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ORIGINAL ARTICLE

Effect of CLA and Other C18 Unsaturated Fatty Acids on DGAT in Bovine Milk Fat Biosynthetic Systems

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Abstract Production of dairy products with increased amounts of nutraceutic FA such as conjugated linoleic acid (CLA) represents a recent approach for dairy producers and processors to increase the value of their products. The effect of CLA and other FA on the expression of *diacylglycerol acyltransferase-1* (*DGAT-1*) and *DGAT-2*, and DGAT activity were investigated in bovine mammary gland epithelial (MAC-T) cells. DGAT gene expression analyses were also conducted using bovine mammary gland tissue from dairy cows. In the studies with MAC-T cells, there were no significant effects of CLA isomers or other FA on *DGAT1* expression, whereas all FA tested showed enhanced *DGAT2* expression ($P < 0.05$ to $P < 0.001$), with α-linolenic acid (α-18:3) having the greatest effect. Additionally, *DGAT2* expression was coordinated with expression of *lysophosphatidic acid acyltransferase* (*LPAAT*), an observation that was also apparent in mammary gland from lactating dairy cows. In contrast, treatment of MAC-T cells with *trans*-10, *cis*-12 18:2 or α-18:3 resulted in a significant ($P < 0.05$) decrease in overall DGAT enzyme activity, although the mechanisms resulting in these effects are unclear. Competition assays using microsomes from bovine mammary gland tissue and 1-[^{14}C]oleoyl-CoA suggested that DGAT activity was more selective for oleoyl (*cis*-9 18:1)-CoA than *cis*-9, *trans*-11 18:2-, *trans*-10, *cis*-12 18:2- or *cis*-9, *cis*-12 18:2-CoA. Collectively, the results suggest the relationship between *trans*-10, *cis*-12 18:2 and reduced TAG production in bovine milk is not linked to the production of DGAT1 or DGAT2 transcripts, but probably involves effects of this CLA isomer at events beyond transcription, such as post-translational and/or enzyme activity effects.

Keywords Conjugated linoleic acid · Diacylglycerol acyltransferase · Gene expression · Milk fat depression · Cattle · Dairy

Abbreviations

ACAT	Acyl-CoA:cholesterol acyltransferase
DGAT	Diacylglycerol acyltransferase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPAT	*sn*-glycerol-3-phosphate acyltransferase
LPAAT	Lysophosphatidic acid acyltransferase
MFD	Milk fat depression
QRT-PCR	Quantitative real-time polymerase chain reaction
SCD	Stearoyl-CoA desaturase

Introduction

Conjugated linoleic acid (CLA) is a naturally abundant component in ruminant milk fat. These positional and geometric isomers of octadecadienoic acid occur as intermediates during the biohydrogenation of dietary fatty acids (i.e., linoleic and linolenic acid) by rumen microbes. Several isomers, most notably *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2, have demonstrated nutraceutic properties,

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including decreased cancer incidence [1] and body weight [2]. Endogenous synthesis in the mammary gland is estimated to account for up to 78% of *cis*-9, *trans*-11 18:2 in milk from cows on total mixed ration diets [3, 4] and for up to 91% of milk CLA from pasture-fed cows [5].

Dairy producers wish to market products enriched in CLA so that consumers may benefit from these properties. The content of CLA isomers can be readily increased in milk fat by supplementing cattle diets with oilseeds or plant oils enriched in linoleic acid (*cis*-9, *cis*-12 18:2) [6, 7]. Another strategy is to supplement cattle with synthetic mixtures of CLA isomers protected from the rumen microflora by abomasal infusion [8]. The ionophore monensin may also be supplemented to the diet to prevent CLA isomers from being altered through rumem biohydrogenation [9].

In many cases, strategies resulting in elevated milk CLA content have also been shown to result in net milk fat depression (MFD). *Trans*-10, *cis*-12 18:2 has been suggested as the isomer responsible for this phenomenon [10, 11]. Cattle fed diets resulting in increased milk *trans*-10, *cis*-12 18:2 content coinciding with MFD also had decreased mRNA levels representing lipogenic genes, including those encoding *sn*-glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15), and lysophosphatidic acid acyltransferase (LPAAT, EC 2.3.1.51) [12]. GPAT and LPAAT catalyze the first and second acyl-CoA-dependent acylations of the glycerol backbone, respectively, in the Kennedy pathway leading to TAG [13].

Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the transfer of an acyl moiety from acyl-CoA to *sn*-1,2 diacylglycerol to form triacylglycerol (TAG) [13]. cDNAs encoding two isoforms of this enzyme (DGAT1 and DGAT2), which do not share amino acid sequence, have been isolated [14, 15]. An early study suggested that the level of DGAT activity in fat-forming tissues may have a substantial effect on the flow of carbon into TAG [16]. Indeed, over-expression of *DGAT1* and *DGAT2* in mouse liver has been shown to result in a 2.0- and 2.4-fold increase, respectively, in tissue TAG content [17]. In contrast, female mice with an inactivated DGAT1 gene ($DGAT^{-/-}$) are unable to lactate [18] and have impaired mammary gland development [19]. Additionally, a missense mutation in *DGAT1* has been shown to result in decreased milk fat content in dairy cows [20]. Hamsters supplemented with CLA isomers in the diet have been shown to display decreased intestinal acyl-CoA: cholesterol acyltransferase (ACAT EC 2.3.1.26) activity [21], suggesting that CLA may act to down regulate ACAT activity. Since DGAT1 shares some sequence homology with ACAT [14], CLA might regulate both enzymes in a similar fashion. In another study, murine primary preadipocyte cultures (3T3-L1) treated with arachidonic acid (all-*cis*-5,8,11,14 20:4) has been shown to result in repressed DGAT activity [22]. In addition, rats fed mixtures of CLA have been shown to display decreased hepatic DGAT activity compared with control rats fed the same amount of FA [22]. Earlier studies had found no difference in DGAT activity in rats fed similar amounts of *cis*-9 18:1 versus 18:0 [23].

Given the association of *trans*-10, *cis*-12 18:2 with MFD, we hypothesized that *DGAT* expression and/or DGAT activity might be affected by this CLA isomer. The effect of CLA isomers, including *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2, and other FA, on *DGAT1*, *DGAT2*, and *LPAAT* expression levels, and DGAT activity were examined in cultured bovine mammary gland epithelial cells (MAC-T cells). Other FAs tested included *trans*-11 18:1, a substrate of mammalian Δ-9 desaturase, and FA commonly found in dietary plant oils ingested by dairy cattle. In addition, expression of *DGAT1*, *DGAT2*, and *LPAAT* were examined in mammary gland tissue from lactating dairy cows. Furthermore, the direct effects of CLA isomers on DGAT activity were investigated using microsomes prepared from bovine mammary gland tissue.

Materials and Methods

Animals

All procedures involving the use of animals were approved by the University of Alberta Animal Policy and Welfare Committee. Cows were housed in tie-stalls with water available at all times. The diets were fed once per day at 09:00 as total mixed rations consisting of 60% (w/w) forage and 40% (w/w) concentrate. Feed intake was recorded daily and adjusted to maintain 5–10% orts (feed remaining). Diets for the primiparous and multiparous lactating Holstein cows are described elsewhere [24]. Samples of mammary gland tissue were taken from six cows supplemented with safflower oil and monensin and six cows not receiving this treatment using a method adapted from Knight et al. [25]. The animals were sedated by administration of xylazine hydrochloride (10 mg/50 kg body weight) and ketamine (2 mg/kg body weight) via jugular catheter. The animals were maintained in a state of sedation with an intravenous drip of glycerol guaiacolate and ketamine [50 g glycerol guaiacolate, 1,000 mg ketamine, 1,000 ml 50% (w/v) dextrose and 900 ml sterile water]. The drip rate was set at 20 ml/min and maintained during surgery. The udder was shaved around the biopsy site and swabbed with iodine and 70% (v/v) ethanol. A region of the basal portion of the udder free of major subcutaneous blood vessels was chosen for the biopsy site. The area was

anesthetized locally by injection of lidocaine (10–30 ml of 2% v/v lidocaine with epinephrine). An 8-cm incision was made in the skin; a 5–10-g piece of mammary gland tissue was excised using a scalpel and snap frozen in liquid nitrogen and then stored at −80°C.

Chemicals

Both *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2 were obtained from Matreya (Pleasant Gap, PA). [1-^{14}C] *cis*-9 18:1 was from GE Healthcare (Baie d'Urfé, Quebec, Canada). Fatty acyl-CoA was prepared from the above FA as described by Taylor et al. [26]. Silica gel 60 H was from VWR Canlab (Missisauga, Ontario, Canada). Solvents (HPLC grade) were from Fisher Scientific (Ottawa, Ontario, Canada). Ecolite(+) biodegradable scintillation cocktail was from ICN Biomedicals (Irvine, CA). *sn*-1,2 Diolein was from Nu Chek Prep Inc. (Elysian, MN). Dulbecco's Modified Eagle Media (DMEM), fetal bovine serum (FBS), TRIzol® reagent, M-MLV reverse transcriptase, oligo (dT)12–18 primer, Rnase OUT Rnase inhibitor, dNTPs, 5X first strand buffer, and custom primers were from Invitrogen (Burlington, Ontario, Canada). rDNase, 10X buffer, and DNase inactivation agent were included in the Ambion DNA-*free*™ kit (catalog no. 1906, Austin, TX). FAM and VIC labeled probes and TaqMan® Universal PCR Mastermix were from Applied Biosystems (Foster City, CA). All other chemicals were from Sigma (Oakville, Ontario, Canada).

