By Chen et al. Trans 10, cis12-conjugated linoleic acid (**t10, c12-CLA**) is well accepted as one of the fatty acids that can lead to milk fat depression, while its underlying molecular mechanism is not yet fully characterized. This study revealed that t10, c12-CLA reduced sterol response element binding protein-1 (**SREBP1**), a master transcription factor in lipogenesis, through the regulation of insulin induced gene-1 (**Insig1**), an endoplasmic reticulum (**ER**) protein that retains SREBP1 precursor in ER from activation. With t10, c12-CLA slowing Insig1 degradation, stabilized Insig1 continued to suppress SREBP1 activation and reduced lipogenic gene transcription, resulted in reduced milk fat synthesis.

The inhibitory effect of trans10, cis12-CLA on SREBP1 activation in bovine mammary epithelial cells involved reduced proteasomal degradation of Insig1

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ABSTRACT

Tran10, cis12-Conjugated linoleic acid (**t10, c12-CLA**) is well recognized as a key CLA isomer responsible for the reduction in milk fat synthesis that leads to milk fat depression (**MFD**) in dairy cows. Sterol response element binding protein-1 (**SREBP1**) is a key transcription factor in bovine mammary gland coordinating transcription of the genes for fatty acid synthesis. SREBP1 activation requires the removal of insulin induced gene-1 (**Insig1**) that serves as a repressor of SREBP1 in the endoplasmic reticulum (**ER**). We hypothesized that t10, c12-CLA reduced SREBP1 activation by delaying Insig1 degradation. In the present study, we utilized undifferentiated bovine mammary epithelial cells (MAC-T cells) and treated them with t10, c12-CLA for 6 h. We found that SREBP1 protein expression declined over 56 % when cells were treated with 60 μM or greater concentration of t10, c12-CLA. Such inhibitory effects were also observed in the mRNA expression of SREBP1-regulated genes including SREBP1, fatty acid synthetase, Δ9-desaturase, and Insig1. Compared with no CLA group, 60 μM or higher concentration of t10, c12-CLA increased Insig1 protein expression over two-fold in cells transfected with FLAG-tagged Insig1. This stimulatory effect was not specific to t10, c12-CLA but also other polyunsaturated fatty acids including c9, t11-CLA and linoleic acid. Oleic acid had no effect on Insig1 protein expression while palmitic acid decreased Insig1 protein expression. Further investigation revealed that increased abundance of FLAG-Insig1 with t10, c12-CLA was due to the inhibition of the proteasomal degradation of Insig1. T10, c12-CLA delayed the Insig1 decay when protein synthesis was blocked. Immunoprecipitation also confirmed that the interaction between Ubiquitin-like domain-containing protein 8 and Insig1, the key step of removing Insig1 from ER and freeing SREBP1 for proteolytic processing, was inhibited by t10, c12-CLA, but not palmitic acid. These findings suggested that t10, c12-CLA played a role in

regulating SREBP1 activation by reducing proteasomal degradation of Insig1. We concluded that stabilized Insig1 retained SREBP1 in the ER from activation, thus reducing lipogenic gene transcription.

Key words: Conjugated linoleic acid, SREBP1 activation, Insig1

INTRODUCTION

The reduction in milk fat synthesis associated with diet-induced milk fat depression (**MFD**) in dairy cows is similar to the MFD caused by trans10, cis 12 conjugated linoleic acid (t10, c12-CLA) established through abomasal infusion or dietary supplementation with t10, c12-CLA (Bauman et al., 2011). The reduction of milk fat synthesis is associated with the decreased expression of genes involved in milk fat synthesis, i.e. acetyl CoA carboxylase (**ACC**), fatty acid synthase (**FASN**) and Δ9-desaturase (**SCD)** (Angulo et al., 2012; Vyas et al., 2013). We have shown that Sterol regulatory element binding protein-1 (**SREBP1**) is a key transcription factor that mediates the transcription of lipogenic genes (Ma and Corl, 2012).

SREBP1 is synthesized as a 125 kDa precursor protein (**pSREBP1**) and resides in the endoplasmic reticulum (**ER**) in a complex with SREBP cleavage activating protein (**SCAP**) (Sakai et al., 1997). Activation of SREBP1 requires SCAP-mediated transport to the Golgi apparatus (**GA**) and cleavage to a 65 kDa mature form (**mSREBP1**) that migrates to the nucleus and promotes transcription of genes it regulates, including SREBP1 (Cheng et al., 2015). Insulin-induced gene-1 (**Insig1**), an ER protein which anchors the SCAP-SREBP1 complex, prevents SREBP1 activation (Yang et al., 2002). The essential step in the release of SCAP-SREBP1 from the ER is the removal of Insig1 for degradation (Lee and Ye, 2004). It is regulated through a two-part process that includes ubiquitination and removal of Insig1 from the ER membrane.

When cellular sterol levels dropped, ER-residing ubiquitin ligase gp78 ubiquitinated Insig1 in preparation for proteasomal degradation (Joon et al., 2006). Furthermore, in cells deprived of unsaturated fatty acids, ubiquitin-like domain-containing protein 8 (**UBXd8**), a key mediator in triglyceride synthesis, was activated and removed ubiquitinated Insig1 from the ER membrane (Lee et al., 2010). When the interaction of Ubxd8 and ubiquitinated Insig1 was blocked, Insig1 was deubiquitinated and resumed its interaction with SCAP-SREBP1 (Joon et al., 2008).

