Available online at www.sciencedirect.com
ScienceDirect
Domestic Animal Endocrinology xxx (2007) xxx–xxx
DOMESTIC ANIMAL ENDOCRINOLOGY
www.journals.elsevierhealth.com/periodicals/dae

Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression

Aileen F. Keating [a,b], Feng-Qi Zhao [b], Kiera A. Finucane [b], David R. Glimm [a], John J. Kennelly [a,*]

[a] *Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E1*
[b] *Department of Animal Science, University of Vermont, Burlington, VT 05401, USA*

Received 8 May 2007; received in revised form 18 July 2007; accepted 17 August 2007

Abstract

The effects of the primary biologically active conjugated linoleic acid (CLA) isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12; 15–150 μM) on growth and survival of the bovine mammary cell-line, Mac-T, were evaluated using cell enumeration and TUNEL assay. Previous studies have shown that high concentrations of CLA induced severe milk fat depression and have had negative effects on milk yield and composition whereas the impact of lower doses has been a modest depression in milk fat percent. In this study, we show that increasing concentrations of both CLA isomers had negative impacts on cell growth, including reduced cell number at concentrations of 35 μM and above ($P < 0.05$) and a two-fold increase in induction of apoptosis in the mammary cells. Changes in cell morphology occurred with large vacuole-like structures in the cytoplasm, nuclear shrinkage and changes of nuclear shape to kidney shape. Insulin did not significantly affect apoptosis in CLA-treated cells. In addition, the effect of increased doses of CLA and the interaction of CLA and insulin on the bovine *stearoyl Co-A desaturase* (*Scd*) gene promoter was also analyzed. While a significant difference in the *Scd* promoter transcriptional activity was not observed in cells treated with different concentrations of CLA, insulin significantly enhanced *Scd* promoter activity in CLA-treated cells. Our *in vitro* data support the hypothesis that high levels of CLA may induce *in vivo* apoptosis in the mammary gland.

Keywords: Conjugated linoleic acid; Mammary gland; Apoptosis; Stearoyl Co-A desaturase; Insulin

1. Introduction

Conjugated linoleic acid (CLA) consists of a complex mixture of geometrical (*cis* and *trans*) and positional isomers in the fat of ruminant animals which have been shown to have anti-mutagenic and anti-carcinogenic activities. The two major isomers in dairy animals are *cis*-9, *trans*-11 and *trans*-10, *cis*-12. These two isomers, however, appear to have different mechanisms of action and physiological effects on human health. The *cis*-9, *trans*-11 isomer has been shown to be a potent cancer-preventative agent in animal models of chemical carcinogenesis [1–4], while the *trans*-10, *cis*-12 isomer of CLA is largely associated with effects on body mass [5,6].

CLA is formed in the rumen as an intermediate product in the digestion of dietary fat primarily through biohydrogenation of linoleic acid by the rumen microflora. The initial reaction involves the isomerization of the

Abbreviations: CLA, conjugated linoleic acid; PUFA, polyunsaturated fatty acids; SCD, stearoyl Co-A desaturase; BSA, bovine serum albumin

* Corresponding author. Tel.: +1 780 492 0102; fax: +1 780 492 8524.
E-mail address: john.kennelly@ualberta.ca (J.J. Kennelly).

0739-7240/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.domaniend.2007.08.005

cis-12 double bond to *trans*-11 by *cis*-9, *trans*-11 isomerase. The next step is the conversion of this diene to the *trans*-11 monoene (*trans*-11 18:1; vaccenic acid). These initial steps occur rapidly. The conversion of *trans*-11 18:1 to 18:0 appears to involve a different group of organisms and occurs at a slower rate [7]. For this reason, *trans*-11 18:1 typically accumulates in the rumen and is transported to the mammary gland where the action of the enzyme stearoyl Co-A desaturase (SCD) (also known as δ9 desaturase) converts it to CLA. Biohydrogenation of linolenic acid also contributes to the rumen pool of vaccenic acid, however, the isomerization step does not occur and CLA is not formed as a transient intermediary in the rumen. Endogenous synthesis in the mammary gland is estimated to account for up to 78% of CLA in milk from cows on total mixed ration (TMR) diets [8,9] and for up to 91% of milk CLA from pasture-fed cows [10].

In the dairy cow, the influence of CLA on milk composition has been evaluated in a number of studies [11–14]. It has been shown that feeding rumen-protected, or abomasally infusing CLA, results in milk fat depression and thus the potential to alter milk fat synthesis for economic reasons, to alleviate the negative energy balance (NEBAL) problems seen in transition and heat-stressed cows [15] have been explored. The *trans*-10, *cis*-12 isomer in particular has been implicated as the isomer responsible for milk fat depression. Abomasal infusion of 10 g/day of either skim milk control, *cis*-9, *trans*-11 CLA, or *trans*-10, *cis*-12 CLA resulted in both milk fat yield and percentage being substantially reduced in cows receiving the *trans*-10, *cis*-12 supplement, but not in the cows receiving the *cis*-9, *trans*-11 isomer. There was no effect on milk yield [11]. In a study where 150 g/day of either control, CLA (31.7% *cis*-9, *trans*-11; 30.4% *trans*-10, *cis*-12), safflower oil or tallow were abomasally infused, there were dramatic effects of CLA infusion on milk production and composition [14] Milk yield was ∼40% lower and concentration and yield of lactose and fat were correspondingly lower in animals receiving the CLA treatment. Animals receiving the CLA treatment had milk somatic cell counts (SCC) between five and seven times higher compared to the other treatments. Sodium and chloride levels were increased similarly to levels seen during involution. Thus, it was speculated that the high-CLA dose affected mammary epithelial cell growth and survival and initiated dry-off mechanisms in the udder [14]. Other studies also reported effects on milk yield when severe milk fat depression was induced through CLA supplementation [16,17]. A number of *in vitro* studies have shown that both the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers have suppressive effects on mammary tumor cell growth and invasiveness [18–20].

