1-casein (CSN1S1) and genes associated with lipid and protein synthesis in bovine mammary epithelial cells (BMECs). Primary cells were isolated from the mammary glands of Holstein dairy cows, and were passaged twice. Then cells were cultured with different levels of palmitate or stearate (0, 200, 300, 400, 500, and 600 μM) for 48 h and fetal bovine serum in the culture solution was replaced with fatty acid-free BSA (1 g/L). The results showed that cell proliferation tended to be increased quadratically with increasing addition of stearate. Treatments with palmitate or stearate induced an increase in TAG contents at 0 to 600 μM in a concentration-dependent manner, and the addition of 600 μM was less effective in improving TAG accumulation. The expression of acetyl-coenzyme A carboxylase alpha, fatty acid synthase and fatty acid-binding protein 3 was inhibited when palmitate or stearate were added in culture medium, whereas cluster of differentiation 36 and *CSN1S1* mRNA abundance was increased in a concentration-dependent manner. The mRNA expressions of peroxisome proliferator-activated receptor gamma, mammalian target of rapamycin and signal transducer and activator of transcription 5 with palmitate or stearate had no significant differences relative to the control. These results implied that certain concentrations of saturated LCFA could stimulate cell proliferation and the accumulation of TAG, whereas a reduction may occur with the addition of an overdose of saturated LCFA. Saturated LCFA could up-regulate *CSN1S1* mRNA abundance, but further studies are necessary to elucidate the mechanism for regulating milk fat and protein synthesis. (**Key Words:** Saturated Long-chain Fatty Acid, Bovine Mammary Epithelial Cells, mRNA Expression, Milk Fat, Milk Protein)

INTRODUCTION

Milk protein and fat content as well as milk fatty acids (FA) composition are the important signals for milk quality measurement. Approximately fifty percent of milk FA, including short- and medium-chain FA (SMCFA) (C4:0 to C14:0) as well as ca. one-half of the C16:0 are synthesized *de novo* from acetate and β-hydroxybutyrate in the mammary gland of dairy cows. The remaining C16:0 and almost all of the longer chain FA are considered to be derived from the diet, depending on the diet composition (Palmquist, 2006). Bionaz and Loor (2008) reported that peroxisome proliferator-activated receptor **g**amma (PPARG) which is one of members of the nuclear receptor transcription factors was up-regulated and the expression of genes involved in *de novo* fatty acid synthesis (acetyl-coenzyme A carboxylase alpha (ACACA) and fatty acid synthase (FASN), fatty acid uptake and transport (Cluster of differentiation 36 (CD36) and Fatty acid-binding protein 3 (FABP3)) and desaturation (Stearoyl-CoA desaturase [SCD]) was stimulated during lactation (Bionaz and Loor, 2008b). The results suggested that the expression of genes related to milk fat synthesis could be regulated though PPARG. Kadegowda et al. (2009) demonstrated that the role of PPARG and long-chain fatty acids (LCFA) in regulating milk fat synthesis. Studies indicated that LCFA significantly suppressed *de novo* synthesis of SMCFA (Banks et al., 1976; Jenkins, 1999; Warntjes et al., 2008) and inhibited

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ACACA and FASN mRNA expression (Kadegowda et al., 2009). Therefore, the expression of genes involved in *de novo* synthesis of FA could probably be regulated by PPARG, and further affected the synthesis of milk fat.