Unsaturated fatty acids including t10, c12-CLA are known to block SREBP1 maturation in cultured cells (Hannah et al., 2001). And this is analogous to the inhibition of SREBP1 activation observed with MFD and the reduction in transcription of SREBP1 target genes (Gervais et al., 2009). A recent report has showed manipulation of Insig1 expression in goat mammary epithelial cells reduced lipogenic gene expression and triglyceride accumulation (Li et al., 2019). On the other hand, t10, c12-CLA appeared to exhibit proteasomal degradation inhibitor-like property that caused reduction in SREBP1 expression in bovine mammary epithelial cells (Shi et al., 2020). An essential step in the release of SCAP-SREBP1 from the ER is the removal of Insig1 for degradation. T10, c12-CLA could inhibit SREBP1 activation through a mechanism involving blockage of Insig1 degradation. Our goal was to evaluate if the inhibitory effect of t10, c12-CLA on SREBP1 activation occurs via Inisg1 signaling. We hypothesized that t10, c12-CLA, not other fatty acids, regulated inhibition of SREBP1 through delayed removal of Insig1 for proteasomal degradation and blocking the Ubxd8-Insig1 interaction.

MATERIALS AND METHODS

Cell culture and treatments

A bovine mammary epithelial cell line, MAC-T cells, was used in this experiment (Huynh et al., 1991). Cells were routinely cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals), 100 U penicillin, 0.01 mg streptomycin, and 0.25 μg/mL amphotericin B (1× antibiotic/antimycotic solution; Sigma). Cells were routinely maintained at 37°C in 5% CO_2.

A 100 mM stock solution of each fatty acid (t10, c12-CLA, c9, t11-CLA, linoleic acid, oleic acid, and palmitic acid) was prepared by diluting each fatty acid in NaOH in equal molar ratio and dissolved completely above 68 °C until clear, then complexed to fatty acid free-BSA (>99 %, sigma) in a 2.5 to 1 ratio (5 mM fatty acid to 2 mM BSA). Fatty acid-BSA complexes were filtered through a 0.22 μm filter before adding to cells. Fatty acid concentration was adjusted to designated concentrations with BSA and added to cells at equal volume. For control, only BSA was added. All solutions were prepared fresh and used on the same day.

Expression vector construction and transfection

Expression vector for C-terminal FLAG-tagged bovine Insig1 protein was constructed by RT-PCR amplifying a 951-base pair (**bp**) product of bovine Insig1 (NM_001077909). The amplifying primers were as following: F: 5'-ACTCCTCCGCGTCCCTCCT-3'; R: 5'-CCCAAACCTTGCCACTCTTC-3'. The resulting amplicon was cloned into the pCR4-TOPO vector (Invitrogen) and then subcloned into the pFLAG-CMV5b expression vector (Invitrogen) using the flanking EcoRI restriction sites in the pCR4-TOPO vector and the EcoRI site in the expression vector. Site-directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene) to destroy the stop codons and create an in-frame fusion protein containing a C-terminal FLAG-tag. The stop codon was changed to glycine

(**t917g**) using the primers as following: F: 5'-GTGCGCCTGAGGATTCCATCACTGTGTGGCTTT-3'; R: 5'-AAAGCCACACAGTGATGGAATCCTCAGGCGCAC-3'. 84 nucleotides following the stop codon intervening the expression of FLAG tag was excised using BamHI restriction site. Successful cloning, mutagenesis and correct orientation were confirmed by sequencing at each step.

Another expression vector coding for an N-terminal Myc-tagged bovine Ubxd8 protein was constructed by RT-PCR amplifying a 1653 bp product encompassing the coding region of bovine Ubxd8 (XM_005209156) with the primers as following: F: 5'-CGGGTCAGAAGCGTAGAGG-3'; R: 5'-CTGGGTTGGAGGTGAAGAGT-3', which was cloned into the pCR4-TOPO vector. The coding region of Ubxd8 was then subcloned into the pCMV-Tag3A expression vector using PCR primers containing BamHI and HindIII restriction sites and cloning into the in the BamHI and HindIII restriction sites in the Tag3a expression vector, to create an in-frame fusion protein containing an N-terminal Myc-tag. Successful cloning and correct orientation were confirmed by sequencing.

For transient transfection of FLAG-Insig1, MAC-T cells were seeded in 6-well plates at 3 $\times 10^5$ cells /well for 24 h to reach 80 % confluency. Fresh medium containing 10% serum was replenished 1 h before transfection. Cells were transfected with 2 μg FLAG-Insig1 plasmids using jetPEI transfection reagent (PolyPlus Transfection). After 24 h, the cells were treated with indicated fatty acids at designated concentrations and time for further study.

Insig1 decay assay

MAC-T cells were transfected with 2 μg FLAG-Insig1 plasmids for 24 h, and then cultured with 75 μM t10, c12-CLA for 6 h prior to administration of 60 μg/mL cycloheximide to stop further protein synthesis for another 4.5 h. Cells were harvested 0, 1.5, 3 and 4.5 h after cycloheximide addition and lysed for immunoblot analysis of Insig1 to measure protein decay. On the other hand, another set of MAC-T cells were cultured with 75 μM t10, c12-CLA or 10 μM MG132, an inhibitor of proteasomal degradation, alone or in combination for 6 h and harvested for immunoblot analysis.