Maintenance and Treatment of Cultured Mammary Gland Epithelial Cell Line

MAC-T cells were originally established by Huynh et al. [27] and were kindly provided by Nicholas Lemee of Nexia Biotechnologies, Inc. (Montreal, Quebec, Canada). Cells were maintained essentially as described by Keating et al. [28]. The cells were cultured in high glucose (4.5 g/l) DMEM containing L-glutamine, sodium pyruvate, and pyroxidine-HCl and supplemented with 10% (v/v) FBS and 5 μg bovine insulin/ml. FAs used to treat MAC-T cells were complexed to BSA as potassium salts. Twelve milligrams of FA was treated with 1 ml of 0.1 M KOH. The solution was vortexed and incubated at 50°C, and then added dropwise to 9 ml of 7.5% (w/v) BSA. The resulting FA-BSA stock solution was incubated at room temperature for 3 h and then overnight at 4°C, and finally stored in small aliquots at −20°C. FA-BSA stock solution was added to DMEM to produce the final FA concentration used in FA treatment experiments. Cells were maintained in a humidified incubator at 37°C and 5% CO_2.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Total RNA was extracted from confluent MAC-T cells treated for 24 h with various exogenous FA and from the mammary gland tissue using TRIzol® reagent as described in the manufacturer's protocol. The total RNA was then treated with DNAase using the DNA-*free*™ kit from Ambion. cDNA was synthesized from 1 μg of total RNA in a 20-μl reaction volume with a final concentration of 0.5 mM dNTP mix, 0.5 μg Oligo $(dT)_{12–18}$, 5 μM DTT, 4 μl 5X first strand buffer, 40 U RNase out RNase inhibitor, and 200 U of M-MLV reverse transcriptase. The reaction mixture was incubated at 37°C for 60 min and then at 70°C for 15 min.

QRT-PCR was performed using the ABI Prism® 7900HT FAST QRT-PCR system with 9600 Emulation (Applied Biosystems) in a total volume of 25 μl reaction mixture following the manufacturer's protocol, using TaqMan® 2X Universal PCR Mastermix, 0.1 μM each of forward and reverse primer, and 0.2 μM of probe. Primers and TaqMan–MGB probes were designed against mRNA sequences specific for *DGAT1* (GenBank NM_174693), *DGAT2* (NM_205793), *LPAAT* (NM_177518), and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (NM_001034034) from *Bos taurus* using Primer Express® 3.0 Software (Applied Biosystems; Table 1).

Relative gene expression for *DGAT1*, *DGAT2*, or *LPAAT* was normalized to a calibrator that was chosen to be the basal condition (BSA control) for each treatment. Results were calculated with the $2_T^{-\Delta\Delta C}$ method [29] where $\Delta\Delta C_T = (C_{T,\ \text{Target}} - C_{T,\ \text{GAPDH}})_{\text{Time } x} - (C_{T,\ \text{Target}} - C_{T,\ \text{GAPDH}})_{\text{Time } 0}$ and expressed as n-fold differences in gene expression relative to GAPDH mRNA and calibrator (BSA). Standard errors of the average $C_{T,\ \text{Target}}$ and average $C_{T,\ \text{GAPDH}}$ were pooled. C_T is defined as the number of cycles required until the specific dye-reporter emission reached the defined intensity threshold.

Extraction of Lipid from MAC-T Cells and Analysis of FA Composition

MAC-T cells were grown in 75-cm^2 flasks in DMEM containing 20 μM FA-BSA and incubated at 37°C. Upon confluence, cells were treated with trypsin-EDTA and harvested and transferred to acid-washed glass tubes. The vials were centrifuged at 1,500×*g*. The supernatant was removed, and the cells were resuspended in 1 ml phosphate buffered saline. The resuspended cells were probe sonicated for 30 s, and a sample was removed for protein determination. Lipids were extracted using a modified Folch [30] protocol. The following were added in sequence to the cell suspension, mixing by vortex for 30 s after each

Table 1 Primer and probe sequences for real-time polymerase chain reaction

Primers/detection probes	Sequence
DGAT1 forward primer	5′-GAACTCCGAGTCCATCACCTACTT-3′
DGAT1 reverse primer	5′-TCTGATGCACCACTTGTGAACA-3′
DGAT1 detection probe	5′-6FAM-TGGCAGAACTGGAACAT-MGBNFQ-3
DGAT2 forward primer	5′-GCCCTGCGCCATGGA-3′
DGAT2 reverse primer	5′-TACACCTCATTCTCCCCAAAGG-3′
DGAT2 detection probe	5′-6FAM-CCTGGTTCCCACCTAC-MGBNFQ-3
LPAAT forward primer	5′-GGACGCAACGTCGAGAACA-3′
LPAAT reverse primer	5′-CCGTACAGGTATTTGATGTGGAGTA-3′
LPAAT detection probe	5′-6FAM-AAGATCTTGCGTCTGATG-MGBNFQ-3
GAPDH forward primer	5′-TGCCGCCTGGAGAAACC-3′
GAPDH reverse primer	5′-CGCCTGCTTCACCACCTT-3′
GAPDH detection probe	5′-6FAM-CCAAGTATGATGAGATCAA-MGBNFQ-3

addition: 0.1 M KCl to bring suspension to total volume of 1.6 ml, 0.8 ml methanol, 2 ml chloroform/methanol (1:1, v/v), 2.7 ml chloroform, and 2.5 ml chloroform/methanol (2:1, v/v). The samples were incubated overnight at 4°C. Phase separation was achieved by centrifugation, and the chloroform layer was transferred to a glass vial and dried under N_2 (g). Prior to extraction, 100 μg of tritricosanoin (tri-23:0) was added to the cell suspension as an internal standard.