A previous study by this group [21] showed that both the main biologically active isomers of CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) reduced transcriptional activity of the bovine *Scd* gene promoter *in vitro*. In addition to its role in converting vaccenic acid to CLA, *Scd* expression has effects on the fatty acid composition of membrane phospholipids, triglycerides and cholesterol esters, resulting in changes in membrane fluidity and lipid metabolism [22]. Thus, *Scd* gene activity may have a role in cell apoptosis.

In this study, both the main biologically active isomers of CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) were evaluated for their effects on mammary epithelial cell growth, morphological changes and apoptosis in an *in vitro* system. Secondly, the role of *Scd* gene expression changes on mammary cell growth was evaluated by treating transfected *Scd* gene promoter constructs with increasing concentrations of the two main CLA isomers. Finally, the effect of insulin inclusion with CLA treatment on cell growth and the *Scd* gene promoter activity was evaluated as a previous study [21] showed that insulin upregulated the *Scd* gene promoter.

2. Materials and methods

2.1. Cell culture

Mac-T cells were obtained from Nexia Biotechnologies (Quebec, Canada) and cultured routinely in Dulbecco's modified eagle media (Invitrogen, CA) containing 5% fetal calf serum (Invitrogen) and 5 μg/ml bovine insulin (Sigma, St. Louis, MO) and incubated in a humidified incubator at 37 °C in the presence of 5% CO_2. Fatty acids (99% pure isomers) (Matreya, PA) were conjugated to fatty acid-free bovine serum albumin (BSA) (Sigma, St. Louis, MO) at 4:1 ratio using 1 mM BSA stocks (M. McIntosh, University of North Carolina, personal communication). The effect of fatty acids on cell growth was assayed by seeding one well of a six-well plate per treatment at a concentration of 1×10^4 cells/well in the presence of increasing doses of fatty acids. This was repeated three times with a new batch of cells each time. The BSA carrier control was increased with increased concentration of fatty acids. Living cell number was evaluated using Trypan blue staining and white (living) cells were counted using a haemocytometer after 2 days. The effect of 30 μM concentrations of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA on Mac-T growth over a longer time period

Please cite this article in press as: Keating AF, et al., Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression, Domest Anim Endocrinol (2007), doi:10.1016/j.domaniend.2007.08.005

was assayed by seeding six-well plates (three wells per treatment) at a concentration of 1×10^4 cells/well and counting daily using a haemocytometer over a 6-day period. This was repeated twice with three wells per treatment in each experiment. The effect of 15 μM and 30 μM concentrations of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA on cell morphology by immunofluorescence was determined by culturing cells on 12 mm glass coverslips (Fisher Scientific, USA) in 24-well culture plates.

2.2. Cell fixation and TUNEL assay

DNA fragmentation measurement was carried out using the APO-BrdU assay kit as described by the manufacturers (Phoenix Flow Systems, San Diego, CA). Briefly, cells were incubated with 30 μM of either CLA isomer in the presence and absence of insulin (10 μg/ml). Media was removed to a 15 ml tube to ensure cells which were floating were included in the assay. Cells were washed twice with 1× phosphate buffered saline (PBS) (Invitrogen) and detached from the culture plates with trypsin–EDTA (Invitrogen). Cells were combined with the previously removed media and pelleted by centrifugation at $200 \times g$ for 5 min. The supernatant was removed and the pellet washed twice with 1× PBS. The pellet was resuspended in 1% paraformaldehyde (Sigma) in PBS, pH 7.4 at a concentration of 1.2×10^6 cells/ml and incubated on ice for 60 min. Cells were centrifuged at $300 \times g$ for 5 min and the supernatant discarded. The pellet was washed twice with 1× PBS and pelleted by centrifugation at $300 \times g$ for 5 min. The pellet was stored in 70% ethanol (1 ml) at −20 °C until TUNEL assay. For the TUNEL assays, cells were pelleted by centrifugation at $300 \times g$ for 5 min and washed twice with the kit provided wash buffer. The cell pellet was resuspended in 50 μl of DNA-labelling solution containing terminal deoxynucleotide transferase (TdT) reaction buffer, TdT enzyme and bromodeoxyuridine triphosphate (Br-dUTP) and incubated in a 37 °C waterbath for 60 min with shaking every 15 min. Cells were washed twice with kit provided rinse buffer and the cell pellet resuspended in 100 μl antibody solution containing fluorescein-labelled anti-BrDU antibody and incubated at room temperature for 30 min in the dark. Propidium iodide/RNase A solution (500 μl) was added, incubated at room temperature for 30 min in the dark and analyzed by flow cytometry (Beckman Coulter Epic-XL four color analytical flow cytometer), using the parameters outlined in the APO-BrDU protocol as per manufacturer's instructions. Four independent experiments were carried out.