Protein extraction and Immunoprecipitation (IP)

For immunoprecipitation assay, MAC-T cells were seeded in 60 mm plates at 3×10^6 cells /plate. After reaching 80 % confluency, cells were co-transfected with 5 μg FLAG-Insig1 and 5 μg Myc-Ubxd8, or with 5 μg FLAG-Insig1/Myc-Ubxd8 alone as single transfection controls. After 24 h, cells were treated with 75 μM of t10, c12-CLA or palmitic acid for another 6 h. Cells were lysed and prepared for immunoprecipitation.

Whole cell lysates were prepared with cold lysis buffer (50 mM Hepes-HCl, pH at 7.4, 150 mM NaCl, 1.5 M $MgCl_2$, 1 % Triton X-100 and protease inhibitors) and passed through a 23g needle several times before centrifugation. Protein concentrations were determined using the Bradford Assay (Bio-Rad). After the proteins were normalized to the same level (800 μg per treatment), 2 μg rabbit anti-Myc antibody (Sigma) or rabbit control IgG, and 20 μL protein G plus agarose beads (#sc2002, Santa Cruz Biotechnology) were added to 1 mL cell lysates and incubated in 4 °C overnight. Beads were washed with PBS four times and eluted with 2× sample buffer. Protein interaction was analyzed by immunoblotting.

RNA extraction and real-time PCR (qPCR)

Total RNA was extracted from cells using TRI Reagent (Molecular Research Center) according to the manufacturer's instructions. RNA pellets were resuspended in RNase-free H_2O and concentrations were quantified at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). 2 μg RNA per reaction were reversed transcribed to its cDNA using the Omniscript RT kit (Qiagen) according to the manufacturer's instructions and Oligo-dT (Eurofins MWG/Operon) as the primer. Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) in an Applied Biosystems 7300 Real-time PCR machine. Reactions were as follows: 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 30 secs, 58°C for 30 secs, and 72°C for 1 min. Each reaction was performed in duplicate wells. Relative quantification of gene transcript was determined using three pairs of endogenous controls: beta-2-microglobulin (**B2M**), eukaryotic translation initiation factor 3 subunit K (**EIF3K**) and ribosomal protein S15 (**RPS15**). Primer amplification efficiency was tested with a serial dilution of cDNA, which generated a standard curve, and the slope was used to calculate the efficiency. The amplification efficiency ranged from 95% to 105% for all primers used. The fold change was calculated using $2^{-\triangle\triangle Ct}$ method with control group as the calibrator (Livak and Schmittgen, 2001). Gene-specific primers for the transcripts in the study are shown in **Table 1**.

Protein extraction and Immunoblotting

Whole cell lysates were prepared with cold lysis buffer (50 mM Tris, pH 7.4, 0.5 % Triton X-100, 0.3 M NaCl, 2 mM EDTA, pH 8.0, and protease inhibitors). Protein concentrations were determined using the Bradford Assay (Bio-Rad). Samples were diluted to equal amount of protein and heated at 95 °C for 5 min after adding 2 × Laemmli sample buffer

(Sigma). Proteins were separated by SDS-PAGE using 7.5 to 12 % PAGEr Gold Plus PreCast SDS-polyacrylamide gels (Lonza) and transferred to PVDF membrane (Bio-Rad). Membranes were then blocked in 5 % nonfat milk TBS-T blocking buffer (0.05 M Tris pH 7.4, 0.2 M NaCl, 0.1 % Tween, and 5 % dried non-fat milk) on a rocker for 1 h at room temperature. Membranes were probed with the indicated primary antibodies: anti-FLAG (1:4000, #F3165, Sigma), anti-SREBP1(1:4000, customized antibody from GenScript) and anti-β-Actin (1:10,000, #A2228, Sigma) was used as loading control in blocking buffer at 4 °C overnight, then incubated with secondary antibody anti-goat IgG1 (1:10,000, #sc2354, Santa Cruz Biotechnology) for 1 h at room temperature, and added detection reagents to the blot using ECL-plus Chemiluminescence Subtract (Amersham Biosciences) according to the manufacturer's instructions and incubated for 5 min. Chemiluminescence was measured using a Chemicdoc XRS digital imaging system and densitometry was performed using Quantity One software (Bio-Rad).

Statistical Analysis

All data were analyzed using the MIXED procedure (SAS 9.4, SAS institute). The model included the treatment as fixed effect and the experiment replicate as random effect. For Insig1 decay experiment, the treatment effect at each time point was tested using slice statement. Post-test pairwise treatment comparisons were carried out using Tukey's HSD test. Differences were considered significant when $P < 0.05$ and tendencies were declared at $P < 0.10$. Results were graphed using GraphPad Prism 8.0 (GraphPad). Each experiment was repeated at least three times.

