Animal Nutrition 5 (2019) 202–208

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Short Communication

Effect of different exogenous fatty acids on the cytosolic triacylglycerol content in bovine mammary cells

Einar Vargas-Bello-Pérez [a, 1, *], Juan J. Loor [b], Philip C. Garnsworthy [a]

[a] The University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, United Kingdom
[b] Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, 1207 West Gregory Drive, Urbana, IL 61801, USA

ARTICLE INFO

Article history:
Received 19 April 2018
Received in revised form
13 July 2018
Accepted 5 September 2018
Available online 26 September 2018

Keywords:
Long chain fatty acid
Mammary cells
Trans fatty acid
Triacylglycerol accumulation
Vaccenic acid

ABSTRACT

The objective of this study was to determine how cytosolic triacylglycerols (TAG) are stored in mammary cells and whether this depends on the individual chemical configuration of fatty acids (FA). This objective was accomplished by addition of different FA to a FA-free medium used to culture mammary alveolar cells-large T antigen cells (MAC-T). Treatments consisted of adding FA (palmitate, stearate, oleate, linoleate, rumenic acid [CLA], elaidate and vaccinate) solutions to the medium at 100, 200, 300 and 400 mmol/L concentrations for a 24-h incubation period. At the end of each incubation period, cytosolic TAG, DNA and protein contents were measured. Palmitate, vaccenate, linoleate and CLA increased ($P < 0.05$) cytosolic TAG (μg/mg protein). Palmitate and CLA increased ($P < 0.05$) cytosolic TAG adjusted for DNA content. Overall, effects on cytosolic TAG accumulation depended on individual FA structure (chain length, degree of saturation, and number and orientation of FA double bonds). In addition, the long-chain FA used in this study did not have a detrimental effect on MAC-T cells as indicated by cytosolic protein and DNA contents reflecting their biological role in lipid accumulation.

1. Introduction

Approximately 50% of total milk fatty acids (FA) are derived by *de novo* synthesis from acetate and butyrate (Knutsen et al., 2018) within specialized mammary cells, and the other 50% by absorption from the blood. Lipids in milk are present in fat droplets formed chiefly of triacylglycerols (TAG) enclosed within a membrane derived from the secretory mammary epithelial cells (Shi et al., 2018). Mammary epithelial cells are known to accumulate TAG as lipid droplets in the cytosol (Yonezawa et al., 2004a,b). During lactation, lipid droplet precursors are formed within the endoplasmic reticulum membrane and are used to synthesize milk lipid globules (Yonezawa et al., 2004a). Although the general steps of lipid droplet formation, growth, movement, and secretion are known (Ernens et al., 2007), few studies have been performed on lipid absorption and secretion in isolated mammary epithelial cells.

Previously, Yonezawa et al. (2004b) reported that exogenous octanoate induced cytosolic TAG accumulation and formation of lipid droplets in bovine mammary cells. In another study, Yonezawa et al. (2004a) found that addition of palmitate, stearate, oleate, and linoleate increased cytosolic TAG contents in primary cultured bovine mammary epithelial cells. Jayan and Herbein (2000) treated mammary cells with stearic, oleic and vaccenic acids bound to bovine serum albumin; they found that although all treatments had 18 carbons, the position and geometry of the double bond was a factor deciding the influence of unsaturated FA on lipogenic enzyme activity. Trans FA (TFA) such as vaccenic and rumenic (C18:2 c-9, t-11) acids have been reported to have positive effects on human health (Wu et al., 2018); therefore, increasing these FA in milk fat will be an important dietary source to persons at risk for coronary heart disease and hypercholesterolemia.

Our hypothesis was that long-chain FA and TFA exert differential TAG accumulation and FA profiles in cytosol of mammary epithelial cells. The aim of this study was to elucidate how cytosolic TAG accumulate and whether this is affected by the chemical configuration of FA (i.e., chain length, geometry and location of double bonds).

* Corresponding author.
E-mail address: evargasb@sund.ku.dk (E. Vargas-Bello-Pérez).
[1] Present address: Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Grønnegardsvej 3, DK-1870 Frederiksberg C, Denmark.

Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2018.09.002

These objectives were achieved by culturing mammary alveolar cells-large T antigen (MAC-T) cells with different FA (palmitate, stearate, oleate, linoleate, rumenic acid [CLA], elaidate and vaccinate). We are ultimately interested in manipulating the FA composition of milk by replacing saturated FA with 'healthier' polyunsaturated FA and certain TFA such as vaccenic and rumenic acids. Thus, our results at a cellular level are crucial to accomplish this goal.

2. Materials and methods

2.1. Cell culture

In this study, MAC-T cells (Bovine mammary alveolar cells by stable transfection with SV-40 large T-antigen; Huynh et al., 1991) were chosen since they have normal physiological functions and respond to prolactin by producing milk constituents, consequently, these cells are a useful tool for studying aspects of milk production *in vitro* (Kadegowda et al., 2009). The MAC-T cells represent a suitable *in vitro* model for bovine lactation since they are responsive to manipulations both in extracellular matrix and in lactogenic hormones (Huynh et al., 1991).

Bovine mammary epithelial cells were grown in HyQ RPMI-1640 media (Hyclone) supplemented with foetal bovine serum (10%, F7524, Sigma) and antibiotic-antimycotic solution (ABAM; 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL; A5955, Sigma) in sterile 75 mL flasks coated with poly-D-lysine or collagen I (to ensure cell attachment and facilitate growth) (156472-Nunclon, Fisher Scientific). Cultures were maintained in a water-jacket incubator at 37 °C in the presence of 5% CO_2. Culture medium was changed every 48 h and cells were sub-cultured to 70% to 80% confluence by rinsing once with 3 mL of 0.25% trypsin, and incubated at 37 °C until there was evidence of cell detachment. Trypsin activity was inhibited by addition of 25 mL of fresh culture media at 37 °C (Kadegowda et al., 2009).