Production and GC analysis of FAME was based on methods described by Cruz-Hernandez et al. [31]. To the dried extract was added 100 μg of trinonadecanoin (tri-19:0), 1.7 ml of hexane, 40 μl of methyl acetate, and 100 μl of $NaOCH_3$ (0.5 M in methanol) followed by mixing for 2 min and incubation at room temperature for 20 min. The vial was then cooled at −20°C for 20 min, and 60 μl of oxalic acid (saturated in diethyl ether) was added. The vials were centrifuged to collect the oxalate precipitate, and the supernatant was spotted to a TLC plate. The plate was developed in one ascension of hexane/diethyl ether (80:20, v/v) using methyl-oleate as a standard. The plates were then visualized by spraying with 0.2% (w/v) 2,7' dichlorofluorescein. The area corresponding to methyl-oleate was scraped and transferred to a column containing sodium sulfate. The FAMEs were eluted using 2 ml of chloroform, and 50 μg of methyl-hexacosanoate (methyl-26:0) was added.

FAMEs were analyzed by GC with a Varian 3800 GC (Varian Inc., Mississauga, Ontario, Canada), equipped with splitless injection port flushed after 0.3 s, a flame ionization detector (FID), autosampler (Model 8200, Varian Inc.), 100-m CP-Sil 88 fused capillary column (Varian Inc.), and a Hewlett-Packard ChemStation software system (version A.07). Operating conditions included: injector and detector temperatures both at 250°C; H_2 as carrier gas (1 ml/min) and for the FID (40 ml/min), N_2 (g) as makeup gas (100 ml/min), and purified air (250 ml/min). The initial temperature of 45°C was held for 4 min, increased at a rate 13°C/min to 175°C, and held for 27 min, and finally increased at a rate 4°C/min to 215°C and held for 35 min. FAMEs were identified by comparison with a reference standard.

Assay of DGAT Activity in MAC-T Cells

DGAT activity was measured in MAC-T cells based on the protocol described by Geelen [32]. MAC-T cells were grown in DMEM containing 20 μM FA-BSA. Confluent cells were treated with trypsin-EDTA and centrifuged for 10 min at 1,500 × *g*. The pelleted cells were incubated in 96 mM MES (pH 6.5) containing 9.6 mM EDTA, 1.9 mM DTT, 125 mM NaCl, 15 μM [1-^{14}C]*cis*-9 18:1-CoA, 30 μg BSA, and 18 μg of digitonin for 10 min at 37°C. The reaction was quenched with 10 μl of 12.4 M HCl, and lipids were extracted as described by Bligh and Dyer [33]. Lipids were spotted to silica gel 60 H TLC plates, which were developed with a single ascension of hexane/diethyl ether (80:20, v/v) using triolein as a standard. Standards were visualized using iodine vapor, and corresponding areas were scraped into vials followed by the addition of scintillant and the determination of [1-^{14}C] *cis*-9 18:1 incorporated into TAG. The protein content of sonically disrupted cells was determined using the Bio-Rad protein microassay based on the method of Bradford [34] using BSA as a standard.

Assay of DGAT Activity in Microsomes from Mammary Gland Tissue

Microsomes were prepared from bovine mammary gland tissue as previously described for muscle and adipose tissue [35], and DGAT was assayed as described in Lozeman et al. [36] with a final concentration of 15 μM [1-^{14}C] *cis*-9 18:1-CoA (56 Ci/mol) in the reaction mixture. Reactions

were allowed to proceed for 10 min at 30°C in the presence of increasing concentrations (0–20 μM) of unlabeled *cis*-9, *trans*-11 18:2-; *trans*-10, *cis*-12 18:2-; *cis*-9, *cis*-12 18:2- or *cis*-9 18:1-CoA.

Statistical Analysis

For effect of exogenous FA treatment on expression of genes encoding lipogenic enzymes in cultured MAC-T cells, statistical analysis was performed using the mixed procedure in SAS (version 9.1), with variance components covariance structure and Kenward-Roger adjustment for the denominator degrees of freedom. The statistics are based on duplicates from three independent trials. Gene and FA treatments were considered fixed effects, and the trial was considered as a random effect in the mixed model analysis. For the effect of various FA treatments on the mole percentages of various FA in the acyl lipid of MAC-T cells, the mixed procedure (SAS) was used, with FA treatment as fixed effect. Spearman's rho correlation coefficients (JMP® IN statistical software, version 4, Duxbury Press, Toronto, ON, Canada) were generated for *DGAT1*, *DGAT2*, and *LPAAT* expression data for mammary gland tissue obtained from lactating Holstein cows. For the experiment measuring the effect of increasing concentrations of various unlabeled acyl-CoA species on the incorporation of radiolabeled-18:1 (from radiolabeled-18:1-CoA) into TAG using bovine mammary gland microsomes, statistical analysis was performed using the mixed procedure in SAS using a diagonal covariance structure and residual degrees of freedom method, considering substrate and substrate concentration as fixed effects in the model.