2.3. Immunofluorescence

Mac-T cells were grown on 12 mm glass coverslips (two wells per treatment with one repeat). Cell morphology was observed by staining cells with anti-GLUT1 antibody as previously described [23], using a Goat anti-GLUT1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and an Alexa-fluor 647 labelled donkey anti-goat IgG secondary antibody (Molecular Probes, Eugene, Oregon). Cell nuclei were stained using Sytox nuclear stain (Molecular Probes) at a dilution of 1:1000. Cell images were captured using a Zeiss confocal laser scanning imaging system with Lasersharp 2000 software (Zeiss).

2.4. Reporter vector construction and transient transfection

A 1523 bp fragment of the bovine *stearoyl Co-A desaturase* gene promoter was constructed as previously described [21]. Mac-T cells were transiently co-transfected with luciferase reporter vectors using Fugene 6^{TM} transfection reagent (Roche, Basel, Switzerland). Transfection plates (24-well) (Nunc Nalge International, Rochester, NY) were seeded at a concentration of 2×10^4 cells/well and incubated overnight at 37 °C in a humidified incubator with 5% CO_2. On the day of transfection, 0.0125 μg of a pRL-TK Renilla (Promega, Madison, WI) vector was mixed with 0.2 μg of the pGL3-experimental constructs. Fugene 6^{TM} transfection reagent (0.6 μl) was added to 20 μl of serum free media. The plasmid DNA mix was then added slowly to the media–Fugene 6^{TM} mix and incubated at room temperature for 20 min. This mix was then added to the cells drop-wise, swirled gently to disperse the mix and incubated at 37 °C in a humidified incubator with 5% CO_2. Experimental treatment media was either 15 μM or 30 μM of one of *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA in the presence or absence of 10 μg/ml insulin. Media was changed to the experimental treatment media 5 h after transfection and further incubated at 37 °C in a humidified incubator with 5% CO_2. After 72 h, media was removed from the wells and 100 μl of 1× passive lysis buffer (Promega, Madison, WI) added. Three independent experiments were carried out with $n = 3$ per experiment.

2.5. Luciferase assay

A Dual Luciferase Assay Kit (Promega, Madison, WI) was used according to manufacturer's instructions to measure both Renilla and Firefly luciferase expres-

Please cite this article in press as: Keating AF, et al., Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression, Domest Anim Endocrinol (2007), doi:10.1016/j.domaniend.2007.08.005

sion by the reporter vectors on a luminometer (20/20^n, Turner Biosystems, Sunnyvale, CA). Firefly luciferase values were normalized to the internal control and relative light unit (RLU) values were compared using the Microsoft^{TM} Excel chart function.

2.6. Statistical analysis

Statistical analysis was carried out using the one-way ANOVA or unpaired *t*-test function of SAS software (Statview). The effect of fatty acid treatments on cell growth, apoptosis and *Scd* gene expression were compared to the BSA control for all experiments. The effect of insulin in combination with CLA was compared to the CLA only treatment. Mean standard errors are presented. Where $P < 0.05$ in an experiment, individual effects between treatments were examined and significant differences ($P < 0.05$) are noted with either numbers or symbols in figure. *P*-values between 0.05 and 0.1 were considered a tendency for significant difference.

3. Results

Mac-T cells were grown in the presence of increasing doses of fatty acids as shown in Fig. 1a. Fatty acids were conjugated to BSA as a vehicle and BSA only was used as a vehicle control. The effects of linoleic acid, linolenic acid, vaccenic acid and the primary biologically active CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis* 12) on cell growth were evaluated. Living cell number was determined using Trypan blue staining. At a concentration of 15 μM there was no difference in cell growth between treatments. At 30 μM there tended to be fewer cells due CLA treatment compared to the BSA vehicle control ($P = 0.07$ for *cis*-9, *trans*-11 and $P = 0.06$ for *trans*-10, *cis*-12). At 35 μM and higher CLA isomer inclusion, cell numbers were substantially lower ($P < 0.05$) compared to other fatty acid treatments. There was no difference from the BSA treatment (control) in cell number due to linoleic, linolenic or vaccenic acid inclusion at any concentration. Cells were unable to survive at 150 μM of CLA, but grew in the other fatty acid treatments.

In addition to examining the effect of increasing doses of CLA on cell growth, the effect of 30 μM concentrations of CLA on cell growth over 6 days was also examined. Fig. 1b shows that cell number for CLA treatment were continually lower than the control BSA only treatment, and showed significant difference after day 3 for both isomers.

An assay to measure DNA fragmentation in the nuclei, a characteristic of apoptosis, was employed to determine if the cells were undergoing apoptosis due to CLA treatment. BSA only was again used as a vehicle control. Cells were grown in the appropriate media until they reached approximately 80% confluency, then were harvested and subjected to TUNEL assay. Fig. 2 shows the percentage apoptotic cells due to CLA inclusion. Both CLA isoforms induced Mac-T cell apoptosis at a concentration of 30 μM, consistent with the cell growth data above. Incubation of cells with CLA iso-