2.2. Basal medium

Approximately 48 h prior to the final subculture before initializing the experiment, cells were allowed to grow in a basal medium similar to that of Peterson et al. (2004). The basal medium was composed of minimum essential medium/Earle's balanced salts (HyQ; MEM/EBSS, HyClone) with insulin (5 mg/L, I6634, Sigma, St. Louis, MO), hydrocortisone (1 mg/L, H0888, Sigma), transferrin (5 mg/L, T1428, Sigma), ascorbic acid (5 mmol/L, A4544, Sigma), sodium acetate (5 mmol/L, S5636, Sigma), and ABAM (10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL; A5955, Sigma). The basal medium was supplemented with foetal bovine serum (10%, F7524, Sigma) and growth-promoting hormones (1 mg/L of progesterone, P8783, Sigma; 0.05% lactalbumin, L5385, Sigma; 0.05% α-lactose, 47287-U, Sigma). All basal medium components were filtered before use (sterile syringe filter; pore size: 0.20 μm). Cells were seeded at a concentration of 1.0×10^4 cells/well (Yonezawa et al., 2004a,b; Keating et al., 2008) in sterile 12-well (well diameter: 22.1 mm) cell culture clusters with flat bottom (Costar 3513, Corning Incorporated, USA).

2.3. Lactogenic medium

Approximately 24 h before applying treatments (cells were at approximately 90% confluence), cells were cultured in a lactogenic medium as reported by Kadegowda et al. (2009). The lactogenic medium was prepared as the basal medium, except that high-glucose Dulbecco's modified Eagle's medium (HG-DMEM, HyClone) was used in order to stimulate lactogenesis. The lactogenic medium was devoid of foetal bovine serum and was supplemented with bovine serum albumin (BSA; 1 g/L) and prolactin (2.5 mg/L). All lactogenic medium components were filtered before use (sterile syringe filter; pore size: 0.20 μm).

2.4. Preparation of long-chain fatty acids (LCFA) solutions

Stock solutions of 30 mmol/L LCFA were prepared in 13 mm × 100 mm Pyrex glass tubes with screw caps at 42 °C with an equimolar solution of NaOH in water. Sodium salts of the individual FA were suspended in ethanol to obtain a final solution of 95% ethanol (Table 1). After preparation, the LCFA stock solutions were stored at −20 °C until analyses (Olofsson et al., 2004; Kadegowda et al., 2009) (Table 2).

2.5. Treatment of MAC-T cells with exogenous fatty acids

Treatments consisted of the following LCFA: palmitic, stearic, oleic, linoleic, rumenic (C18:2 c-9, t-11), elaidic and vaccenic acids. Experimental solutions were added to the lactogenic medium at increasing concentrations and cultured for a 24-h period. Ethanol was used as a control. Concentrations of individual FA in the lactogenic medium were 100, 200, 300 and 400 mmol/L. At the end of each incubation, lipid from media and cell lysates were extracted *in situ* using hexane:isopropanol (3:2, vol/vol).

2.6. Measurement of the TAG, DNA and protein contents in cytosol

Cultured cells on 12-well plates (COSTAR 3513) were washed once with ice-cold phosphate buffered saline (PBS) + albumin (to

Table 1
Preparation of fatty acid (FA) treatment solutions.

FA	Molecular weight, g/mol	FA, mg	One mol/L NaOH, μL	Absolute ethanol, mL[1]
C16:0[2]	256.43	25.7	112.2	3.34
C18:0[3]	284.40	21.1	83.10	2.47
C18:1 t-9[4]	282.46	23.5	92.96	2.76
C18:1 t-11[5]	282.46	28.8	114.24	3.40
C18:1 c-9[6]	282.47	28.5	112.89	3.36
C18:2n c-6[7]	280.46	26.6	94.80	3.16
C18:2 c-9, t-11[8]	280.45	25.4	101.36	3.01

[1] Absolute ethanol from Fishers Scientific E/0650DF/P17.
[2] P0500-SIGMA, ≥99%.
[3] S4751-SIGMA, ≥99%, gas chromatography grade.
[4] Elaidic donated by Nestle.
[5] ≥95%, donated by INRA, France.
[6] SIGMA O1008, ≥99%, gas chromatography grade.
[7] L1376-SIGMA, ≥99%.
[8] 16413-SIGMA, Conjugated (9Z, 11E) linoleic acid analytical standard, ≥96%.

Table 2
Concentration of fatty acid (FA) solutions.

Concentration, mmol/L[1]	Amount, μL/mL[2]
100	3.3
200	6.6
300	9.9
400	13.2

[1] Concentration of stock FA is 30 mmol/L (30 nmol/μL) and the stock FA solutions were not sterile.
[2] Amount required per milliliter of media; FA solutions were filtered (sterile syringe filter; pore size: 0.20 μm) along with the lactogenic medium.

remove FA traces) and twice with PBS, scraped off into 200 μL of 0.05 mol/L trisodium citrate (Fishers S/3320/60) for DNA and protein quantification and stored at −20 °C until analysis. Cells were sonicated (Soniprep 150, MSE, UK, Ltd.) at 5 microns for 10 s to disrupt cell membranes and release cellular contents. DNA quantification was performed by the Hoechst fluorometric method. Sensitivity of the Hoechst 33258 Dye assay was approximately 10 ng/mL. The linear dynamic range extended over 3 orders of magnitude from 10 ng/mL to 1 μg/mL DNA. Protein was determined by the Lowry et al. (1951) protein assay. Optical density was read by a microplate reader (Microplate Manager Bio-Rad Laboratories, Inc.). The TAG in the cell lysates was extracted by using hexane:isopropanol (3:2, vol/vol) and quantified by using a Triglyceride reagent (Infinity Triglyceride, Thermo Scientific, TR22421). Optical density was read by using a microplate reader (Microplate Manager Bio-Rad Laboratories, Inc.). The measurement wavelength range was 550 to 655 nm for reference wavelength. Each assay was performed in triplicate and repeated at least 3 times.