Results

Effect of Exogenous FA on Expression of Genes Encoding Lipogenic Enzymes in MAC-T Cells

Results from QT-PCR of *DGAT1*, *DGAT2*, and *LPAAT* expression are shown in Fig. 1. None of the FA isomers had a substantial effect on *DGAT1* expression relative to the control, and none resulted in significant change in the expression of *DGAT1*. Treatment with oleic acid (*cis*-9 18:1), vaccenic acid (*trans*-11 18:1); linoleic acid (*cis*-9, *cis*-12 18:2); *cis*-9, *trans*-11 18:2; *trans*-10, *cis*-12 18:2; or α-linolenic acid (α-18:3), however, resulted in increases in *DGAT2* expression ($P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively). CLA isomers, *cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2 did not have a differential effect on *DGAT1* versus *DGAT2* expression, but *trans*-11 18:1 treatment resulted in more enhanced *DGAT2* expression ($P < 0.05$) than for treatment with *cis*-9, *trans*-11 18:2. All FA tested for enhanced *LPAAT* expression ($P < 0.001$) in the MAC-T cells.

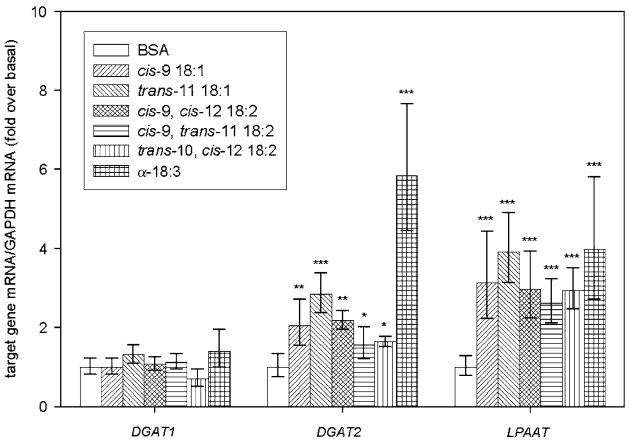

Fig. 1 Expression of genes encoding lipogenic enzymes in MAC-T cells treated with exogenous FA. Data points were calculated using the $2_T^{-\Delta\Delta CT}$ method [30]. Duplicates from three separate trials were used. *Error bars* represent upper and lower intervals. *, **, *** represent significant differences between treatment and control *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$)

Expression of Genes Encoding Lipogenic Enzymes in Mammary Gland

Spearman rho coefficients for *DGAT1*, *DGAT2*, and *LPAAT* expression in mammary gland samples from 12 lactating cows are shown in Table 2. Expression of *DGAT2* demonstrated significant positive correlation to that of *LPAAT*.

Effect of Exogenous FA on Lipid Accumulation and DGAT Activity in MAC-T Cells

None of the exogenous FA tested affected lipid accumulation in MAC-T cells (Fig. 2). The FA composition of lipid extracted from treated MAC-T cells is shown in Table 3. SFA, MUFA, and PUFA proportions are shown

Table 2 Spearman ρ coefficients for expression[a] of genes encoding lipogenic enzymes from mammary gland tissue of lactating Holstein cows ($n = 12$)

	DGAT2	*LPAAT*
DGAT1	0.37	0.37
DGAT2		0.59*

DGAT-1 diacylglycerol acyltransferase-1, *DGAT-2* diacylglycerol acyltransferase-2, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *LPAAT* lysophosphatidic acid acyltransferase

*$P < 0.05$

[a] ΔC_T value (i.e., $C_{T\ Target} - C_{T\ GAPDH}$)

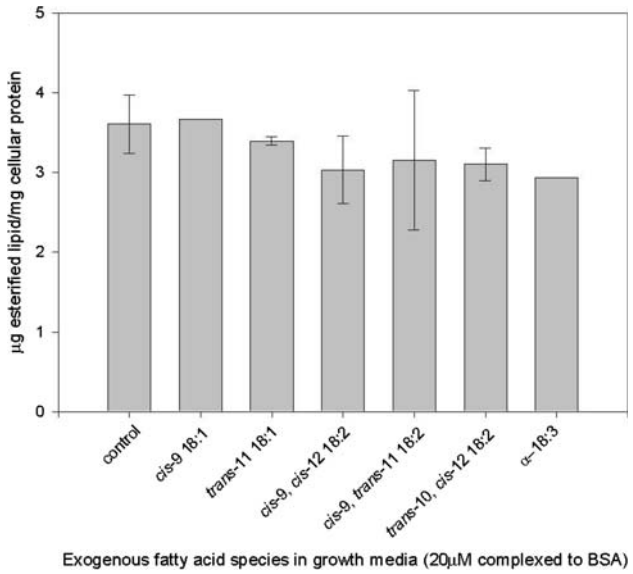

Fig. 2 Acyl lipid content of MAC-T cells treated with exogenous FA. Esterified lipid (fatty acyl lipid) content was obtained from the FA composition. Mean values are presented for the results of two experiments