Studies indicated that the change of milk fat was usually accompanied by a decrease in milk protein content when fat was added in the cow diets (Cant et al., 1991; Jenkins and Mcguiret, 2006; Weisbjerg et al., 2008). Weisbjerg et al. (2008) reported that when medium and high yielding cows were fed the diets with 29, 40, and 52 g palm fatty acid distillate fat by substituting barley, general linear responses per 10 g increase in FA ration were 0.039 ($p = 0.07$) and −0.071 ($p<0.0001$) for fat and protein concentration, respectively. These data suggested that milk fat synthesis was improved by addition of exogenous LCFA whereas milk protein synthesis was inhibited. However, Yonezawa et al. (2004) indicated that exogenous LCFA such as palmitate, stearate, oleate, or linoleate stimulated the accumulation of triacylglycerol (TAG) as well as αs1-casein (CSN1S1) mRNA expression in bovine mammary epithelial cells (BMECs). Little data regarding the mechanism of the effect of LCFA on milk fat and protein synthesis is available. Some studies suggested that the mammalian target of rapamycin (mTOR) played a role in FA and TAG synthesis (Soliman, 2011), together with mammary protein synthesis (Burgos et al., 2010). Rapamycin inhibited the expression and the transactivation activity of PPARG by blocking mTOR (Kim and Chen, 2004). Milk fat and protein synthesis might be co-regulated by signal transducer and activator of transcription 5 (STAT5) (Bernard et al., 2008).

The present study examined the effects of exogenous saturated LCFA on cell proliferation and the accumulation of TAG, together with mRNA expression of *CSN1S1* and genes involved in lipid and protein synthesis in BMECs, to provide a theoretical basis for further elucidating the mechanism by which LCFA regulates milk fat and protein synthesis.

MATERIALS AND METHODS

Cell culture and treatments

Primary cells were isolated from the mammary glands of Holstein dairy cows at a local abattoir. Several pieces of approximately 1 cm^3 mammary gland tissues were aseptically removed and washed with cold phosphate buffered solution (PBS) (HyClone, NWJ0467, China) containing 100 U/mL penicillin and 100 μg/mL streptomycin. The mammary tissue fragments were minced with sterile scissors and digested by collagenase Ⅱ for 1 h at 37°C and 5% CO_2, and were shaken every 20 min. The digesta were filtered through 200 μm nylon mesh to remove large tissue fragments, the filtered liquid was centrifuged at 1,300 rpm for 5 min and the supernatant liquid was removed. The cell pellet was resuspended in the culture medium contained Dulbecco's modified Eagle's medium/F12 (DMEM/F12) media supplemented with 10% fetal bovine serum (FBS)(GIBCO), 0.5% insulin, 4 μg/mL prolactin, 1 μg/mL hydrocortisone, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO) under 5% CO_2 and air at 37°C. Cells were passaged twice and then cryopreserved. For all treatments, FBS in the culture solution was replaced with fatty acid-free BSA (1 g/L), and cells were treated with various concentrations of sodium salts of palmitate or stearate (0, 200, 300, 400, 500, and 600 μM) for 48 h in the culture solution at 37°C and 5% CO_2, and the control culture solution contained only fatty acid-free BSA without palmitate or stearate (0 μM). Each treatment was performed in Sextuple.

Preparation of palmitate and stearate

Palmitate and stearate were prepared essentially as described by Cousin et al. (2001) with minor modifications. In brief, the stock solution of 60 mM LCFA was prepared in an equimolar solution of NaOH by saponification at 70°C. Meanwhile, a 10% (wt/vol) Fatty acid-free BSA solution was prepared in ultrapure water at 55°C. Various concentrations of sodium palmitate or stearate were complexed to BSA, then mixed with shaking. The mixture was incubated at 55°C for 10 min, cooled down to room temperature and then sterile-filtered. The resulting solutions were added to the cell culture medium to attain the desired final concentrations (200, 300, 400, 500, and 600 μM) and 0.1% BSA before the experiments. The stock solutions were kept at −20°C until use.

Cell proliferation assay

Proliferation of cells was determined by MTT assay. Cells were plated in 96-well plates at 1×10^4 cells/well. Briefly, after 48 h incubation, 20 μL of 5 mg/mL MTT was added to each well and incubated for 4 h at 37°C. Then the formazan crystals were dissolved in 100 μL DMSO. The absorbance of each well was read at 490 nm using a microplate reader (BioTek, USA).