2.7. Fatty acid analysis from cells and media

Lipid from cells and media were extracted using an adaptation of the method of Bligh and Dyer (1959) and methylated according to the method of Christie (1982) with the modifications reported by Chouinard et al. (1999). All chemicals and solvents used for these methods were of analytical grade. For analysis of FA in cells and media, a gas chromatograph (GC) system (Agilent, GC 6890 series) equipped with a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm i.d., with 0.2 μm film thickness; Varian Inc., Oxford) was used. The GC conditions were as follows: the oven temperature was initially set at 110 °C for 4 min after injection, and then increased to 240 °C with equilibration time of 2 min. The inlet and flame-ionization detector temperatures were 260 °C, the split ratio was 15:1 and a 2 μL injection volume was used. The hydrogen carrier gas flow to the detector was 25 mL/min, airflow was 400 mL/min, and the flow of nitrogen makeup gas was 40 mL/min. Fatty acid peaks were identified by using a fatty acid methyl ester standard (FAME; Supelco 37 Component FAME mix, Bellefonte, PA) and a TFA reference standard (C18:1 t-11, methyl ester, Supelco, Bellefonte, PA). The concentration of each FA (g/100 g) was calculated for C16:0 to C22:6n3 FA with concentrations higher than 0.3 g/100 g.

2.8. Statistical analysis and design

Using the GenStat 12th statistical package (VSN International Ltd., Oxford), data from cytosolic TAG corrected by protein and DNA were analyzed as linear mixed models (restricted maximum likelihood) with fixed effects of LCFA and concentration; random effects were experimental run, plate, strips and wells. Multiple runs (of which there were 8) were carried out to obtain replication for each of the measurements. Treatments were added randomly to wells (of which there were 408), strips (of which there were 3 in each 12-well plate) and plates (of which there were 35) for each run. Cytosolic FA profile from cell and media data were analyzed as linear mixed models with fixed effects of LCFA and concentration; the random effect was plate. Multiple means comparison for FA and concentration effects were performed using Tukey's honest significant difference test. Probability of $P < 0.05$ was used to determine significant differences among means.

3. Results and discussion

The design of the current study allowed analysis of cytosolic and media FA profiles which has not been reported previously. There are cell culture studies that have examined the effect of FA on transcriptional regulation (Dan et al., 2018) and FA profile of cytosolic TG (Jacobs et al., 2013). Other studies have focused on different aspects of mammary physiology such as enzymatic activity and gene expression (Peterson et al., 2004; Yonezawa et al., 2004a,b; Keating et al., 2008; Sorensen et al., 2008).

The current study also allowed analysis of effects of LCFA including TFA on cytosolic TAG accumulation, and protein and DNA concentrations. In the experiments of Keating et al. (2008) and Yonezawa et al. (2004b, 2008), FA were bound to bovine serum albumin. Albumin is the most abundant serum protein and one of its main physiological functions is to bind and transport LCFA (Spector, 1986). Based on previous studies (Olofsson et al., 2004; Kadegowda et al., 2009; Thering et al., 2009), in this current study, ethanol was used to dissolve sodium salts of FA in preference to albumin in order to avoid additional limitations on LCFA uptake by mammary epithelial cells (Spector, 1975). Ethanol was used as a control to determine if there was any effect of its increasing amounts on MAC-T cell cultures. Concentrations of individual FA (100, 200, 300 and 400 mmol/L) were greater than those used by Jayan and Herbein (2000) (25, 50 and 100 mmol/L of FA) and Keating et al. (2008) (15, 20, 30, 35, 37.5 and 150 mmol/L of FA), but similar to those used by Yonezawa et al. (2004a, 2008) (50, 100, 200, 300 and 400 mmol/L of FA). In a pilot experiment, we tested lower concentrations (< 100 mmol/L of FA), and cytosolic TAG accumulation was undetectable.

3.1. Accumulation of cytosolic triacylglycerol

The hypothesis of the current study was that cytosolic TAG accumulation would be stimulated by addition of LCFA at different concentrations and that type of LCFA would affect the FA profile of cytosolic free FA (FFA). In general, our results (Tables 3 and 4) showed that unsaturated FA (UFA) treatments induced greater ($P < 0.05$) cytosolic TAG accumulation than saturated FA (SFA) treatments suggesting that SFA might be metabolized via different routes (Yonezawa et al., 2004a). Treatment concentrations increased cytosolic TAG accumulation with significant linear and quadratic effects; this agrees in part with observations of Yonezawa et al. (2004a) where primary cultured bovine mammary epithelial cell (bMEC) cells incubated with palmitic, stearic, oleic and linoleic FA increased (in a concentration dependent manner) cytosolic TAG accumulation.

3.2. Cytosolic triacylglycerol accumulation normalized for protein and DNA contents

Cytosolic TAG accumulation was normalized for protein (μg/mg protein) and DNA contents (μg of TAG/μg of DNA). Protein content is commonly used in cell culture experiments as an indicator of cell proliferation to eliminate variances among cell cultures and to allow data comparison with greater confidence. Normalization is a process by which data are corrected to remove sample-to-sample variability caused by factors other than those being tested in the

experiment. These factors included variability in cell plating, and pipetting inconsistencies (Schagat et al., 2007). Because MAC-T cells synthesise protein for inclusion in milk, protein content will not reflect only cell proliferation, thus, cytosolic TAG accumulation normalized for protein may not be appropriate for this study. However, each cell has a fixed amount of DNA, thus DNA content per well reflects the number of cells per well. Hence, when cytosolic TAG is corrected for DNA, a measure of TAG per cell can be obtained, which is more accurate than normalising for protein content. In this study, both types of normalization are reported and discussed in order to be able to compare them with previous reports.