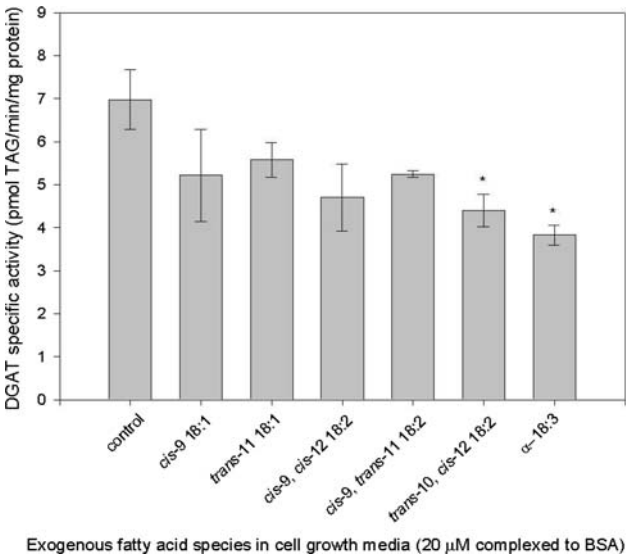

Fig. 3 DGAT activity of MAC-T cells treated with exogenous FA. Values represent means ± SEM ($n = 6$). *Values are significantly different from control ($P < 0.05$)

along with specific proportions for C18 FA. In each case, there was an enhanced incorporation of exogenous FA into the cellular lipid. Treatment of the cells with *trans*-10, *cis*-12 18:2 resulted in increased proportions of saturated FA and stearic acid (18:0) ($P < 0.01$) and a decreased proportion of *cis*-9 18:1.

The effect of exogenous FA on DGAT-specific activity in MAC-T cells is shown in Fig. 3. Compared to control cells (treated only with BSA), the DGAT-specific activities of cells treated with *trans*-10, *cis*-12 18:2, and α-18:3 were decreased ($P < 0.05$).

Competition Assays of DGAT Activity Using Mammary Gland Microsomes

The effect of increasing concentrations of unlabeled molecular species of acyl-CoA on the incorporation of [1-^{14}C] *cis*-9 18:1 from radiolabeled *cis*-9 18:1-CoA is shown in Fig. 4. Incorporation of [1^{14}-C]18:1 into TAG was decreased at all concentrations of *cis*-9, *trans*-11 18:2-; *trans*-10, *cis*-12 18:2- or *cis*-9, *cis*-12 18:2-CoA into TAG was decreased ($P < 0.001$) compared to *cis*-9 18:1-CoA.

Table 3 FA composition (mole %) of acyl lipid extracted from MAC-T-cells treated with exogenous FA

	Treatment						
	BSA	*cis*-9 18:1	*cis*-9, *cis*-12 18:2	α-18:3	*trans*-11 18:1	*cis*-9, *trans*-11 18:2	*trans*-10, *cis*-12 18:2
SFA	38.4 ± 1.1	38.5 ± 0.7	39.9 ± 0.4	45.7 ± 2.7*	37.8 ± 2.0	40.6 ± 0.8	52.2 ± 1.1***
MUFA	42.2 ± 2.4	43.9 ± 0.7	19.9 ± 1.1***	26.5 ± 2.6**	45.5 ± 1.8	34.9 ± 1.2*	25.4 ± 1.5**
PUFA	19.3 ± 1.7	17.7 ± 1.0	40.2 ± 0.7***	27.8 ± 0.6**	16.6 ± 1.2	24.6 ± 0.8*	22.4 ± 2.1
18:0	12.3 ± 1.0	10.0 ± 0.5	12.0 ± 0.5	14.6 ± 1.2	10.6 ± 1.9	11.6 ± 0.3	17.7 ± 0.4**
cis-9 18:1	25.0 ± 1.4	32.1 ± 0.3**	10.9 ± 0.5***	14.7 ± 1.0**	21.4 ± 1.0	20.0 ± 0.5*	15.6 ± 1.4**
cis-9, *cis*-12 18:2	4.2 ± 0.6	4.0 ± 0.3	24.1 ± 0.2***	4.4 ± 0.4	3.4 ± 0.1	3.9 ± 0.1	3.1 ± 1.0
α-18:3	0.4 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	9.2 ± 1.7***	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
cis-9, *trans*-11 18:2	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	1.9 ± 0.7	3.6 ± 1.7	0.3 ± 0.0
trans-10, *cis*-12 18:2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	3.0 ± 1.5

Values represent means ± SEM ($n = 4$)

*, **, *** Represent significant differences between treatment and control (BSA) within a row, *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$)