RESULTS

Increasing t10, c12-CLA reduced SREBP1 protein activation and lipogenic gene expression

The effect of t10, c12-CLA (**CLA**) on SREBP1 and the transcription of its target genes were investigated. Undifferentiated MAC-T cells were treated with increasing concentrations of CLA and SREBP1 protein expression was measured for a short-term incubation of 6 h. The bovine specific anti-SREBP1 customized antibody was sensitive enough to detect two clear bands representing the precursor form (**pSREBP1**) as well as the mature form (**mSREBP1**) at about 125 kDa and 65 kDa, respectively. Both pSREBP1 and mSREBP1 responded to t10, c12-CLA treatment in a dose dependent pattern between 0 to 100 μM of concentration. As CLA concentration increased, the expression of both proteins decreased (**Figure 1A**). The protein expression of pSREBP1 remained stabilized until CLA concentration increased to at least 60 μM ($P < 0.05$), while the mSREBP1 dropped significantly by 48 % at an initial CLA concentration of as low as 20 μM ($P < 0.05$). Both proteins continued to decrease and leveled off at 20 % as CLA concentration increased to 100 μM (**Figure 1B**). Furthermore, the dose-challenge effect of t10, c12-CLA on mRNA expression of SREBP1 and its target lipogenic genes was examined, MAC-T cells were treated with a series of increasing CLA concentrations (0, 20, 40, 60, 80, 100 μM) for 24 h. The mRNA abundance of SREBP1, FASN and SCD1 was significantly reduced at 40 μM CLA and continued to decrease in a dose-dependent pattern (**Figure 2A-C**). Unexpectedly, the mRNA expression of Insig1 increased at a lower CLA concentration (20 μM). As CLA dose increased, Insig1 progressively declined and differed significantly at 60 μM CLA ($P < 0.05$) (**Figure 2D**). Insig2 failed to respond to CLA treatment between 0-100 μM (**Figure 2E)**. These results showed that increasing CLA exhibited an inhibitory effect on SREBP1 protein activation and its target gene transcription involved in lipogenesis.

t10, c12-CLA increased Insig1 protein expression

Insig1 is the key regulator of SREBP1 activation in the ER membrane, and whether t10, c12-CLA blocked SREBP1 activation through Insig1 is of interest. A FLAG-tagged-Insig1 plasmid was constructed to facilitate Insig1's measurement due to the unavailability of a valid antibody against the bovine protein, as well as the low expression of endogenous Insig1 (not shown). MAC-T cells were transfected with FLAG-Insig1 and then treated with t10, c12-CLA for 6 h. CLA increased FLAG-Insig1 protein expression in a dose-dependent pattern and was significantly different from 0 μM at 40 μM (**Figure 3**). Further investigation showed that Insig1 protein was regulated by t10, c12-CLA as well as by other polyunsaturated fatty acid treatments (**Figure 4A**). C9, t11-CLA and linoleic acid (c9, c12 18:2) also increased FLAG-Insig1 protein expression but linoleic acid to a lesser extent compared with t10, c12-CLA treatment (**Figure 4B**). In contrast, palmitic acid and oleic acid reduced or did not affect FLAG-Insig1 expression compared with control, respectively (**Figure 4B**).

t10, c12-CLA reduced Insig1 degradation

To determine whether the increase of FLAG-Insig1 protein expression was due to upregulation of protein synthesis or decreased protein degradation, cycloheximide (**CHX**), a protein synthesis inhibitor, and MG132, a proteasomal degradation inhibitor, along with t10, c12-CLA were applied to measure FLAG-Insig1 expression. Without new Insig1 protein being synthesized, t10, c12-CLA treatment significantly slowed FLAG-Insig1 decay rate after CHX addition (**Figure 5**). After 1.5 h and 3 h CHX incubation, 85 % and 67 % of proteins remained in the CLA treatment group compared to 59 % and 44 % of the CHX-only group ($P < 0.05$). The

CLA effect diminished to less than 20 % at 4.5 h of CHX incubation. On the other hand, MAC-T cells treated with MG132 for 6 h effectively blocked FLAG-Insig1 degradation. Cells treated with t10, c12-CLA also achieved the positive effect but an even higher protein expression of FLAG-Insig1 (**Figure 6**). Cells treated with combination of MG132 and t10, c12-CLA for the same period, failed to produce an additive effect of further increase in FLAG-Insig1 expression.

t10, c12-CLA reduced interaction between Insig1 and Ubxd8

Next the possible mechanism of CLA blocking Insig1 degradation was investigated using immunoprecipitation (**IP**). A second expression vector coding for Myc-tagged-Ubxd8, which is part of protein complex required to remove Insig1 from ER, along with FLAG-Insig1, were co-transfected with MAC-T cells for 24 h. Then the cells were treated with either 75 μM t10, c12-CLA or 75 μM palmitic acid for another 6 h before IP with anti-Myc antibody. The interaction between Ubxd8 and Insig1 was confirmed through detection of FLAG-Insig1 in the precipitates that were brought down with anti-Myc (**Figure 7, Lane 1)**. When cells treated with 75 μM t10, c12-CLA, less FLAG-Insig1 was detected compared with control with no fatty acid added (**Figure 7, Lane 1 and 4**), while cells treated with palmitic acid (**PA**) resulted in higher FLAG-insig1 expression compared with control and CLA (**Figure 7, Lane 1, 4 and 5**).