Oil red O staining and assay

Intracellular triglyceride accumulation was measured by Oil Red O staining according to the method of Ramirez-Zacarias (1992). The lipid droplets staining in cells were done with Oil Red O method. Cells were seeded (1×10^5 cells/well) in 24-well plates and treated with palmitate and stearate for 48 h. After two washes with PBS, cells were fixed with 4% paraformaldehyde for 1 h. Fixed cells were washed with PBS, and stained with 0.5 mL of Oil Red O for 2 h. Then cells were rinsed three times with PBS. Images were taken using an inverted microscope at ×400

magnification. For quantification, cells were incubated with 0.3 mL of isopropanol for 20 min to extract Oil Red O staining, and optical density (OD) was measured at 510 nm by a microplate reader (BioTek, USA).

RNA extraction and Real-Time PCR

Cells were plated in 6-well plates at 2×10^5 cells/well in lactogenic medium. After 48 h, Culture media was changed to DMEM/F12 induction medium containing different concentrations of LCFA, and FBS was replaced with fatty acid-free BSA (1 g/L). Cultures were maintained at 37°C and 5% CO_2 for 48 h. Total RNA was harvested from cells using RNAprep pure Cell Kit (TIANGEN, DP430, China) according to the manufacturer's instructions. RNA integrity and purity were assessed by 2% agarose gel electrophoresis and microplate reader.

cDNA was generated for real-time PCR in a 10-μL volume using PrimeScript RT Master Mix (TaKaRa, No.DRR036A). Real-time PCR reactions were carried out in 20-μL reactions containing 10 μL of 2×SYBR Premix Ex Taq II, 2 μL cDNA, 0.4 μL each of 10 μM forward and reverse primers, and 0.8 μL RNase free water. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the internal controls. Primers were included in Table 1. Reactions were performed in a MxPro-Mx3000P (Agilent) Real-time PCR machine with an initial denaturing step of 95°C for 30 s followed by forty cycles of 95°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 20 s(extension). Quality and specificity of PCR product were assessed by melt curve analysis and subsequent agarose gel electrophoresis. The quantitative real-time PCR data were calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Data were analyzed using General Linear Model procedure of SAS software (SAS Version 9.0), and to test the statistical significance among treatments. Regression analysis was carried out to evaluate linear and quadratic effects of saturated LCFA on the various response criteria. A level of $p<0.05$ was regarded as significant, while difference of $p<0.10$ was discussed as a statistical trend.

RESULTS

Cell proliferation

The present study examined the effects of saturated LCFA on BMECs proliferation by MTT assay. BMECs were cultured with palmitate or stearate at the concentrations of 0, 200, 300, 400, 500, and 600 μM for 48 h. The results indicated that cell proliferation tended to be suppressed when the addition of palmitate was above 500 μM. Regression analysis showed that cell proliferation tended to be increased quadratically with increasing addition of stearate (Table 2, $p = 0.052$). Cell proliferation was promoted more effectively when cells were treated with stearate from 200 to 500 μM in the culture medium than the

Table 1. Summary of genes, primer sequences and amplicon size

Gene	Primer sequences	Amplicon size (bp)
GAPDH	F. 5'-GGGTCATCATCTCTGCACCT	177
	R. 5'-GGTCATAAGTCCCTCCACGA	
FASN	F. 5'- AGGACCTCGTGAAGGCTGTGA	85
	R. 5'-CCAAGGTCTGAAAGCGAGCTG	
ACACA	F. 5'- CATCTTGTCCGAAACGTCGAT	101
	R. 5'- CCCTTCGAACATACACCTCCA	
SCD	F. 5'- TCCTGTTGTTGTGCTTCATCC	101
	R. 5'- GGCATAACGGAATAAGGTGGC	
CD36	F. 5'- CCTCTTGGCAACCACTTTCA	113
	R. 5'- GCTTTGACACCCGAGTAACG	
FABP3	F. 5'- GAACTCGACTCCCAGCTTGAA	102
	R. 5'- AAGCCTACCACAATCATCGAAG	
PPARG	F. 5'- CCAAATATCGGTGGGAGTCG	101
	R. 5'- ACAGCGAAGGGCTCACTCTC	
CSN1S1	F. 5'-ACATCCTATCAAGCACCAAGGACTC	192
	R. 5'-GACGAAATGCTTTCAGCTTCCA	
STAT5	F. 5'-AAGACCCAGACCAAGTTCGC	422
	R. 5'-AGCACCGTGGCAGTAGCAT	
MTOR	F. 5'- TGAACTGGAGGCTGATGGACAC	83
	R. 5'- TGACTGGCCAGCAGAGTAGGAA	