When cytosolic TAG was normalized for protein content (μg/mg protein), vaccenate, linoleate and CLA increased ($P < 0.05$) cytosolic TAG content (Table 3) compared with control incubation with palmitate. This is in agreement with Yonezawa et al. (2004a), who reported that palmitate, and linoleate increased ($P < 0.05$) cytosolic TAG (μg/mg protein) in primary culture bovine mammary epithelial cells isolated from the mammary gland of a 102-d pregnant Holstein heifer. In the current study, however, cytosolic TAG accumulation (from 221.9 to 919.4 μg/mg protein) in MAC-T cells was greater than that of Yonezawa et al. (2004a; from 54.8 to 122.6 μg/mg protein). Cytosolic TAG (μg/mg protein) accumulation increased ($P < 0.05$) quadratically with increasing treatment concentration; this agrees in part with the increase in cytosolic TAG accumulation in a concentration-dependent manner observed in primary cultured bMEC cells by Yonezawa et al. (2004a). Compared with the control, palmitate and CLA increased ($P < 0.05$) cytosolic TAG adjusted for DNA content (μg of TAG/μg of DNA) and cytosolic TAG increased ($P < 0.05$) quadratically with treatment concentration (Table 4). Previous studies (Jayan and Herbein, 2000; Yonezawa et al., 2004a,b; 2008) did not quantify cytosolic DNA content.

In the current study, LCFA did not decrease ($P > 0.05$) cytosolic protein or DNA content. However, it is well documented, that accumulation of excess lipids in tissues leads to cell dysfunction or cell death (Schaffer, 2003). When cells accumulate more FFA than required for anabolic or catabolic processes, excess lipid is esterified and stored as TAG in lipid droplets. Although TAG accumulation is essentially a condition of lipid overload, cellular TAG accumulation may initially serve a protective role. Accumulation of excess FFA in TAG pools diverts these molecules from pathways that lead to cytotoxicity and may thus serve as a buffer against lipotoxicity (Schaffer, 2003).

3.3. Cytosolic and media fatty acid profiles

Changes in cytosolic FA profiles were consistent with most of the treatments added to MAC-T cell cultures; oleate increased ($P < 0.05$) oleic acid from 100 to 300 mmol/L of FA; elaidate increased ($P < 0.05$) elaidic acid (EA) from 200 to 400 mmol/L of FA; linoleate increased ($P < 0.05$) linoleic acid from 100 to 400 mmol/L of FA; and CLA increased ($P < 0.05$) rumenic acid (CLA) from 100 to 400 mmol/L of FA. Effects of added FA on cytosolic FA concentrations were not consistent within FA classes (Table 5); however, palmitate decreased ($P < 0.05$) UFA; elaidate increased ($P < 0.05$) cytosolic monounsaturated FA (MUFA) and TFA; oleate increased ($P < 0.05$) MUFA and UFA; linolate increased ($P < 0.05$) SFA; CLA increased ($P < 0.05$) polyunsaturated FA (PUFA) and UFA and decreased ($P < 0.05$) SFA.

In media samples, stearate increased ($P < 0.05$) stearic acid (from 100 to 300 mmol/L of FA), oleate increased ($P < 0.05$) oleic acid (from 200 to 400 mmol/L of FA), linoleate increased ($P < 0.05$) linoleic acid (from 100 to 400 mmol/L of FA) and CLA increased ($P < 0.05$) α-linoleic acid (from 100 to 400 mmol/L of FA). In the media, only TFA class was affected by treatments (Table 6); media TFA concentrations were increased ($P < 0.05$) by elaidate and vaccenate (from 100 to 400 mmol/L of FA). These results may be explained by the media enrichment of each individual FA added and perhaps the FA extraction method (Bligh and Dyer, 1959), which included some of the FFA added as well as TAG. This study made an emphasis on TFA because previous data suggest that the

Table 3
Effects of fatty acids (FA) added to culture media on accumulation of triacylglycerol by mammary alveolar cells-large T antigen cells (μg/mg protein).

Item	Concentration, mmol/L				SED[1]	*P*-value	Linear effect	Quadratic effect
	100	200	300	400				
Control	138	200	158	235	120.0	0.002[2]		
Palmitate	352	393	590	817				
Stearate	314	343	209	292				
Oleate	189	146	339	512				
Elaidate	228	323	340	394				
Vaccenate	285	365	427	508				
Linoleate	222	275	510	919				
CLA	294	281	499	583				
FA effect								
Control			183[d]		71.96	<0.001[3]		
Palmitate			538[a]					
Stearate			290[c]					
Oleate			296[c]					
Elaidate			321[c]					
Vaccenate			396[b]					
Linoleate			482[a]					
CLA			414[b]					
Control								
Concentration effect								
Concentration, mmol/L	100		253[c]		39.58	<0.001[4]	<0.001	0.020
	200		291[c]					
	300		384[b]					
	400		533[a]					

CLA = conjugated linoleic acid.
[a–d] Means with the same letter were not significantly different at $P < 0.05$.
[1] Standard errors of differences between means (calculated on variance scale).
[2] *P*-value represents the probability of a treatment–concentration interaction.
[3] *P*-value represents the probability of a treatment effect.
[4] *P*-value represents the probability of a concentration effect.

Table 4
Effects of fatty acids (FA) added to culture media on accumulation of triacylglycerol in mammary alveolar cells-large T antigen cells (μg of TAG/μg of DNA).