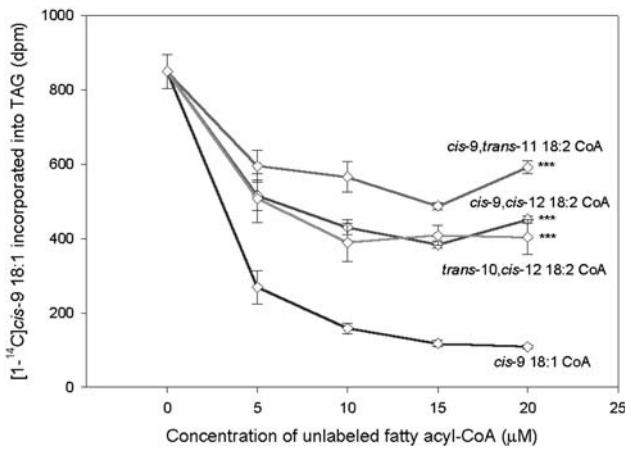

Fig. 4 Effect of increasing concentrations of various unlabeled acyl-CoA species on the incorporation of [1-^{14}C] *cis*-9 18:1 from [1-^{14}C] *cis*-9 18:1-CoA into TAG using bovine mammary gland microsomes. Values represent mean ± SEM ($n = 3$). ***Represents significant differences between *cis*-9 18:1-CoA and other acyl donors at all concentrations, ***($P < 0.001$)

Discussion

In an early study, Hansen and Knudsen [37] found that the addition of long chain FA, namely 18:0 and *cis*-9, *cis*-12 18:2, inhibited the incorporation of FA into the TAG of mammary gland epithelial cells. Peterson et al. [12] reported that treating MAC-T cells with *trans*-10, *cis*-12 18:2 resulted in reduced lipid synthesis. In the current study, this CLA isomer had no effect on lipid accumulation in cultured MAC-T cells (Fig. 2). Peterson et al. [12], however, measured lipid synthesis by monitoring the incorporation of [^{14}C] acetate into total lipid and noted inhibition of lipid accumulation at increasing concentrations of *trans*-10, *cis*-12 18:2. The effects of other isomers of CLA were not investigated. Our studies of lipid accumulation in MAC-T cells were based on the methods used in the recent report by Keating et al. [28] to study bovine mammary cell growth, apoptosis, and stearoyl-CoA (SCD) gene expression. In this recent report, various concentrations of CLA and other FA were applied to MAC-T-cells for 48 h. CLA (*cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2) concentrations of 30 μM or greater resulted in MAC-T-cell apoptosis. Twenty micromolar CLA was the highest concentration that could be applied before the cells began to do poorly. In the current study, a 20-μM FA treatment for 24 h was used to minimize the chances of apoptosis. The MAC-T cell line presents limitations for the study of a functioning mammary gland as it has abnormal characteristics in vitro. The in vitro data of Keating et al. [28], however, may help to clarify the effect of relatively high doses of fatty acids in vivo on the mammary gland. Eventually these investigations could be extended to evaluate the effects of CLA on differentiated mammary gland cells in vitro, such as the bovine mammary epithelial cell collagen cell culture system [38].

In the current study, treatment of MAC-T-cells with *trans*-10, *cis*-12 18:2 resulted in an increased proportion of 18:0 and decreased proportion of *cis*-9, 18:1 in total lipid compared to the control treatment (Fig. 3). This is consistent with evidence that this CLA isomer results in decreased expression and activity of SCD [39]. The decreased production of *cis*-9 18:1 and ultimately *cis*-9 18:1-CoA, a readily utilized substrate of DGAT, might be a factor in the MFD effect. The ability of DGAT to effectively utilize *cis*-9 18:1-CoA is confirmed in our competition studies using mammary gland microsomes where radiolabeled *cis*-9 18:1-CoA was incubated separately with unlabeled *cis*-9 18:1-; *cis*-9, *trans*-11 18:2-; *trans*-10, *cis*-12 18:2- or *cis*-9, *cis*-12 18:2.-CoA (Fig. 4).

Degrace et al. [40] have reported that feeding *trans*-10, *cis*-12 18:2 to mice increases the abundance of hepatic DGAT1 mRNA. All mice received control diet for 1 week and then control verus CLA isomer diets for 4 weeks. In the current study, treating of MAC-T-cells with various FA, however, had no effect on the expression of *DGAT1*. Peterson et al. [12] reported that cattle fed a MFD-inducing diet (i.e., high concentrate/low forage) resulted in decreased expression of *LPAAT*, whereas our data indicate that that *trans*-10, *cis*-12 18:2 increased expression of this gene to the same extent as any other FA tested. In the current study, incubation of MAC-T cells with *trans*-10, *cis*-12 18:2 or α-18:3 resulted in decreases ($P < 0.05$) in DGAT activity. In the competitive assay, however, the addition of *cis*-9 18:1-CoA decreased the incorporation of radiolabeled [1-^{14}C] *cis*-9 18:1 into TAG more than for other acyl-CoA tested. Although *cis*-9 18:1 may be preferentially used by DGAT, it is possible that one or more of the other molecular species of acyl-CoA tested is inhibitory. For example, *trans*-10, *cis*-12 18:2 might inhibit microsomal DGAT activity and not serve as an effective substrate for the enzyme. Berge et al. [41] incubated rat liver microsomes in the presence of various acyl-CoA derivatives, including *cis*-9, *cis*-12 18:2-CoA in the presence of 20 μM *cis*-9 18:1-CoA, and demonstrated that TAG synthesis was decreased. Eicosapentaenoyl-CoA (all *cis*-5,8,11,14,17 20:5-CoA) has also been shown to cause a substantial decrease in hepatic DGAT activity, but other acyl-CoAs tested were not inhibitory [42].