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; FASN = Fatty acid synthase; ACACA = Acetyl-CoA Carboxylase; SCD = Stearoyl-CoA desaturase; CD36 = Cluster of differentiation 36; FABP3 = Fatty acid-binding protein 3; PPARG = Peroxisome proliferator-activated receptor-γ; CSN1S1 = αs1-casein; STAT5 = Signal transducer and activator of transcription 5; Mtor = Mammalian target of rapamycin.

Table 2. Effect of palmitate and stearate on BMECs proliferation

LCFA	Treatment (μM)						SEM	p-value	
	0	200	300	400	500	600		Linear	Quadratic
Palmitate	100.00	109.44	102.58	102.36	97.28	96.00	0.014	0.287	0.148
Stearate	100.00	116.38	112.04	111.81	111.69	103.32	0.008	0.770	0.052

control and the 600 μM stearate addition.

Accumulation of intracellular TAG

As shown in Table 3, intracellular TAG accumulation increased significantly ($p<0.05$) and quadratically with increasing addition of palmitate in culture medium. TAG contents increased significantly ($p<0.05$) and linearly or tended to increase quadratically ($0.05<p<0.1$) with increasing addition of stearate to the culture medium. The addition of palmitate (400 to 500 μM) or stearate (400 to 600 μM) caused greater TAG contents than the control and other treatments. TAG contents tended to decrease with the addition of palmitate at 600 μM.

mRNA abundance of CSN1S1 and genes associated with lipid and protein synthesis in BMECs

FASN, *ACACA*, and *FABP3* mRNA expressions in BMECs were significantly ($p<0.05$) down-regulated linearly or quadratically with the addition of palmitate (Table 4), and treatments with addition of 500 to 600 μM palmitate had lower *FASN*, *ACACA* mRNA expressions than the control and those with addition of 200 to 400 μM palmitate. In contrast, expression of *CD36* was markedly up-regulated in a dose-dependent manner from 0 to 600 μM when palmitate was supplemented in culture medium ($p<0.05$). The expression of *CD36* mRNA in the cells cultured with palmitate at 400 to 600 μM was 19.57-, 21.07-, and 39.05-fold greater than the control, respectively. Expression of *CSN1S1* was significantly increased linearly ($p<0.05$) or tended to increase quadratically ($p<0.10$) with increasing contents of palmitate relative to the control. The expression of *CSN1S1* in the cells incubated with palmitate at 400 to 600 μM was greater than those observed in the control and other treatments. Expressions of *SCD*, *PPARG*, *mTOR*, and *STAT5* were not affected by the addition of palmitate ($p>0.05$), but expression of *SCD* and *mTOR* mRNA tended to be depressed in value with the increasing addition of palmitate versus the control, while *PPARG* mRNA expression had the opposite tendency.