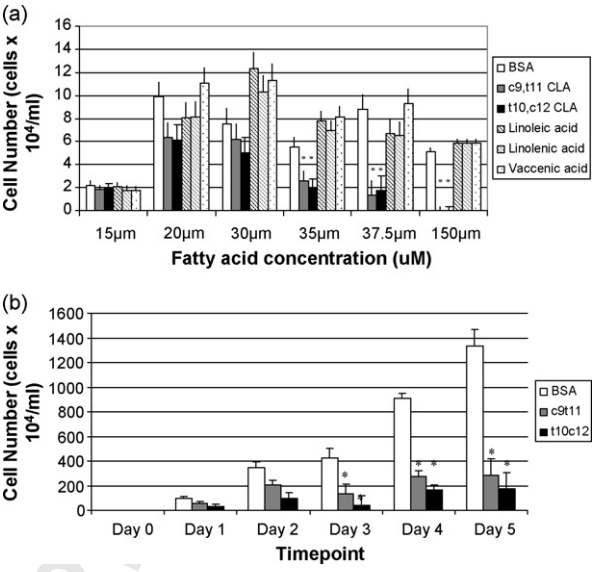

Fig. 1. Effect of CLA on Mac-T cell growth. (a) Mac-T cells grown in the presence of fatty acid treatments for 2 days at each concentration (data presented is a mean of three independent experiments). Fatty acids were fused to BSA as a vehicle. BSA only was used as a control. Living cell number was counted. Statistical differences are indicated by the * symbol ($P < 0.05$). (b) Mac-T cells were grown in 30 μM/ml of either BSA, *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA ($n = 3$). Living cell number was counted daily. Statistical differences are indicated by the * symbol ($P < 0.05$). Data presented is one representative experiment.

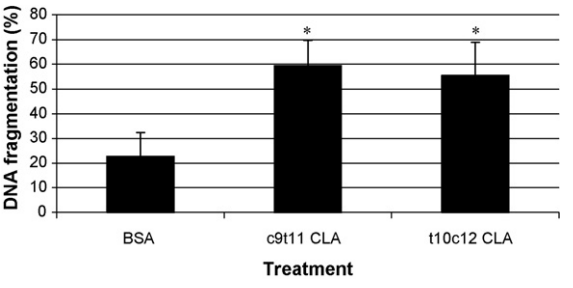

Fig. 2. Effect of CLA on Mac-T cell apoptosis using TUNEL assay. Cells were incubated in 30 μM of either *cis*-9, *trans*-11 or *trans*-10, *cis*-12 CLA. Apoptotic cells were determined by antibody labelling of DNA fragments. Combined data of effect of CLA on apoptosis at 30 μM concentration from four independent experiments. Statistical differences are indicated by the * symbol ($P < 0.05$).

Please cite this article in press as: Keating AF, et al., Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression, Domest Anim Endocrinol (2007), doi:10.1016/j.domaniend.2007.08.005

mers resulted in an approximate 2.5-fold increase in the amount of DNA fragmentation taking place and there appeared to be no difference in apoptosis induction between isomers.

From previously unpublished observations in our lab, the GLUT1 glucose transport protein has been found to have a plasma membrane and cytoplasmic location in Mac-T cells. For this reason we used this protein as a marker of cell morphology and studied the effect of CLA treatments on cell morphology. In comparison to the control (Fig. 3), CLA at the 30 μM concentration (c: *cis*-9, *trans*-11, e: *trans*-10, *cis*-12) caused dramatic