Item	Concentration, mmol/L				SED[1]	*P*-value	Linear effect	Quadratic effect
	100	200	300	400				
Control	138	126	140	184	28.59	0.026[2]		
Palmitate	146	158	167	284				
Stearate	127	115	115	140				
Oleate	112	113	148	165				
Elaidate	132	129	163	226				
Vaccenate	106	110	130	165				
Linoleate	127	115	175	236				
CLA	122	190	223	299				
FA effect								
Control			147[c]		15.60	<0.001[3]		
Palmitate			189[b]					
Stearate			124[d]					
Oleate			135[d]					
Elaidate			162[c]					
Vaccenate			128[d]					
Linoleate			163[c]					
CLA			208[a]					
Concentration effect								
Concentration, mmol/L	100		126[c]		9.81	<0.001[4]	<0.001	<0.001
	200		132[c]					
	300		158[b]					
	400		212[a]					

CLA = conjugated linoleic acid.
[a–d] Means with the same letter were not significantly different at $P < 0.05$.
[1] Standard errors of differences between means (calculated on variance scale).
[2] *P*-value represents the probability of a treatment–concentration interaction.
[3] *P*-value represents the probability of a treatment effect.
[4] *P*-value represents the probability of a concentration effect.

physiological effects of TFA are isomer-dependent, as well as the carbon chain length that determines metabolic responses to saturated FA (Mozaffarian, 2016). In an epidemiological study, Jakobsen et al. (2006) showed the association between intake of TFA from ruminant-derived foods and risk of coronary heart disease was rather harmless or with protective effects.

The effects of individual FA observed in the current experiment may be due to specific activity of each FA on enzymes (acetyl-CoA carboxylase [ACC] and FA synthetase [FAS]) involved in biosynthesis of SFA and stearoyl-CoA desaturase (SCD) responsible for catalyzing biosynthesis of UFA from SFA precursors in MAC-T cells (Jayan and Herbein, 2000). The ability of PUFA to down-regulate expression of genes for enzymes involved in FA synthesis, including ACC, FAS and SCD, has been recognised in studies of membrane lipid composition (Salter and Tarling, 2007). However, we did not analyse enzymatic activity.

Table 5
Effects of fatty acids (FA) added to culture media on cytosolic concentration of major FA classes in mammary alveolar cells-large T antigen cells (g/100 g total FA).

Item	Concentration, mmol/L				SED[1]	*P*-value[2]
	100	200	300	400		
Saturated						
Control	61.9	61.6	63.3	72.3	8.952	<0.001
Palmitate	22.6	32.6	37.6	35.2		
Stearate	65.7	66.8	69.2	76.1		
Oleate	22.5	13.3	9.44	10.7		
Elaidate	24.6	40.0	21.8	19.2		
Vaccenate	43.4	31.8	20.6	32.7		
Linoleate	31.6	33.1	36.6	37.5		
CLA	49.6	42.9	28.8	22.2		
Trans						
Control	4.26	5.22	2.27	1.56	4.589	<0.001
Palmitate	9.47	8.51	5.87	7.50		
Stearate	1.62	4.63	3.13	2.88		
Oleate	7.12	7.00	4.16	4.72		
Elaidate	36.7	20.5	42.8	50.9		
Vaccenate	12.1	28.7	43.0	33.9		
Linoleate	6.86	5.25	5.53	4.56		
CLA	6.26	4.37	3.60	4.34		
Unsaturated[3]						
Control	27.9	28.6	30.4	20.8	7.462	<0.001
Palmitate	54.4	46.8	37.7	44.6		
Stearate	31.8	25.4	25.9	19.8		
Oleate	60.1	73.1	81.2	81.3		
Elaidate	46.9	46.6	69.7	72.6		
Vaccenate	38.4	32.9	32.4	33.1		
Linoleate	52.7	55.5	52.6	53.8		
CLA	38.4	42.8	55.3	63.4		

CLA = conjugated linoleic acid.
[1] Standard errors of differences between means (calculated on variance scale).
[2] *P*-value represents the probability of a treatment–concentration interaction.
[3] Monounsaturated + polyunsaturated FA.

3.4. Cytosolic versus media fatty acid profiles

Media FA profiles revealed disappearance of some FA and may indicate which FA were not incorporated and absorbed into cytosol of MAC-T cells. Only changes in media FA profiles of stearate, oleate, linoleate and CLA were detected:

Table 6
Effects of fatty acids (FA) added to culture media on media trans FA concentration (g/100 g total FA).

FA	Concentration, mmol/L				SED[1]	*P*-value[2]
	100	200	300	400		
Control	0.83	1.09	0.85	1.80	1.529	0.006
Palmitate	1.09	0.65	0.43	1.45		
Stearate	0.67	1.55	2.13	4.13		
Oleate	1.12	2.22	0.25	1.69		
Elaidate	2.77	6.15	6.64	11.5		
Vaccenate	5.49	6.25	7.96	12.8		
Linoleate	1.25	1.28	1.36	2.28		
CLA	2.24	0.18	0.59	0.00		

CLA = conjugated linoleic acid.
[1] Standard errors of differences between means (calculated on variance scale).
[2] *P*-value represents the probability of a treatment–concentration interaction.