As shown in Table 5, expressions of *FASN*, *ACACA*, and *SCD* and *FABP* mRNA were decreased linearly or quadratically with incremental addition of stearate ($p<0.05$). The treatments with addition of 500 to 600 μM stearate in culture medium had lower expression of *FASN*, *ACACA*, *SCD*, and *FABP*3 than the control and those with addition of 200 to 400 μM stearate. Stearate induced linearly or quadratically *CD36* and *CSN1S1* mRNA expression ($p<0.05$) with the increasing addition of stearate, and the

Table 3. Effect of palmitate and stearate on TAG contents in BMECs

LCFA	Treatment (μM)						SEM	p-value	
	0	200	300	400	500	600		Linear	Quadratic
Palmitate	0.078	0.091	0.094	0.102	0.096	0.089	0.004	0.23	0.04
Stearate	0.089	0.093	0.094	0.096	0.101	0.098	0.002	0.01	0.05

Table 4. Effect of palmitate on the mRNA expression of genes involved in lipid synthesis in BMECs

Gene[1]	Treatment (μM)						SEM[2]	p-value	
	0	200	300	400	500	600		Linear	Quadratic
FASN	1.00	0.92	0.87	0.80	0.76	0.65	0.16	0.00	0.00
ACACA	1.00	0.79	0.75	0. 72	0.67	0.66	0.07	0.00	0.00
SCD	1.00	0.64	0.68	0.72	0.79	0.67	0.12	0.22	0.20
CD36	1.00	2.00	3.58	19.57	21.07	39.05	4.21	0.02	0.01
FABP3	1.00	0.70	0.68	0.63	0.60	0.53	0.10	0.01	0.01
PPARG	1.00	1.63	1.57	2.69	1.78	1.57	0.33	0.32	0.29
CSN1S1	1.00	1.15	1.18	1.96	2.26	2.02	0.50	0.02	0.09
STAT5	1.00	0.90	0.95	1.31	0.83	0.91	0. 17	0.86	0.90
MTOR	1.00	0.82	0.83	0.86	0.93	0.84	0.12	0.40	0.34

[1] FASN = Fatty acid synthase; ACACA = Acetyl-CoA Carboxylase; SCD = Stearoyl-CoA desaturase; CD36 = Cluster of differentiation 36; FABP3 = Fatty acid-binding protein 3; PPARG = Peroxisome proliferator-activated receptor-γ; CSN1S1 = αs1-casein; STAT5 = Signal transducer and activator of transcription 5; Mtor = Mammalian target of rapamycin.

[2] SEM = Standard error of the mean.

Table 5. Effect of stearate on the mRNA expression of genes involved in lipid synthesis in BMECs

Gene[1]	Treatment (μM)						SEM[2]	p-value	
	0	200	300	400	500	600		Linear	Quadratic
FASN	1.00	0.99	0.99	0.84	0.60	0.61	0.09	0.02	0.03
ACACA	1.00	0.97	0.83	0. 73	0.69	0.61	0.13	0.00	0.01
SCD	1.00	0.89	0.56	0.54	0.49	0.44	0.09	0.01	0.03
CD36	1.00	1.68	5.41	7.52	11.71	15.03	2.17	0.00	0.00
FABP3	1.00	0.75	0.74	0.72	0.34	0.28	0.04	0.01	0.03
PPARG	1.00	2.07	2.57	1.95	1.35	1.23	0.58	0.60	0.38
CSN1S1	1.00	4.44	5.29	6.10	9.46	8.26	2.50	0.00	0.02
STAT5	1.00	1.06	1.22	1.55	1.33	1.16	0. 37	0.26	0.33
mTOR	1.00	0.90	1.37	1.19	1.10	1.00	0.14	0.75	0.61

[1] FASN = Fatty acid synthase; ACACA = Acetyl-CoA Carboxylase; SCD = Stearoyl-CoA desaturase; CD36 = Cluster of differentiation 36; FABP3 = Fatty acid-binding protein 3; PPARG = Peroxisome proliferator-activated receptor-γ; CSN1S1 = αs1-casein; STAT5 = Signal transducer and activator of transcription 5; mTOR = Mammalian target of rapamycin.
[2] SEM = Standard error of the mean.