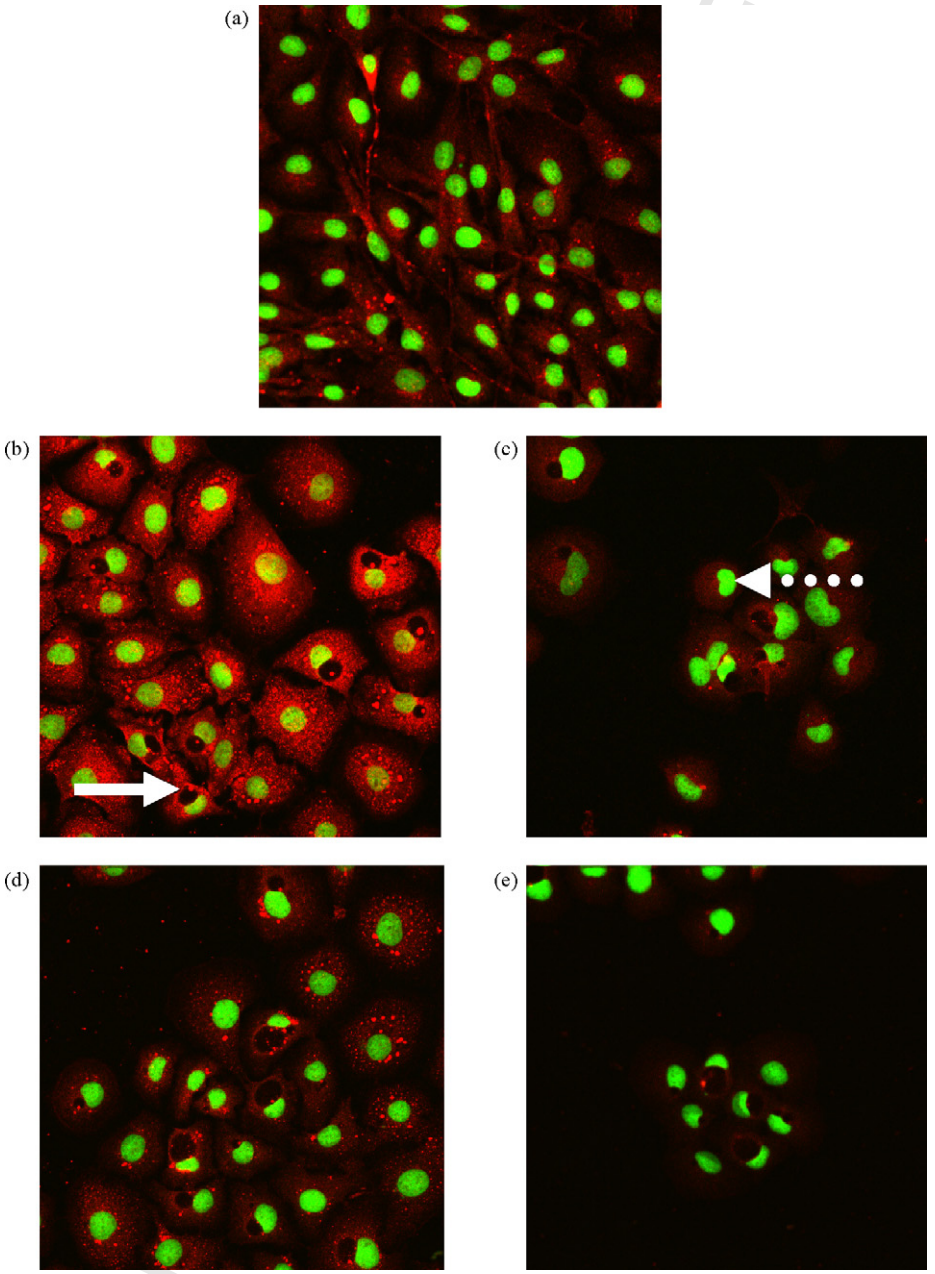

Fig. 3. Evaluation of CLA on cell morphology using immunofluorescence. Mac-T cells were incubated in increasing doses of CLA as indicated, fixed on coverslips and stained with an anti-GLUT1 antibody and fluorescently labelled secondary antibody. Nuclei were labelled using Sytox (green): (a) control cells; (b) 15 μM *cis*-9, *trans*-11 CLA; (c) 30 μM *cis*-9, *trans*-11 CLA; (d) 15 μM *trans*-10, *cis*-12 CLA; (e) 30 μM *trans*-10, *cis*-12 CLA. Note the vacuole-like structures in cytosol indicated by block arrow and half-moon-shaped nuclei in 30 μM CLA-treated cells indicated by broken arrow. Data presented is one representative experiment.

Please cite this article in press as: Keating AF, et al., Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression, Domest Anim Endocrinol (2007), doi:10.1016/j.domaniend.2007.08.005

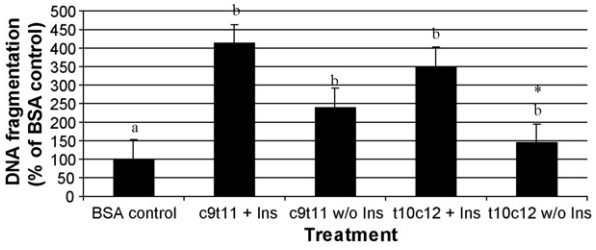

Fig. 4. Effect of insulin on the percentage DNA fragmentation due to CLA treatment. Apoptotic cells were determined by antibody labelling of DNA fragments. Level of DNA fragmentation in Mac-T cells cultured in one of 30 μM *cis*-9, *trans*-11 CLA in the presence and absence of 10 μg/ml insulin or 30 μM *trans*-10, *cis*-12 CLA in the presence and absence of 10 μg/ml insulin. Different superscripts represent $P < 0.05$, * symbol represents $P = 0.08$. Data presented is one representative experiment.

changes in cell cytology. CLA induced large vacuole-like structures in the cytosol and changed the cell nuclei shape to a kidney-like or half-moon-shaped structures.

A second experiment evaluated whether the inclusion of insulin could partially alleviate the effect of CLA on apoptosis. In this experiment, although no significant difference was observed for both CLA isoforms in the presence or absence of insulin, inclusion of insulin tended to increase DNA fragmentation in the cells treated with the *trans*-10, *cis*-12 isomer ($P = 0.08$) (Fig. 4).

To determine whether the effect of CLA on cell growth was associated with the action of the *Scd* gene, the effect of increasing concentrations of CLA on *Scd* transcription was determined (Fig. 5). Both CLA isoforms did inhibit transcriptional activity of the *Scd* gene at 15 μM but did not show a significant further reduction at 30 μM although the *cis*-9, *trans*-11 isomer tended ($P = 0.08$) to be inhibitory.

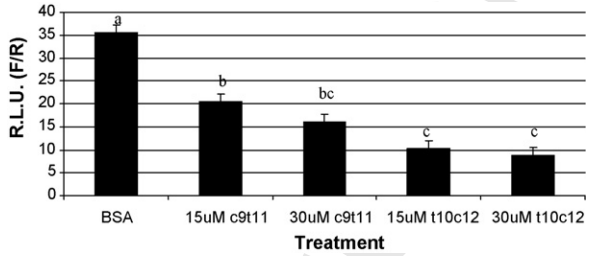

Fig. 5. Effect of increasing concentrations of CLA on *Scd* transcriptional activity in Mac-T cells. Reporter constructs containing the bovine *Scd* gene promoter fused to a luciferase-encoding cDNA were transiently transfected into bovine Mac-T cells and incubated with (a) *cis*-9, *trans*-11 CLA or (b) *trans*-10, *cis*-12 CLA. Reporter firefly luciferase activity was quantified, normalized by control Renilla luciferase activity (F/R) and expressed as RLU. Different superscripts indicate significant differences ($P < 0.05$). Data presented is one representative experiment.