DISCUSSION

Milk fat depression in dairy cows occurs in response to dietary factors that influence biohydrogenation in the rumen and results in changes in fatty acid intermediates including CLA. Administering t10, c12-CLA to dairy cows can cause MFD and can be used to examine mechanisms regulating milk fat synthesis *in vivo* and *in vitro*. In this study, we investigated the

molecular mechanism of t10, c12-CLA on bovine SREBP1 activation through Insig1. It is the first to measure the effect of t10, c12-CLA on Insig1 degradation through proteasomal degradation and immunoprecipitation assays in bovine mammary epithelial cells. Both the precursor and mature forms of SREBP1 protein decreased significantly (>50 %) in response to t10, c12-CLA treatment in a brief incubation of 6 h. Peterson et al (2004) reported that no reduction of pSREBP1 while mSREBP1 was significantly reduced when mammary epithelial cells were treated with 75 μM t10, c12-CLA for 48 h. The discrepancy on pSREBP1 is likely due to the antibody sensitivity and different incubation time. The customized antibody we used to measure SREBP1 detects total SREBP1. It should be noted that there are two SREBP1 isoforms. SREBP1a is predominantly expressed in cultured cells while SREBP1c is mainly found in tissues especially liver of rodents (Shimomura et al., 1997). Furthermore, SREBP1a is largely regulated in the protein cleavage process while SREBP1c is regulated at the transcriptional level (Eberlé et al., 2004). The reduction of mSREBP1 was consistent when treated with a relatively high concentration of 75 μM of t10, c12-CLA. We also observed that both SREBP1 proteins declined at different rates in response to t10, c12-CLA incubation. One possible explanation for the differential decline is that PUFAs blocked SREBP1 proteolytic processing, which resulted in less mSREBP1 being produced and lowered lipogenic gene transcription, including SREBP1 mRNA that in turn reduced SREBP1 protein synthesis (Takeuchi et al., 2010). In another study using MAC-T cells and treating with t10, c12-CLA for different lengths of time, the decline of SREBP1 mRNA expression was first observed at 3 h, continued to decline and reached nadir at 12 h, and decreased no further beyond 12 h (Kadegowda et al., 2009). The 6 h time point selected in the present study was within the ideal incubation window for an effective CLA response. SREBP1 activation responded to CLA treatment in a dose-dependent pattern in this

study agreeing with a previous study in MAC-T cells that used t10, c12-CLA at a lower range (0-300 nM) and for a longer period (24 h) (Shi et al., 2020). Cells were starved for 12 h prior to CLA treatment, as a lipid deprivation step, which might increase SREBP1 sensitivity to CLA treatments (Chen et al., 2012).

SREBP1 regulates its target genes, mainly involved in lipogenesis, by binding to their sterol response elements located in the promoter regions, such as acetyl-CoA carboxylase (**ACC**), fatty acid synthase (**FASN**), and SREBP1, and activates their transcription (Shimano, 2001; Seo et al., 2009). The downregulation of lipogenic genes by t10, c12-CLA has been well characterized in cows (Peterson et al., 2003) as well as cultured cells (Wang et al., 2014). The results from current study were consistent with previous reports that mRNA expression of SREBP1, FASN and SCD1 responded to CLA and started declining at a lower dose of 40 μM after 24 h incubation. Particularly, the mRNA expression of SREBP1 repressor Insig1 responded dose-dependently to CLA treatments, different from Insig2, another SREBP1 regulator, which appeared insensitive to CLA treatment (Harvatine and Bauman, 2006). Yabe et al. (2002) showed that human Insig1 and 2 possessed 59 % identical sequence, while higher expression and affinity to SCAP were observed with Insig1. Insig1 required mSREBP1 for its gene expression but Insig2 mRNA expression was unaffected by SREBP1 (Yabe et al., 2002). Furthermore, Insig2, degraded slowly and appeared uninfluenced by sterol regulation (Ikeda et al., 2009). These discrepancies between Insig1 and Insig2 possibly indicated complimentary regulation to cholesterol synthesis in the human body, which might indicate a similar mechanism in regulating fatty acid synthesis in bovine mammary epithelial cells. A recent study using goat mammary epithelial cells showed that overexpression of Insig1 and 2 reduced total triacylglycerol and lipogenic gene expression while knockdown of Insig1 and Insig2 increased SREBP1 mRNA

316 expression (Li et al., 2019). It is not clear whether Insig1 and 2 work synergistically in the
317 presence of CLA in bovine mammary epithelial cells. The exact relationship between bovine
318 Insig1 and 2 and fatty acid metabolism would benefit from further investigation.
319 Insig1 protein, in contrast to its mRNA expression, increased dose-dependently in
320 response to t10, c12-CLA treatment. This result was in line with repletion of sterol increasing
321 Insig1 in sterol deficient medium in CHO-7 cells (Gong et al., 2006). Insig1 protein expression
322 was not inhibited by t10, c12-CLA at a low level (20 μM), which might explain the increase in
323 its mRNA expression at the same concentration of CLA while remaining mSREBP1 continued to
324 stimulate Insig1 mRNA expression. However, with further increased t10, c12-CLA
325 concentration, Insig1 protein decreased accordingly, consistent with the reciprocal change
326 previously observed in CLA-induced SREBP1 inhibition. Further investigation found that
327 supplementing with 75 μM t10, c12-CLA, or other polyunsaturated fatty acids (**PUFAs**)
328 including c9, t11-CLA, increased Insig1 protein expression compared to no fatty acid
329 supplement, oleic acid (C18:1), or palmitic acid. The higher 75 μM dose was chosen to represent
330 the maximal MFD induced by t10, c12-CLA in cow studies. This finding is consistent with
331 previous studies in CHO-7 cells that Insig1 protein expression responded to various PUFAs but
332 not saturated fatty acids (Joon et al., 2008). Similar effect was seen in PUFAs inhibition of
333 SREBP1 activation where polyunsaturated fatty acids decreased mSREBP1 expression (Hannah
334 et al., 2001). Considering the regulatory effect of Insig1 on SREBP1 activation, we could argue
335 that PUFAs block SREBP1 activation through increased Insig1 protein. However, this response
336 to various fatty acids on Insig1 protein was at odds with the concept of t10, c12-CLA specific
337 induced-downregulation of lipogenic gene transcription, given c9, t11-CLA failed to elicit a
338 similar inhibitory effect on mRNA expression of fatty acid synthesis genes (Peterson et al., 2004)