treatments with addition of 500 to 600 μM stearate in culture medium had higher expression of *CD36* and *CSN1S1* mRNA than the control and those with addition of 200 to 400 μM stearate. The *CSN1S1* mRNA expressions in the cells cultured with stearate (200 to 600 μM) were 4.44-, 5.29- , 6.10-, 9.46-, and 8.26-fold greater than the control, respectively. CD36 mRNA expressions with addition of 200 to 600 μM stearate were 1.16, 5.41, 7.52, 11.71, and 15.03 fold greater than the control, respectively (Table 5). Expression of *PPARG*, *mTOR*, and *STAT5* were not affected by the addition of stearate ($p>0.05$), but *PPARG* and *STAT5* expression had a tendency to be improved compared with the control in value.

DISCUSSION

Compared with the control, all LCFA had no significant effects on cell proliferation at 0.78 to 100 μM, and a significant suppression was observed at 200 to 400 μM when BMECs were cultured with 0.78 to 400 μM stearate, oleate, linoleate, or linolenate (Cui et al., 2012). Yonezawa et al. (2008b) indicated that there was no significant differences in cell proliferation when BMECs were incubated with or without 100 μM palmitate or stearate, whereas a significant increase occurred compared with the control when incubated with 100 μM oleate or linoleate. However, the present study indicated that BMECs proliferation tended to increase quadratically with increasing addition of palmitate or stearate in a dose-dependent manner, and that the addition of 200 to 500 μM palmitate or 200 to 400 μM stearate promoted more effectively than the other groups, but the positive effects tended to be suppressed when the addition of palmitate or stearate was increased to 500 and 600 μM, respectively. It would therefore appear that a low dose of palmitate or stearate had a stimulatory effect on BMECs proliferation, and that overdose of palmitate or stearate had adverse effects, and inhibited the proliferation of BMECs *in vitro*. The reason LCFA could mediate cell proliferation and survival was unknown, and it is probably related to bGPR40, GPR40 is one of the G protein-coupled receptors, and it could be activated by medium and long-chain fatty acids (Briscoe et al., 2003), and then regulated cell proliferation and survival in mammary epithelial cells (Yonezawa et al., 2008b). The reason the present result was inconsistent with previous research was probably that the action of LCFA on cell proliferation was dependent on the kinds and amount of exogenous LCFA. There is very little data that examines the relationship between LCFA and cell proliferation in BMECs and further studies are needed to investigate the mechanism.

Milk fat is composed mainly of TAG secreted mammary epithelial cells in the form of droplets of variable size. Cells cultured with LCFA (palmitate, stearate or oleate) at 200 to 400 μM or linoleate at 50 to 400 μM markly increased TAG accumulation (Yonezawa et al., 2004). Yonezawa et al. (2008a) showed that 100 μM oleate and linoleate caused a significant increase in the accumulation of TAG, whereas palmitate and stearate failed to do so. In contrast, intracellular TAG contents were 140, 80, and 250% greater than the control, respectively, when 100 μM palmitate, stearate or t10c12 CLA was supplemented in culture medium (Kadegowda et al., 2009). These results suggested that addition of exogenous LCFA could accelerate the accumulation of intracellular TAG in BMECs. The present experiment observed similar results with palmitate or stearate, and indicated that TAG contents were increased at 0 to 600 μM in a concentration-dependent manner, and the addition of 600 μM was less effective in improving TAG accumulation.