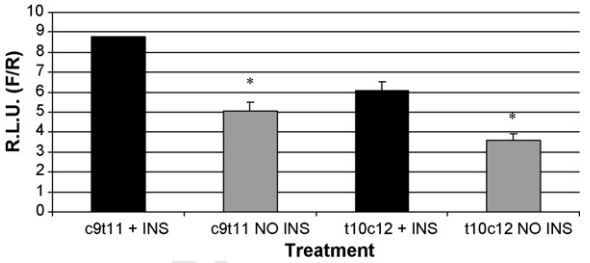

Fig. 6. Effect of insulin on the *Scd* gene transcriptional down-regulation induced by CLA in Mac-T cells. Reporter constructs containing the bovine *Scd* gene promoter fused to a luciferase-encoding cDNA were transiently transfected into bovine Mac-T cells and incubated with (a) 30 μM *cis*-9, *trans*-11 CLA in the presence and absence of 10 μg/ml insulin or (b) 30 μM *trans*-10, *cis*-12 CLA in the presence and absence of 10 μg/ml insulin. Reporter firefly luciferase activity was quantified, normalized by control Renilla luciferase activity (F/R) and expressed as RLU. Significant differences are represented by the * symbol ($P < 0.05$). Data presented is one representative experiment. Q1

An additional experiment was carried out to determine if insulin could antagonise the effect of the CLA isomers on the *Scd* gene promoter transcriptional activity. The inclusion of insulin (10 μg/ml) did indeed show an increased transcriptional activity of the *Scd* gene in the cells treated with both CLA isomers (Fig. 6).

4. Discussion

CLA has been studied as a tool to alter milk fat composition in lactating dairy cattle, to aid the energy balance of the transition cow, and to increase the overall level of CLA in bovine milk in order to promote milk as a functional food. However, diets designed to increase milk CLA levels have also been shown to cause milk fat depression and, in some instances, have had negative effects on milk yield [14,16,17,24]. The purpose of this study was firstly to investigate the effect of increasing doses of both of the main biologically active CLA isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12, on mammary epithelial cell growth *in vitro*, secondly to determine whether alteration in *Scd* gene expression is associated with changes in mammary cell growth, and finally, to examine whether insulin could play a role in modulating the effect of CLA on both cell growth and *Scd* transcriptional activity.

The effect of increasing doses of CLA on cell growth was determined by counting the living cell number after 2-day CLA treatments (Fig. 1a). In addition to the two CLA isomers, vaccenic, linoleic and linolenic acids were also included to determine whether any effect might be due to simply a high dose of any fatty acid and not CLA specifically. Results seen in Fig. 1a demonstrate quite

Please cite this article in press as: Keating AF, et al., Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression, Domest Anim Endocrinol (2007), doi:10.1016/j.domaniend.2007.08.005

clearly that at lower levels of CLA (15 μM/ml) that there was no difference in cell proliferation between control, CLA and fatty acid treatments. As the dose of fatty acid added increased, however, it is evident that at higher doses of CLA (35 μM and above), cell growth is inhibited or cells are no longer viable. To determine if the cells were merely growing slower or if they were undergoing apoptosis in CLA treatment, a TUNEL assay approach was employed. Previous studies have shown that camptothecin can induce apoptosis in human mammary cells [25,26], thus it was employed in this study as a positive control for apoptosis. Treatment of cells with 30 μM CLA resulted in an approximate 2.5-fold increase in the amount of DNA fragmentation taking place (Fig. 2). This is consistent with cell morphology change under CLA treatment observed in Fig. 3. Visualization of the nuclei demonstrated the effects of high-dose CLA incubation. The nuclei at higher levels of CLA treatment form kidney bean- or half-moon-shaped structures, which have previously been noted to be a morphological sign of apoptosis, due to chromatin condensation in the nucleus [27–29]. In addition, the cells display large vacuole-like structures, which may indicate some form of cellular damage.

These results are in agreement with physiological studies *in vivo* showing detrimental effects of high doses of abomasally infused CLA on mammary cell function and survival [14], while low doses of orally delivered rumen-protected CLA show little or no negative effects [11–13,15]. Our data support the hypothesis that high levels of CLA can induce apoptosis of mammary epithelial cells *in vivo*. A recent study also reported the growth inhibitory effects of a number of CLA isomers on human colon cancer cell lines and found an apoptotic effect in a dose- and time-dependent manner [30]. While the Mac-T cell line presents limitations for the study of a functioning mammary gland as it has abnormal characteristics, our *in vitro* data are suggestive of a possible effect of high doses of CLA *in vivo* on the mammary gland. In addition, these data could be extended to evaluate the effects of CLA on differentiated mammary cells *in vitro* such as the bovine mammary epithelial cell collagen gel culture system [31].

Our experiments also showed that insulin did not significantly alter the effect of CLA on cell apoptosis. It is likely that insulin and CLA have their effect on mammary epithelial cells through different signalling pathways. While both insulin and CLA signalling take place through the mitogen-activated protein kinase pathways [32,33], it has been reported that CLA also mediates an effect on the mitochondrial pathway to induce apoptosis in a mammary epithelial cell line, MDA-MB-231 [34].