- The C18:0 was decreased ($P < 0.05$) by palmitate, linolate, oleate, CLA, elaidate, and vaccenate from 37.57 to 28.78, 35.26 to 33.10, 34.66 to 27.76, 37.74 to 29.98, 35.99 to 28.90, 33.32 to 29.40 ± 3.07 g/100 g at 100 to 400 mmol/L of FA and increased by stearate from 24.13 to 30.61 ± 3.07 g/100 g at 100 to 400 mmol/L of FA.
- The C18:1 c-9 was decreased ($P < 0.05$) by linolate from 2.71 to 2.45 ± 1.44 g/100 g at 100 to 400 mmol/L of FA and increased ($P < 0.05$) by palmitate, stearate, oleate, CLA, elaidate and vaccenate from 2.71 to 3.55, 0.91 to 1.12, 5.73 to 14.85, 1.89 to 2.63, 2.10 to 3.12, 1.80 to 1.91 ± 1.44 g/100 g at 100 to 400 mmol/L of FA and control hardly changed from 1.01 to 1.05 ± 1.44 g/100 g at 100 to 400 mmol/L of FA.
- The C18:2n c-6 was increased ($P < 0.05$) by linolate from 1.69 to 11.47 ± 0.31 g/100 g at 100 to 400 mmol/L of FA; however, other FA were below 2.5 g/100 g at 100 to 400 mmol/L of FA.
- The C18:3n3 was increased ($P < 0.05$) by CLA from 2.61 to 12.77 ± 2.60 g/100 g at 100 to 400 mmol/L of FA; however, other FA and control were not detectable.

Our results represent the FA that were incorporated and secreted within complex lipids and they may not reflect the composition of cytosolic FA. In order to confirm this phenomenon, additional pre-processing techniques (e.g., solid-phase extraction) for extraction of analytes from complex matrices will be needed. Results from this study, may be attributed to different roles of LCFA in cellular metabolism. Long-chain fatty acids are taken up by cells and used for a large number of biological functions such as energy generation and storage; LCFA also contribute to phospholipid synthesis, which is necessary for the structure, integrity and function of plasma membranes. FA modulate the function of enzymes and regulate expression of multiple genes involved in FA metabolism (Hajri and Abumrad, 2002).

Cellular uptake of LCFA may be due to passive diffusion through the lipid bilayer and protein-facilitated transport. The relative contribution of each component depends on concentrations and molar ratios of FA and albumin in the circulation (Hajri and Abumrad, 2002). In the circulation and extracellular medium, FA are carried quantitatively bound to albumin. Complexes of FA and albumin are used in *in vitro* uptake assays because FA adsorption to assay tubes and pipette walls, and FA aggregation, may create confounding effects. In the absence of albumin or other FA binding protein, the concentration of LCFA that can be used is low and is depleted by cells instantaneously; meaning that the linear portion of the uptake time course would be too short to measure (Hajri and Abumrad, 2002). In the current experiment, ethanol used to dissolve sodium salts of FA probably affected FA uptake of LCFA (palmitic, stearic, oleic, linoleic and conjugated linoleic acids) and TFA (elaidic and vaccenic acids).

3.5. Fatty acid groups in lipid metabolism

Long-chain fatty acids have numerous roles in biological functions of cells, not only as an energy substrate but also as substrates for cell membrane synthesis and as precursors for intracellular signalling molecules (McArthur et al., 1999). Excess LCFA are stored as TAG in lipogenic tissues such as the adipose, liver, testis, ovary and mammary gland. During onset of lactation, energy metabolism of cells is dramatically modified to enable synthesis of milk components (Nayeri and Stothard, 2016). Animals accordingly diminish storage of nutrients, particularly of TAG, in adipose tissue. The lactating mammary gland, thus, uses LCFA derived from both dietary fats and TAG released from adipose tissue. Lactating mammary epithelial cells are highly active in synthesis of FA *de novo* (Zhang et al., 2018). Bovine mammary epithelial cells have the ability to produce a remarkable amount of lipid (Johnson et al., 2010); this lipogenic capacity probably affected results from the current study and influenced transfer of some FA from cytosol to media by decreasing TAG accumulation in cytosol.

In the current study, MAC-T cells incubated with SFA (palmitic and stearic acids), UFA (oleic acid), PUFA (linoleic and conjugated linoleic acids), TFA (elaidic and vaccenic acids), increased cytosolic TAG content; however, when TAG content was normalised for cytosolic DNA, contents of SFA (palmitic acid) and PUFA (CLA) noticeably increased cytosolic TAG content. Hence, these findings may indicate that mammary epithelial cells accumulate lipids depending on chain length of the FA, degree of unsaturation and configuration.

Although activity of lipogenic enzymes in mammary gland cells was not evaluated, the findings (cytosolic and media FA profiles) from the present study complement previous studies. For example, Jayan and Herbein (2000) studied exogenous factors related to synthesis of SFA and UFA in MAC-T cells. They analyzed effects of oleic and vaccenic acids on activities of ACC, FAS and SCD. Acetyl-CoA carboxylase and FAS are major enzymes involved in biosynthesis of SFA in eukaryotic cells and SCD is responsible for biosynthesis of UFA from their saturated precursors. Jayan and Herbein (2000) reported that vaccenic acid (VA) depressed activity of ACC and FAS, along with simultaneous enhancement of mammary desaturase activity and explained that effects of exogenous FA on ACC and FAS in MAC-T cells were related to biological function of FA in cells and tissues. For example, FA are components of cell membranes, and FA provided in the media of cultured mammary epithelial cells are primarily incorporated into cell membrane PL. Inhibition of lipogenesis in various tissues by LC-UFA has been reported; in rats, oleic acid and VA inhibited hepatic FAS activity and inhibited conversion of glucose to lipids (Clarke et al., 1990).

Fatty acids are components of cell membranes, and FA provided in the media of cultured mammary epithelial cells are primarily incorporated into cell membrane phospholipids (Mida et al., 2012). The relative amounts of SFA and UFA, as well as chain length of FA that constitute membranes, are important factors that determine membrane fluidity. In general, fluidity has an inverse relationship to chain length and a direct relationship to degree of unsaturation of FA. Jayan and Herbein (2000) noted that excess incorporation of unsaturated oleic acid and VA into cell membranes, especially at the expense of the corresponding saturated isomer, would increase membrane fluidity above normal limits. Short-chain SFA have the same effect on membrane fluidity as long-chain UFA, meaning that both these groups enhance membrane fluidity when compared with long-chain SFA. Therefore, inhibition of ACC and FAS activities by the monounsaturated isomers could be a cellular response to reduce synthesis of short-chain SFA.