ACACA and *FASN* are two important genes implicated in *de novo* synthesis of milk fatty acid in bovine mammary

tissues. *De novo* synthesis of fatty acid, via acetyl-CoA and butyryl-CoA, is executed by *ACACA* and *FASN* (Bionaz and Loor, 2008). The present results suggested that expressions of *ACACA* and *FASN* were suppressed markedly when palmitate was added in the culture medium. The results explained the previous observations by Noble et al. (1969), who found that the supplementation of 10% palmitic acid in the cow diet decreased the concentrations and yields of 6:0, 10:0, 12:0 and 14:0 and the concentrations of 8:0 and 14:1, but increased the concentrations of 16:0 and 16:1, as well as the yields of 4:0, 16:0 and 16:1 in the milk fat. Similarly, the concentrations of short and medium chain FA (C6:0-C15:0) were decreased or tended to be decreased when C16:0 was supplemented in the diet of lactating dairy cows *in vivo* (Warntjes et al., 2008). So, the present study proposed that palmitate could inhibit *de novo* synthesis of milk fatty acid through suppressing genes (*ACACA* and *FASN*) associated with *de novo* fatty acid synthesis. A previous study reported that palmitic acid suppressed *de novo* synthesis of fatty acids from acetate in bovine mammary homogenate and the possible reason was an allosteric effect on ACACA or FASN (Wright et al., 2002). However, opposite results were observed by Hansen and Knudsen (1987), who reported that addition of palmitic acid to the incubation medium stimulated synthesis and incorporation of fatty acids synthesized *de novo* into triacylglycerols. They presumed that palmitic acid acted as a "primer" for triacylglycerol synthesis by acytation of the sn-1 position; thereby it allows medium-chain and short-chain fatty acids to be incorporated into the sn-2 and sn-3 positions (Hansen and Knudsen., 1987). Therefore, further studies are necessary to solve this inconsistency.

Noble et al. (1969) reported that the addition of 5% or 10% stearic acid instead of starch in the diets of cows decreased the concentrations and yields of 10:0, 12:0, 14:0, 14:1, 16:0, and 16:1 in the milk fat but increased the concentrations and yields of 18:0 and 18:1. In the present study, the suppressing effects of stearate on *ACACA* and *FASN* mRNA abundance were shown, which was also in agreement with previous observations by Kadegowda et al. (2009), who reported that treatment with stearate decreased the expression of *ACACA* and *FASN* in MacT relative to the control. Three possible reasons for inhibitory effect of *de novo* fatty acid synthesis by stearate are as follows: i) Stearate inhibited *de novo* synthesis of fatty acid by down-regulating the mRNA expression of *ACACA* and *FASN*; ii) Long-chain acyl-CoA competed with newly synthesized medium-chain acyl-CoA for the sn-2 and sn-3 positions of the triacylglycerol backbone; iii) long-chain acyl-CoA inhibited *de novo* synthesis of fatty acid by suppression of acetyl-CoA carboxylase (Hansen and Knudsen., 1987).

FABP participates in the uptake and intracellular transport of fatty acid in many tissues (Lehner and Kuksis, 1996). The coexpression of *FABP* and *CD36* in the bovine mammary gland implied that these two proteins were indeed involved in a close functional relationship (Spitsberg et al., 1995). FABP3 involved in intracellular LCFA transport and *FABP3* mRNA abundance was up-regulated during lactation in the bovine mammary gland (Bionaz and Loor, 2008a). Kadegowda et al. (2009) reported that palmitate and stearate up-regulated the *FABP3* mRNA expression, whereas t10c12 CLA and cis-9 18:1 had the opposite effect. However, in the present results showed that the expression of FABP3 was inhibited by addition of palmitate or stearate in the culture medium. Less data are available regarding effect of saturated LCFA on *FABP3* mRNA expression and the reason for the discrepancy is still unknown. CD36 played an important role in FA importation in bovine mammary cells (Bionaz and Loor, 2008b). Previous studies indicated that mRNA abundance of *CD36* was markedly increased when cells were cultured with palmitate, stearate, oleate, linoleate, 20:5 or t10c12 CLA (Yonezawa et al., 2004; Kadegowda et al., 2009). This trend was confirmed by the present study.