Previously we have shown that treatment with 30 μM/ml of both CLA isomers resulted in down-regulation of *Scd* transcriptional activity [21]. It was also reported that simian virus 40-transformed human lung fibroblasts bearing a knockdown of human *Scd* showed a considerable reduction in monounsaturated fatty acids, cholesterol, phospholipid synthesis, along with high-cellular levels of saturated free fatty acids and triacylglycerol, compared with control cells [35]. In addition, *Scd*-deficient cells were more sensitive to palmitic acid-induced apoptosis compared with control cells. These data suggested that, by globally regulating lipid metabolism, *Scd* activity modulated cell proliferation and survival [35]. To study if the effect of CLA on *Scd* transcription could be involved in the effect of mammary cell growth at high doses, we examined the dose effect of CLA on *Scd* promoter activity. Our data showed that while *Scd* transcriptional activity was significantly reduced by CLA at 15 μM, cell growth was not significantly inhibited at this concentration. Furthermore, increased doses of CLA did not result in a significant dose-dependent decrease in the transcriptional activity of the *Scd* gene although there was a tendency for the *cis*-9, *trans*-11 isomer ($P < 0.1$) to have this effect. These data indicate that the effect on *Scd* gene expression may not be a major factor influencing the CLA effect on cell growth.

Our previous study showed that insulin results in induction of transcriptional activity of the *Scd* gene [21]. In this study, we further demonstrate that insulin had the ability to act as an antagonist to the down-regulation of the *Scd* gene promoter by CLA. Again, if the *Scd* gene was involved in the cell death induced by high doses of CLA, it would also be expected that inclusion of insulin would result in less DNA fragmentation. However, a significant effect of insulin on CLA-induced apoptosis was not observed. Previously we identified that the effect of CLA on the *Scd* gene promoter was mediated through a region of 36 bp in length, termed the STE, where the sterol response element binding protein (SREBP) transcription factor was predicted to bind [21]. Insulin has been shown to regulate SREBP transcription in the liver and adipocytes [36] and in addition, insulin may increase activity of SREBP by inducing phosphorylation of specific serine and threonine residues [37]. The opposing effects of CLA and insulin on the *Scd* gene promoter may be mediated through SREBP. This provides further support for the hypothesis that the effect of CLA on *Scd* gene expression is unlikely the main mechanism by which apoptosis is induced and is likely a secondary effect of the apoptotic pathways induced by high levels of CLA.

Please cite this article in press as: Keating AF, et al., Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression, Domest Anim Endocrinol (2007), doi:10.1016/j.domaniend.2007.08.005

5. Conclusion

At high doses, the two main CLA isomers have detrimental effects on mammary epithelial cell growth and survival *in vitro*, however it is unlikely that the down-regulation of the *Scd* gene transcription plays a major role in this effect. This correlates well with the negative effects seen on milk yield and composition when animals were abomasally infused with high doses of CLA. At lower doses of CLA there was no effect on cell growth, likely mirroring the amounts of CLA used in the majority of animal studies.

Acknowledgements

We kindly acknowledge the help of Dr. J. Folsom for providing both advice and camptothecin for apoptosis induction. We also acknowledge the help of Scott Tighe at Vermont Cancer Center flow cytometer facility.

Financial support was from Dairy Farmers of Canada (DFC), the Natural Sciences and Engineering Research Council (NSERC) and the Alberta CLA network.