3.6. Trans fatty acids and lipid metabolism

In the present study, at concentrations of 100, 200, 300 and 400 mmol/L, EA and VA increased ($P < 0.05$) cytosolic TAG content (μg/mg of protein and μg/μg of DNA). Jayan and Herbein (2000) found a concentration-dependent uptake of VA complexed to FA-free bovine serum albumin at concentrations of 25, 50 and 100 mmol/L; however, the current study is the first to use EA with MAC-T cells. Jayan and Herbein (2000) reported that the position and geometry of the double bond is a factor determining the influence of UFA on lipogenic enzyme activity. When compared with cis double bonds, trans double bonds have a more rigid structure and are less fluid in nature. It is possible that VA competed with oleic acid for incorporation into cell membranes (oleic acid will be produced in bovine mammary epithelial cells by desaturation of stearic acid). Excess incorporation of VA at the expense of oleic acid would reduce fluidity of cell membranes and therefore enhanced synthesis of cis-unsaturated FA from available substrates or precursors. Our data

indicated that vaccenic and rumenic acids can be increased in cytosolic TAG content. However, in on-farm conditions, in order to avoid the effects of an extensive biohydrogenation of dietary unsaturated lipids (Vargas-Bello-Perez et al., 2016), cows will need to be fed appropriate feedstuffs that can increase the contents of both TFA (such as rumen protected or by-pass lipid sources) in milk. The changes observed with vaccenate and CLA at concentrations between 300 and 400 mmol/L are comparable in intensity to vaccenic and rumenic acids concentrations found in plasma of cows supplemented (2.6% of dry matter) with soybean oil (Vargas-Bello-Pérez et al., 2016) or fish oil (Vargas-Bello-Pérez et al., 2017) in total mixed rations (56:44 forage:concentration ratio).

Some additional aspects need to be considered for interpretation of the present results: 1) data suggest that palmitic, elaidic, linoleic and CLA all stimulate TAG accumulation, whereas stearic, vaccenic and oleic do not. However, due to our methodological approach it is difficult to elucidate whether this is stimulation of synthesis or inhibition of secretion; 2) in this study, cytosolic FA are presented as TAG + phospholipid, and thus, further research will be needed to determine what proportion is associated with each lipid class. Further studies should consider analyzing cell vitality, proliferation and cytotoxicity of MAC-T cells, and expression of genes involved in lipid metabolism.

4. Conclusions

Overall, the results of this study demonstrate that palmitic, elaidic, linoleic and CLA all stimulate cytosolic TAG accumulation whereas stearic, vaccenic and oleic acids do not. The observed effects on cytosolic TAG accumulation are not consistent within FA classes (TFA, SFA and UFA), but depend on individual FA structure (chain length of FA, degree of saturation, and number and orientation of FA double bonds). At concentrations of 100, 200, 300 and 400 mmol/L, LCFA (palmitic, stearic, oleic, linoleic, elaidic, vaccenic and conjugated linoleic acids) did not have detrimental effects on MAC-T cells as determined by cytosolic protein and DNA contents, presumably reflecting their biological role in lipid accumulation.

Conflicts of interest

There was no conflict of interest with this research.

Acknowledgements

Einar Vargas-Bello-Pérez was supported by a PhD studentship (196739) from Consejo Nacional de Ciencia y Tecnología (CONACYT-México). We thank Linda Sheldrick, Dongfang Li, and Zoë Daniel for technical assistance. Statistical advice was provided by Jim Craigon.