or cause MFD in cows (Baumgard et al., 2000). This result from the current study would also provide support that challenges the notion of the trans10 shift in ruminal biohydrogenation as an indicator of MFD. In one meta-analysis study that evaluated MFD in dairy ewes, different responses in milk fat yield resulted in similar trans10 shifts (Toral et al., 2020), and the pattern of trans C18:1 isomers, rather than the content of any specific isomer, better explained the alternative biohydrogenation pathway that leads to MFD (Conte et al., 2018). Considering the evidence from the current study, Insig1 is less specific, or more sensitive to the inhibitory effect of CLA, and reaffirmed that the inhibitory effect on SREBP1 activation started from PUFAs and not oleic acid or palmitic acid. Since there are multiple steps involved in the process of SREBP1 activation, it is possible that some regulatory pathways are specifically induced by t10, c12-CLA, such as altering proteasome activity (Shi et al., 2020), but not other fatty acids that lead to collective inhibition effect on SREBP1. It is worth noting that even oleic acid failed to change Insig1 protein expression but palmitic acid reduced Insig1 which might help to explain why palmitic acid supplementation can increase milk fat yield (Rico et al., 2014). More palmitic acid in the mammary gland might have lowered expression of Insig1 thus removing the suppression on SREBP1 activation which promoted milk fat synthesis.

Migration of the SCAP-SREBP1 complex to the GA requires separation from Insig1 protein-induced retention in the ER (Yang et al., 2002). For separation to occur, Insig1 must first be ubiquitinated by gp78, an ER membrane resident ubiquitin ligase (Joon et al., 2006). In the presence of sterols, SCAP replaced gp78 and bound to Insig1, and thus prevented Insig1 from being ubiquitinated (Song et al., 2005). Depletion of sterols triggered, ubiquitinated Insig1 to bind to Ubxd8, which recruited VCP resulting in Insig1 extraction from the ER membrane for proteasomal degradation (Lee et al., 2010). In current study, CLA-induced elevated Insig1

protein expression was assessed through two independent experiments to determine if the increased Insig1 was due to accelerated protein synthesis or decreased protein degradation. T10, c12-CLA slowed FLAG-Insig1 protein degradation when further protein synthesis ceased. This result is consistent with findings from CHO-7 cells treated with arachidonate that exhibited stabilized Insig1 (Joon et al., 2008). On the other hand, CLA exerted an MG132-like proteasomal degradation inhibition effect on FLAG-Insig1. This is also similar to sterol-dependent Insig1 degradation on controlling cholesterol synthesis (Gong et al., 2006). Taken together, the decay rate of Insig1 protein was reduced in response to t10, c12-CLA treatment. CLA specifically inhibited the removal of Insig1 protein from ER membrane and degradation, thus Insig1 protein levels increased with t10, c12-CLA treatment.

Ubxd8 could function as the sensor for unsaturated fatty acids and in their absence, remove Insig1 proteins from the ER membrane with VCP (Lee et al., 2010). In the current study, interaction between Ubxd8 and Insig1 was disrupted by t10, c12-CLA treatment, while palmitic acid appeared to strengthen the association. This result also supported the previous observation of differential effects of fatty acids on Insig1 protein expression where t10, c12-CLA, among other PUFAs, effectively increased FLAG-Insig1 protein expression, while palmitic acid suppressed FLAG-Insig1 protein expression and palmitic acid promoted the association of Ubxd8 with Insig1, thus facilitating Insig1 degradation. This study is the first to confirm the interaction between Ubxd8 and Insig1 in bovine mammary epithelial cells and the inhibitory effect of t10, c12-CLA on blocking the interaction. However, it is not clear how CLA affected the Insig1-Ubxd8 interaction where further investigation on direct fatty acid-protein interaction is warranted.

In this study, we reported that the effect of t10, c12-CLA on bovine SREBP1 activation is through inhibition of Insig1 degradation in a dose dependent pattern using MAC-T cells. Furthermore, we confirmed that the interaction between Insig1 and Ubxd8 can be interrupted by t10, c12-CLA. Retention of Insig1 protein continued to withhold SREBP1 from further processing resulting in a reciprocal reduction of immature and mature SREBP1 protein expression, in response to the increased t10, c12-CLA treatment. The present study also showed that Insig1 protein expression is less responsive to various PUFAs including c9, t11-CLA, while Insig1 was not responsive to MUFA (oleic acid) or inhibited by palmitic acid.