PPARG is one of members of the nuclear receptor transcription factors. Kadegowda et al. (2008) reported that Lipogenic genes were up-regulated when MAC-T cells were incubated with the PPARG agonist rosiglitazone compared with the control and the results suggested that PPARG played a role in regulating bovine milk fat synthesis. The present findings suggested that both palmitate and stearate had no significant effect on *PPARG* mRNA expression in BMECs, but *PPARG* mRNA expression had a tendency to be increased in value with the increasing addition of palmitate and stearate compared with the control. Kadegowda et al. (2009) indicated that fatty acids including 16:0, 18:0, cis-9 18:1, trans-10 18:1, trans- 10, cis-12 18:2 and 20:5, up-regulated or down-regulated the expression of the lipogenic genes without affecting mRNA abundance of *PPARG* in $BMEC_S$. These results are supported by *in vivo* study that the lipid supplement diets had no effect on the expression *PPARG* in bovine mammary tissue (Invernizzi et al., 2010). Although there is no effect of fatty acids on expression *PPARG*, we cannot neglect the role for this nuclear receptor transcription factor in milk fat synthesis.

Little information is available regarding the regulation of milk protein synthesis by fatty acids. In this study, the findings indicated that palmitate or stearate elevated linearly or quadratically *CSN1S1* mRNA expression. Yonezawa et al. (2004) observed that expression of *CSN1S1* was stimulated when saturated or unsaturated fatty acids were supplemented in culture medium. However, previous observation reported that β-casein levels were decreased in HC11 mouse mammary epithelial cells treated with unsaturated fatty acids, but not saturated fatty acid (Pauloin et al., 2010). There are two possible reasons for this

difference: i) the cells are from different species of animals, and HC11 is a cell line ii) HC11 cells are unable to produce αS1-casein (Pauloin et al., 2010). In addition, the present study examined only the regulation of *CSN1S1* at a transcriptional level by saturated LCFA.

The mTOR protein, a conserved Ser/Thr protein kinase, is comprised of two distinct multi-protein complexes termed mTORC1 and mTORC2 (Laplante and Sabatini, 2009). The mTOR signaling cascade integrated amino acid availability, cellular energy status, and endocrine signals to regulate protein synthesis by phosphorylating eukaryotic initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1), a translational repressor, and p70 ribosomal protein S6 kinase-1 (S6K1) (Burgos et al., 2010). The expression of *SREBP1* target genes *ACACA*, *FASN*, and *SCD* was suppressed by rapamycin, suggesting a role for mTORC1 in *de novo* synthesis of fatty acids (Soliman, 2011). mTOR inhibition with rapamycin reduced protein levels and the activity of PPARG *in vitro* (Kim and Chen, 2004). The present study did not investigate the effect of palmitate or stearate on *SREBP1* expression, however, there were no significant differences in mRNA abundance of *mTOR* and *STAT5* between saturated fatty acid treatments and the control, although they had a tendency to be increased or be decreased in value with increasing addition of palmitate or stearate. Many protein-protein interactions are involved in regulating STAT5 activity at the level of gene expression (Furth et al., 2011). Consequently, the fact that the expressions of *mTOR* and *STAT5* were not affected by saturated LCFA in our study is not against a role for two signaling pathways in the synthesis of milk fat and protein.

Taken together, the present study revealed that BMECs viability and the accumulation of TAG was stimulated in a dose-dependent matter with increamental addition of saturated LCFA in culture medium. Palmitate and stearate suppressed linearly or quadratically the expression of *ACACA*, *FASN*, and *FABP3*, but had the opposite effect on *CD36* and *CSN1S1* mRNA abundance. Our results suggested that saturated LCFA could inhibit *de novo* synthesis of milk fatty acids and accelerate milk protein synthesis by regulating related genes expression. The present results also implied that LCFA regulated milk fat synthesis as well as milk protein synthesis. Significant differences in mRNA expression of *PPARG*, *mTOR*, and *STAT5* were not observed in this study, so future research is needed to elucidate the role of LCFA in regulation of protein translation. Further investigation is required to examine the exact mechanism in which LCFA regulates milk fatty acids and milk protein synthesis.

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