References

Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–7.
Chouinard PY, Corneau L, Barbano DM, Metzger LE, Bauman D. Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows. J Nutr 1999;129:1579–84.
Christie WW. A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. J Lipid Res 1982;23:1072–5.
Clarke SD, Armstrong MK, Jump DB. Dietary polyunsaturated fats uniquely suppress rat-liver fatty-acid synthase and s14 messenger-RNA content. J Nutr 1990;120:225–31.
Dan N, Zhang H, Ao C, Khas-Erdene. Transcriptional regulation of milk lipid synthesis by exogenous C16:0 and C18 fatty acids in bovine mammary epithelial cells. Can J Anim Sci 2018;98:260–70.
Ernens I, Clegg R, Schneider YJ, Larondelle Y. Short communication: Ability of cultured mammary epithelial cells in a bicameral system to secrete milk fat. J Dairy Sci 2007;90:677–81.
Hajri T, Abumrad NA. Fatty acid transport across membranes: relevance to nutrition and metabolic pathology. Annu Rev Nutr 2002;22:383–415.
Huynh HT, Robitaille G, Turner JD. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp Cell Res 1991;197:191–9.
Jacobs A, Dijkstra J, Liesman J, VandeHaar M, Lock A, Van Vuuren A, et al. Effects of short- and long-chain fatty acids on the expression of stearoyl-CoA desaturase and other lipogenic genes in bovine mammary epithelial cells. Animal 2013;7:1508–16.
Jakobsen MU, Bysted A, Andersen NL, Heitmann BL, Hartkopp H, Leth T, et al. Intake of ruminant trans fatty acids and risk of coronary heart disease. Atherosclerosis Suppl 2006;7:9–11.
Jayan GC, Herbein JH. Healthier dairy fat using *trans*-vaccenic acid. Nutr Food Sci 2000;30:304–9.
Johnson T, Fujimoto B, Jiménez-Flores R, Peterson D. Growth hormone alters lipid composition and increases the abundance of casein and lactalbumin mRNA in the MAC-T cell line. J Dairy Res 2010;77:199–204.
Kadegowda AKG, Bionaz M, Piperova LS, Erdman RA, Loor JJ. Peroxisome proliferator-activated receptor-gamma activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents. J Dairy Sci 2009;92:4276–89.
Keating AF, Zhao FQ, Finucane KA, Glimm DR, Kennelly JJ. Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression. Domest Anim Endocrinol 2008;34:284–92.
Knutsen TM, Olsen H, Tafintseva V, Svendsen ML, Kohler A, Kent M, et al. Unravelling genetic variation underlying de novo-synthesis of bovine milk fatty acids. Sci Rep 2018;8:2179.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
Mida K, Shamay A, Argov-Argaman N. Elongation and desaturation pathways in mammary gland epithelial cells are associated with modulation of fat and membrane composition. J Agric Food Chem 2012;60:10657–65.
McArthur M, Atshaves BP, Frolov A, Foxworth WD, Kier AB, Schroeder F. Cellular uptake and intracellular trafficking of long chain fatty acids. J Lipid Res 1999;40:1371–83.
Mozaffarian D. Natural trans fat, dairy fat, partially hydrogenated oils, and cardiometabolic health: the Ludwigshafen Risk and Cardiovascular Health Study. Eur Heart J 2016;37:1079–81.
Nayeri S, Stothard P. Tissues, Metabolic pathways and genes of key importance in lactating dairy cattle. Springer Sci Rev 2016;4:49.
Olofsson CS, Salehi A, Holm C, Rorsman P. Palmitate increases L-type Ca2+ currents and the size of the readily releasable granule pool in mouse pancreatic beta-cells. J Physiol London 2004;557:935–48.
Peterson DG, Matitashvili EA, Bauman DE. The inhibitory effect of trans-10, cis-12 CLA on lipid synthesis in bovine mammary epithelial cells involves reduced proteolytic activation of the transcription factor SREBP-1. J Nutr 2004;134:2523–7.
Salter AM, Tarling EJ. Regulation of gene transcription by fatty acids. Animal 2007;1:1314–20.
Schaffer JE. Lipotoxicity: when tissues overeat. Curr Opin Lipidol 2003;14:281–7.
Schagat T, Paguio A, Kopish K. Normalizing genetic reporter assays: approaches and considerations for increasing consistency and statistical significance. Promega Corp Cell Notes 2007;17:9–12.
Shi HB, Du Y, Zhang CH, Sun C, He Y, Wu YH, et al. Fatty acid elongase 5 (ELOVL5) alters the synthesis of long-chain unsaturated fatty acids in goat mammary epithelial cells. J Dairy Sci 2018;101:4586–94.
Sorensen BM, Kazala EC, Murdoch GK, Keating AF, Cruz-Hernandez C, Wegner J, et al. Effect of CLA and other C18 unsaturated fatty acids on DGAT in bovine milk fat biosynthetic systems. Lipids 2008;43:903–12.
Spector AA. Fatty-acid binding to plasma albumin. J Lipid Res 1975;16:165–79.
Spector AA. Structure and lipid-binding properties of serum-albumin. Meth Enzymol 1986;128:320–39.
Thering BJ, Bionaz M, Loor JJ. Long-chain fatty acid effects on peroxisome proliferator-activated receptor-alpha-regulated genes in Madin-Darby bovine kidney cells: optimization of culture conditions using palmitate. J Dairy Sci 2009;92:2027–37.
Vargas-Bello-Perez E, Cancino-Padilla N, Romero J. Garnsworthy PC Quantitative analysis of ruminal bacterial populations involved in lipid metabolism in dairy cows fed different vegetable oils. Animal 2016;10:1821–8.
Vargas-Bello-Pérez E, Íñiguez-González G, Cancino-Padilla N, Loor JJ. Garnsworthy PC Effect of dietary vegetable oils on the fatty acid profile of plasma lipoproteins in dairy cows. Arch Anim Nutr 2016b;70:322–32.
Vargas-Bello-Pérez E, Íñiguez-González G, Garnsworthy PC, Loor JJ. Transport of fatty acid within plasma lipoproteins in lactating and non-lactating cows fed on fish oil and hydrogenated palm oil. J Anim Physiol Anim Nutr 2017;101:369–77.
Wu W, Lin L, Shi B, Jing J, Cai L. The effects of early life polyunsaturated fatty acids and ruminant trans fatty acids on allergic diseases: a systematic review and meta-analysis. Crit Rev Food Sci Nutr 2018;17:1–14.
Yonezawa T, Yonekura S, Kobayashi Y, Hagino A, Katoh K, Obara Y. Effects of long-chain fatty acids on cytosolic triacylglycerol accumulation and lipid droplet formation in primary cultured bovine mammary epithelial cells. J Dairy Sci 2004a;87:2527–34.
Yonezawa T, Yonekura S, Sanosaka M, Hagino A, Katoh K, Obara Y. Octanoate stimulates cytosolic triacylglycerol accumulation and CD36 mRNA expression but inhibits acetyl coenzyme A carboxylase activity in primary cultured bovine mammary epithelial cells. J Dairy Res 2004b;71:398–404.
Yonezawa T, Sanosaka M, Haga S, Kobayashi Y, Katoh K, Obara Y. Regulation of uncoupling protein 2 expression by long-chain fatty acids and hormones in bovine mammary epithelial cells. Biochem Biophys Res Commun 2008;375:280–5.
Zhang T, Huang J, Yi Y, Zhang X, Loor JJ, Cao Y, et al. Akt serine/threonine kinase 1 regulates de Novo fatty acid synthesis through the mammalian target of rapamycin/sterol regulatory element binding protein 1 Axis in dairy goat mammary epithelial cells. J Agric Food Chem 2018;66:1197–205.