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Table 1 Primer sequences for transcripts used in real-time quantitative PCR

Transcript	Accession number	Primers (5' to 3')	
SREBP1	NM_001113302	Forward	atgccatcgagaaacgctac
		Reverse	gtccgcagactcaggttctc
SCD1	NM_173959	Forward	ccctttccttgagctgtctg
		Reverse	atgctgactctctcccctga
FASN	NM_001012669	Forward	ctgcaactcaacgggaactt
		Reverse	aggctggtcatgttctccag
Insig1	NM_001077909	Forward	gtcatcgccaccatcttctc
		Reverse	agtggaacctctcggtgtgtt
Insig2	XM_003581843	Forward	tccagtgtgatgcggtgtgta
		Reverse	agtgtgaccgacgtgatagtt
EIF3k	NM_001034489	Forward	gcgatgtttgagcagatgag
		Reverse	gcattttctttggcctgtgt
B2M	NM_173893	Forward	tgctgaagaatcgggagaag
		Reverse	ccttgctgttgggagtgaa
RPS15	XM_585783	Forward	ctctgtgcattcgggttttc
		Reverse	gggctctctgggttcctct

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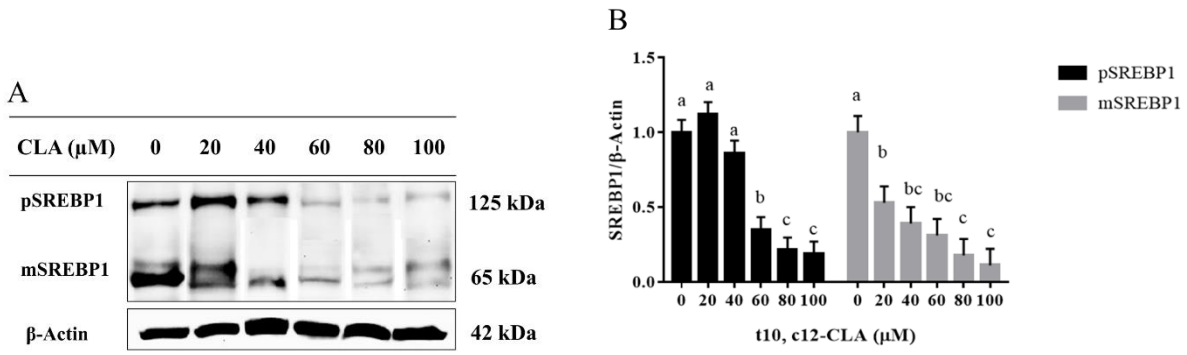

Figure 1. t10, c12-CLA reduced SREBP1 protein expression. Bovine epithelial cells (MAC-T) were treated with t10, c12-CLA at 0, 20, 40, 60, 80 and 100 μM for 6 h. Immunoblots were visualized and quantified using Quality One software (Bio-Rad) (A) Representative of pSREBP1 (125 kDa) and mSREBP1 (65 kDa) of 3 independent experiments with similar results. (B) Relative protein abundance of pSREBP1 and mSREBP1 normalized to β-actin. Data are expressed as mean ± SEM, n=3. Differences were considered significant when $P < 0.05$ and denoted by means lacking a common superscript (a-c).

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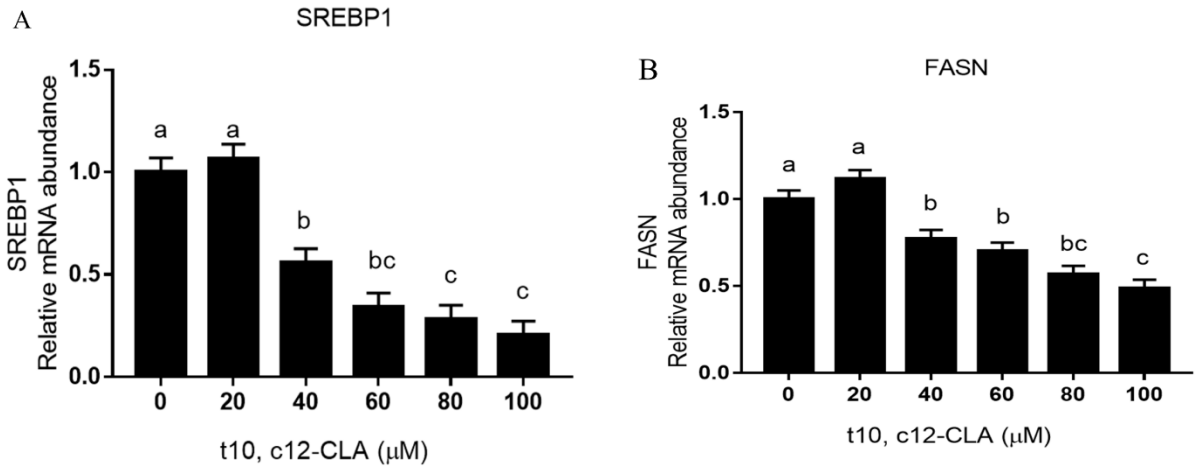