References

[1] Ip C, Chin SF, Scimeca JA, Pariza MW. Mammary cancer prevention by conjugated dienoic derivatives of linoleic acid. Cancer Res 1991;51:6118–24.
[2] Ip C, Singh M, Thompson HJ, Scimeca JA. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res 1994;54:1212–5.
[3] Ip C, Thompson H, Banni S, Zhu Z, Loftus T, Darcy K. Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid: implication for a reduction in mammary cancer risk. Cancer Res 1997;57:5067–72.
[4] Ip C, Banni S, Angioni E, Carta G, McGinley J, Thompson HJ, et al. Conjugated linoleic acid-enriched butterfat alters mammary gland morphogenesis and reduces cancer risk. J Nutr 1999;129:2135–42.
[5] Belury MA, Mahon A, Banni S. The conjugated linoleic acid (CLA) isomer, t10c12-CLA, is inversely associated with changes in body weight and serum leptin in subjects with type 2 diabetes mellitus. J Nutr 2003;133:257S–60S.
[6] Terpstra AH. Effect of conjugated linoleic acid on body composition and plasma lipids in humans: an overview of the literature. Am J Clin Nutr 2004;79:352–61.
[7] Griinari JM, Chouinard PY, Bauman DE. *Trans* fatty acid hypothesis of milk fat depression revised. In: Proc. Cornell Nutr. Conf. Feed Manuf. 1997. p. 208–16.
[8] Griinari JM, Corl BA, Lacy SH, Chouinard PY, Nurmela KVV, Bauman DE. Conjugated linoleic acid is synthesised endogenously in lactating cows by delta-9 desaturase. J Nutr 2000;130:2285–91.
[9] Corl BA, Baumgard LH, Dwyer DA, Griinari JM, Phillips BS, Bauman DE. The role of delta-9 desaturase in the production of *cis*-9, *trans*-11 CLA. J Nutr Biochem 2001;12:622–30.
[10] Kay JK, Mackle TR, Auldist MJ, Thomson NA, Bauman DE. Endogenous synthesis of *cis*-9, *trans*-11 conjugated linoleic acid in dairy cows fed fresh pasture. J Dairy Sci 2004;87:369–78.
[11] Baumgard LH, Corl BA, Dwyer DA, Saebo A, Bauman DE. Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. Am J Physiol Integr Comp Physiol 2000;278:R179–84.
[12] Baumgard LH, Sangster JK, Bauman DE. Milk fat synthesis in dairy cows is progressively reduced by increasing supplemental amounts of *trans*-10, *cis*-12 conjugated linoleic acid (CLA). J Nutr 2001;131:1764–9.
[13] Peterson DG, Baumgard LH, Bauman DE. Short communication: milk fat response to low doses of *trans*-10, *cis*-12 conjugated linoleic acid (CLA). J Dairy Sci 2002;85:1764–6.
[14] Bell JA, Kennelly JJ. Short communication: postruminal infusion of conjugated linoleic acids negatively impacts milk synthesis in Holstein cows. J Dairy Sci 2003;86:1321–4.
[15] Moore CH, Hafliger III HC, Mendivil OB, Sanders SR, Bauman DE, Baumgard LH. Increasing amounts of conjugated linoleic acid (CLA) progressively reduces milk fat synthesis immediately postpartum. J Dairy Sci 2005;87:1886–95.
[16] Chouinard PY, Corneau L, Barbano DM, Metzger LE, Bauman DE. Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows. J Nutr 1999;129:1579–84.
[17] Mackle TR, Kay JK, Auldist MJ, McGibbon AKH, Philpott BA, Baumgard LH, et al. Effects of abomasal infusion of conjugated linoleic acid on milk fat concentration and yield from pasture-fed dairy cows. J Dairy Sci 2003;86:644–52.
[18] O'Shea M, Devery R, Lawless F, Murphy J, Stanton C. Milk fat conjugated linoleic acid (CLA) inhibits growth of human mammary MCF-7 cancer cells. Anticancer Res 2000;20:3591–601.
[19] Hubbard NE, Lim D, Erickson KL. Conjugated linoleic acid alters matrix metalloproteinases of metastatic mouse mammary tumour cells. J Nutr 2007;137:1423–9.
[20] Ou L, Ip C, Lisafeld B, Ip MM. Conjugated linoleic acid induces apoptosis of murine mammary tumor cells via Bcl-2 loss. Biochem Biophys Res Commun 2007;356:1044–9.
[21] Keating AF, Kennelly JJ, Zhao FQ. Characterization and regulation of the bovine Stearoyl-CoA desaturase gene promoter. Biochem Biophys Res Commun 2006;344:233–40.
[22] Ntambi JM. The regulation of stearoyl CoA desaturase (SCD). Prog Lipid Res 1995;34:139–50.
[23] Miller PJ, Finucane KA, Hughes M, Zhao FQ. Cloning and expression of bovine glucose transporter GLUT12. Mamm Genome 2005;16:873–83.
[24] Bell JA, Griinari JM, Kennelly JJ. Effect of safflower oil, flaxseed oil, monensin, and vitamin E on concentration of conjugated linoleic acid in bovine milk fat. J Dairy Sci 2006;89:733–48.
[25] Lamparska-Przybysz M, Galjkowska B, Motyl T. Cathepsins and BID are involved in the molecular switch between apoptosis and autophagy in breast cancer MCF-7 cells exposed to camptothecin. J Physiol Pharmacol 2005;56:159–79.
[26] Gao S, Scott RE. Stable overexpression of specific segments of the P2P-R protein in human MCF-7 cells promotes camptothecin-induced apoptosis. J Cell Physiol 2003;197:445–52.
[27] Earnshaw WC. Nuclear changes in apoptosis. Curr Opin Cell Biol 1995;7:337–43.
[28] Martelli AM, Zweyer M, Ochs RL, Tazzari PL, Tebellini G, Narducci P, et al. Nuclear apoptotic changes: an overview. J Cell Biochem 2001;82:634–46.

Please cite this article in press as: Keating AF, et al., Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression, Domest Anim Endocrinol (2007), doi:10.1016/j.domaniend.2007.08.005

[29] Rogalinska M. Alterations in cell nuclei during apoptosis. Cell Mol Biol Lett 2002;7:995–1018.
[30] Beppu F, Hosokawa M, Tanaka L, Kohno H, Tanaka T, Miyashita K. Potent inhibitory effect of *trans*9, *trans*11 isomer of conjugated linoleic acid on the growth of human colon cancer cells. J Nutr Biochem 2006;17:830–6.
[31] Collier RJ, Steining CM, Pollard BC, VanBaale MJ, Baumgard LH, Gentry PC, et al. Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. J Anim Sci 2006;84. Suppl. E1–13.
[32] Kim SK, Novak RF. The role of intracellular signaling in insulin-mediated regulation of drug metabolizing enzyme gene and protein expression. Pharmacol Ther 2007;113:88–120.
[33] Brown JM, Boysen MS, Chung S, Fabiyi O, Morrison RF, Mandrup S, et al. Conjugated linoleic acid induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signalling by adipocytokines. J Biol Chem 2004;279:26735–47.
[34] Migletta A, Bozzo F, Bocca C, Gabriel L, Trombetta A, Belotti S, et al. Conjugated linoleic acid induces apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway. Cancer Lett 2006;234:149–57.
[35] Scaglia N, Igal RA. Stearoyl-CoA desaturase is involved in the control of proliferation, anchorage-independent growth, and survival in human transformed cells. J Biol Chem 2005;280:25339–49.
[36] Eberle D, Hegarty B, Bossard P, Ferre P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 2004;86:839–48.
[37] Foretz M, Pacot C, Dugail I, Lemarchand P, Guichard C, Le Liepvre X, et al. ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. Mol Cell Biol 1999;19:3760–8.

Please cite this article in press as: Keating AF, et al., Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression, Domest Anim Endocrinol (2007), doi:10.1016/j.domaniend.2007.08.005