Although DGAT activity was decreased in the MAC-T cells as a result of incubation with *trans*-10, *cis*-12 18:2 or α-18:3, it is unclear how DGAT production may have been affected. Application of all FA types led to increased expression of *DGAT2* (Fig. 1), suggesting that the effect of *trans*-10, *cis*-12 and α-18:3 in decreasing DGAT activity production was post-transcriptional in nature. Exogenous application of α-18:3 did not influence lipid accumulation

in the MAC-T cells (Fig. 1), even though treatment of the cells with this FA appeared to have the greatest effect on enhancing *DGAT2* expression (Fig. 2).

Furthermore, treatment with α-18:3 resulted in a decrease in microsomal DGAT-specific activity (Fig. 3). A number of studies have shown that dietary α-18:3 leads to enhanced β-oxidation and reduced fat accumulation [43–46]. The increased *DGAT2* expression brought about by treatment of MAC-T cells with α-18:3 and other FAs suggests that DGAT2 mRNA levels probably do not reflect the activity of this isoenzyme of DGAT in promoting fat accumulation in this system. The occurrence of DGAT2 transcript in the bovine mammary gland, however, is consistent with the previous discovery of this transcript in human mammary gland [15]. CLA may not directly affect the activity of DGAT, but rather the apparent *trans*-10, *cis*-12 18:2-mediated decrease in DGAT activity may be the result of the action of this isomer on other lipogenic enzymes that generate substrates for DGAT. Cattle treated by abomasal infusion of purified *trans*-10, *cis*-12 18:2 emulsified in skim milk have been shown to exhibit a decrease in the expression of *SCD* [47]. In $SCD^{-/-}$ mice, decreased SCD activity has been shown to result in the accumulation of saturated FA, which had an inhibitory effect on acetyl-CoA carboxylase [48]. In turn, decreased acetyl-CoA carboxylase activity has been shown to result in a decrease in the production of malonyl-CoA, and ultimately a decrease in FA synthesis would result in less acyl-CoA available for DGAT. In cattle fed a MFD-inducing diet (i.e., high concentrate/low forage), Peterson et al. [12] also noted a decrease in the abundance of mRNA encoding FA binding protein. In another study, Brown et al. [49] have shown that treatment of human primary preadipocytes with *trans*-10, *cis*-12 18:2 results in a decrease in the expression of acyl-CoA binding protein. Thus, a decrease in the cellular content of these proteins might affect the availability of substrate for DGAT.

It is also possible that *trans*-10, *cis*-12 18:2 may influence the splicing of DGAT mRNA. Indeed, Grisart et al. [50] have shown using a baculovirus expression system that a K232A mutation in DGAT1 increases the occurrence of an alternative splicing variant of *DGAT1* that produces a DGAT1 isoform that is devoid of DGAT activity.

In addition, *trans*-10, *cis*-12 may have caused alterations in the phospholipid composition of the ER of the MAC-T cells leading to changes in the physical properties of the membrane. Ma et al. [51] have shown that *trans*-10, *cis*-12 18:2 is rapidly incorporated into the membrane phospholipid of MDA-MB-123 cells. Changes in SCD activity induced by *trans*-10 *cis*-12 18:2 also resulted in altered membrane composition. Since DGAT is membrane-bound, changes in the membrane environment may affect the activity of the enzyme. Indeed, Mathur et al. have shown that varying the phospholipid composition in incubations of rat liver microsomes alters ACAT activity [52].

In the present study, the fact that *DGAT2* and *LPAAT* showed similar expression patterns in response to the application of various exogenous FAs (Fig. 1) suggests that the two acyltransferases may be coordinated at the transcriptional level. As well, the expression of *DGAT2* and *LPAAT* showed a significant positive correlation in mammary gland tissue obtained from 12 dairy cows (Table 2). A similar association was observed by Kazala et al. [53] who previously reported a significant positive correlation between microsomal DGAT and LPAAT activity in bovine *pars costalis diaphragmitis* muscle tissue. The results reported here suggest this relationship is also reflected at the transcript level. Although Cases et al. [15] were able to differentiate between the activity of murine DGAT1 and DGAT2 by altering the concentration of $MgCl_2$ in the reaction mixture, we were unable to replicate this technique using MAC-T cells or bovine mammary gland tissue. At this point, it remains unclear to which extent DGAT2 contributes to the overall DGAT activity.

In conclusion, *trans*-10, *cis*-12 18:2 does not appear to mediate MFD by decreasing the expression of either *DGAT1* or *DGAT2*. Instead, the results of this study suggest that, upon exposure to *trans*-10, *cis*-12 18:2 or α-18:3, DGAT activity in MAC-T-cells is decreased, but the mechanism is unclear. *Trans*-10, *cis*-12 18:2 may inhibit DGAT activity either directly or indirectly as a result of altered substrate pools that in turn are a result of the action of *trans*-10, *cis*-12 18:2 on enzymes that are located upstream of DGAT in the lipid biosynthetic pathway. MFD caused by the *trans*-10, *cis*-12 CLA isomer probably affects a number of lipogenic enzymes including possible effects on DGAT production and/or activity.

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