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

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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 mammary 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.

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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.

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Abbreviation: TFA, trans fatty acid; CLA, conjugated linoleic acid; FA, fatty acids; TAG, triacylglycerol.
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). 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 situ (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). The basal medium was supplemented with foetal bovine serum (10%, F7524, Sigma; 0.05% a-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 $1.0 \times 10^4$ 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).

2.2. 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).

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). All lactogenic medium components were filtered before use (sterile syringe filter; pore size: 0.20 μm).

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).

| FA       | Molecular weight, g/mol | FA, mg | One mol/L NaOH, μL | Absolute ethanol, mL |
|----------|-------------------------|--------|---------------------|----------------------|
| C16:0$^2$ | 256.43                  | 25.7   | 112.2               | 3.34                 |
| C18:1$^4$ | 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$^4$ | 282.46         | 28.8   | 114.24              | 3.40                 |
| C18:1 c-9$^4$ | 282.47            | 28.5   | 112.89              | 3.36                 |
| C18:2 c-6$^4$ | 280.46            | 26.6   | 94.80               | 3.16                 |
| C18:2 c-9, t-11$^4$ | 280.45    | 25.4   | 101.36              | 3.01                 |

$^1$ Absolute ethanol from Fisher 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%.
$^8$ I6413-SIGMA, Conjugated (9Z, 11E) linoleic acid analytical standard, ≥96%.

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Cultured cells on 12-well plates (COSTAR 3513) were washed once with ice-cold phosphate buffered saline (PBS) + albumin (to remove FA traces) and twice with PBS, scraped off into 200 μL of 0.05 mol/L trisodium citrate (Fishes 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 550 to 655 nm for reference wavelength. Each assay was performed in triplicate and repeated at least 3 times. Optical density was read by using 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.

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

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 n-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.

2.7. Fatty acid analysis from cells and media

Sensitivity of the Hoechst 33258 Dye assay was approximately 550 to 655 nm for reference wavelength. Each assay was performed in triplicate and repeated at least 3 times.

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. (2004a, 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 of FA) 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), linolate increased (P < 0.05)

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 | SED¹ | P-value | Linear effect | Quadratic effect |
|---------------|-----------------------|------|---------|---------------|-----------------|
|               | 100       | 200   | 300     | 400           |                 |
| Control       | 138       | 200   | 158     | 235           | 120.0           | 0.002²          |
| 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       | 183²      |       |         |               |                 |
| Palmitate     | 538²      |       |         |               |                 |
| Stearate      | 290¹      |       |         |               |                 |
| Oleate        | 296¹      |       |         |               |                 |
| Elaidate      | 321¹      |       |         |               |                 |
| Vaccenate     | 396³      |       |         |               |                 |
| Linoleate     | 482¹      |       |         |               |                 |
| CLA           | 414³      |       |         |               |                 |

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 P-value represents the probability of a treatment effect.
4 P-value represents the probability of a concentration effect.

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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

Table 4
Effects of fatty acids (FA) added to culture media on accumulation of triacylglycerol in mammary alveolar cells-large T antigen cells (g/100 g total FA).

| Item          | Concentration, mmol/L | SED | P-value | Linear effect | Quadratic effect |
|---------------|-----------------------|-----|---------|---------------|-----------------|
|               | 100  | 200  | 300  | 400          |                 |
| Control       |      |      |       |              |                 |
| Palmitate     | 138  | 126  | 140  | 184          | 28.59           |
| Stearate      | 146  | 158  | 167  | 284          |                 |
| Oleate        | 127  | 115  | 115  | 140          |                 |
| Elaidate      | 112  | 113  | 148  | 165          |                 |
| Vaccenate     | 132  | 129  | 163  | 226          |                 |
| Linoleate     | 106  | 110  | 130  | 165          |                 |
| CLA           | 127  | 115  | 175  | 236          |                 |
| FA effect     |      |      |       |              |                 |
| Control       | 147  |      | 15.60 | <0.001       |
| Palmitate     | 189  |      |      |              |                 |
| Stearate      | 135  |      |      |              |                 |
| Oleate        | 162  |      |      |              |                 |
| Elaidate      | 128  |      |      |              |                 |
| Vaccenate     | 163  |      |      |              |                 |
| Linoleate     | 208  |      |      |              |                 |
| Concentration | 100  |      | 126  | <0.001       |
|               | 200  |      | 132  | <0.001       |
|               | 300  |      | 158  | <0.001       |
|               | 400  |      | 212  | <0.001       |

CLA – conjugated linoleic acid.

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 | P-value |
|---------------|-----------------------|-----|---------|
|               | 100  | 200  | 300  | 400          |
| Saturated     |      |      |      |              |
| Control       | 61.9 | 61.6 | 63.3 | 72.3          |
| 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          |
| Palmitate     | 9.47 | 8.51 | 5.87 | 7.50          |
| Stearate      | 7.12 | 7.00 | 4.16 | 4.72          |
| Oleate        | 36.7 | 20.5 | 42.8 | 50.9          |
| Elaidate      | 12.1 | 28.7 | 43.0 | 33.9          |
| Vaccenate     | 6.86 | 5.25 | 5.53 | 4.56          |
| Linoleate     | 6.28 | 4.37 | 3.60 | 4.34          |

Unsaturated

| Item          | Concentration, mmol/L | SED | P-value |
|---------------|-----------------------|-----|---------|
|               | 100  | 200  | 300  | 400          |
| Control       | 27.9 | 28.6 | 30.4 | 20.8          |
| 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.

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 | P-value |
|-------|-----------------------|-----|---------|
|       | 100  | 200  | 300  | 400          |
| Control | 0.83 | 1.09 | 0.85 | 1.80          |
| Palmitate | 1.09 | 0.65 | 0.43 | 1.45          |
| Oleate | 0.67 | 1.55 | 2.13 | 4.13          |
| Elaidate | 1.12 | 2.22 | 0.25 | 1.69          |
| Vaccenate | 2.77 | 6.15 | 6.64 | 11.5          |
| Linoleate | 5.49 | 6.25 | 7.96 | 12.8          |
| CLA | 2.24 | 0.18 | 0.59 | 0.00          |

CLA – conjugated linoleic acid.

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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.

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:

- 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 C18:2 n–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 C18:3n–3 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-UFAs 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 ($\mu$g/mg of protein and $\mu$g/$\mu$g 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 and saturated FA. This effect is driven in part by the availability of rumenic acid, which is present in dairy cows milk. Thus, we hypothesize that total rumenic acid content in milk is higher in diets that contain more rumenic acid than diets that contain more vaccenic acid.

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). In 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.

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