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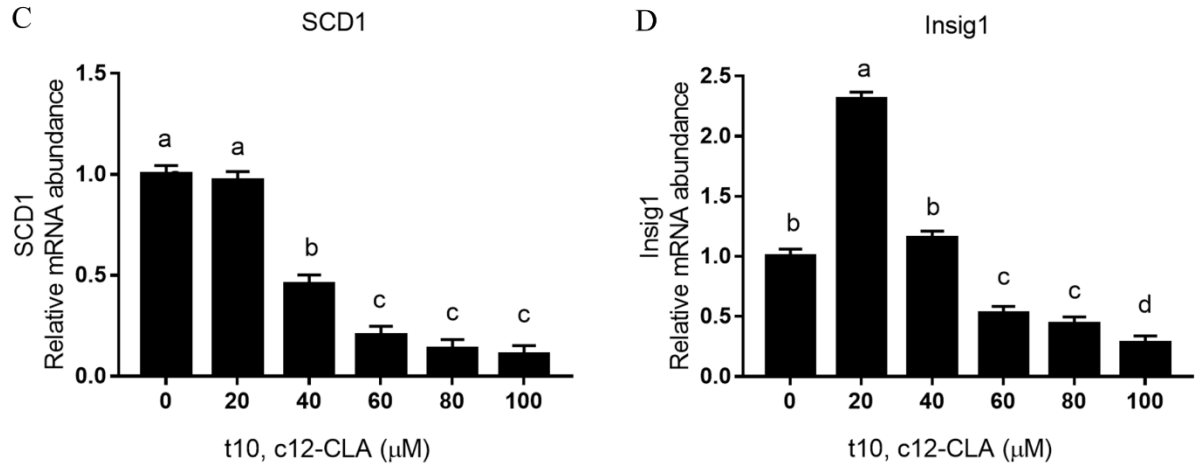

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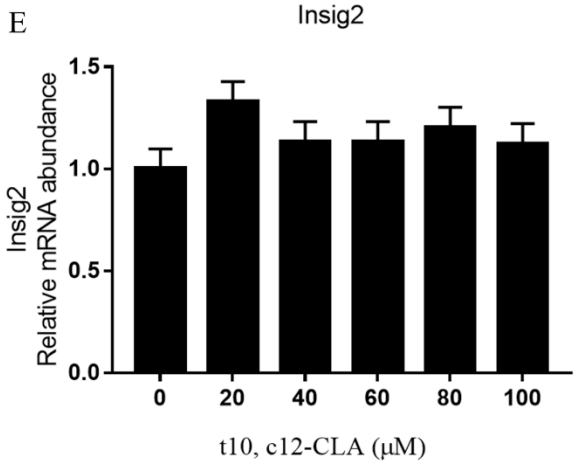

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Figure 2. t10, c12-CLA reduced lipogenic gene expression. Bovine epithelial cells (MAC-T) were treated with t10, c12-CLA at 0, 20, 40, 60, 80 and 100 μM for 24 h. Fold changes of mRNA abundance were measured by qPCR and normalized to 3 endogenous control genes B2M, RPS15 and EIF3K using $2^{-\triangle\triangle Ct}$ method. Values are expressed as mean ± SEM, n=3. Differences were considered significant when $P < 0.05$ and denoted by means lacking a common superscript (a-d). (A) SREBP1; (B) FASN; (C) SCD1; (D) Insig1; (E) Insig2.

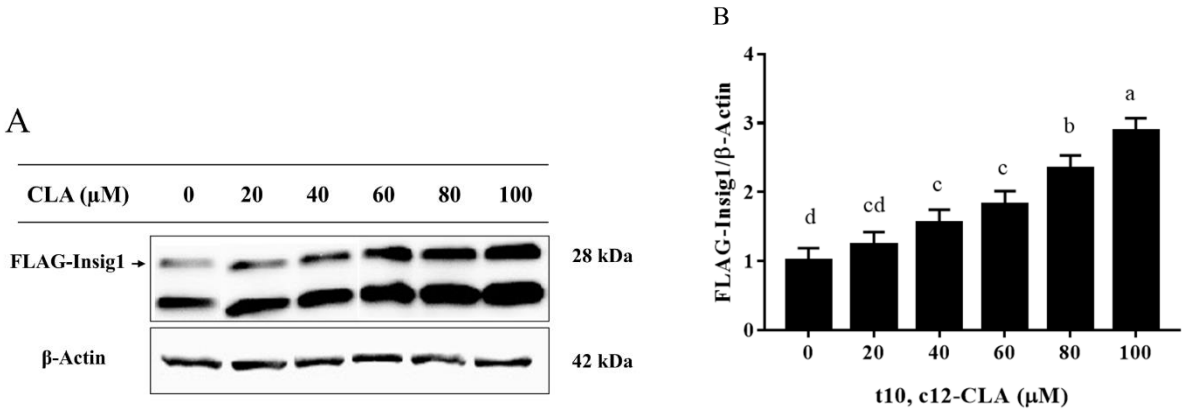

Figure 3. t10, c12-CLA increased Insig1 protein expression. Bovine epithelial cells (MAC-T) were transfected with pCMV-FLAG-Insig1 for 24 h, then incubated with t10, c12-CLA at 0, 20, 40, 60, 80 and 100 μM for another 6 h. Immunoblots were visualized and quantified using Quality One software (Bio-Rad) (A) Representative of immunoblots of FLAG-Insig1 (28 kDa) of 3 independent experiments with similar results. (B) Data represent mean FLAG-Insig1 protein abundance normalized to β-Actin. Data are expressed as mean ± SEM, n=3. Differences were considered significant when $P < 0.05$ and denoted by means lacking a common superscript (a-d).

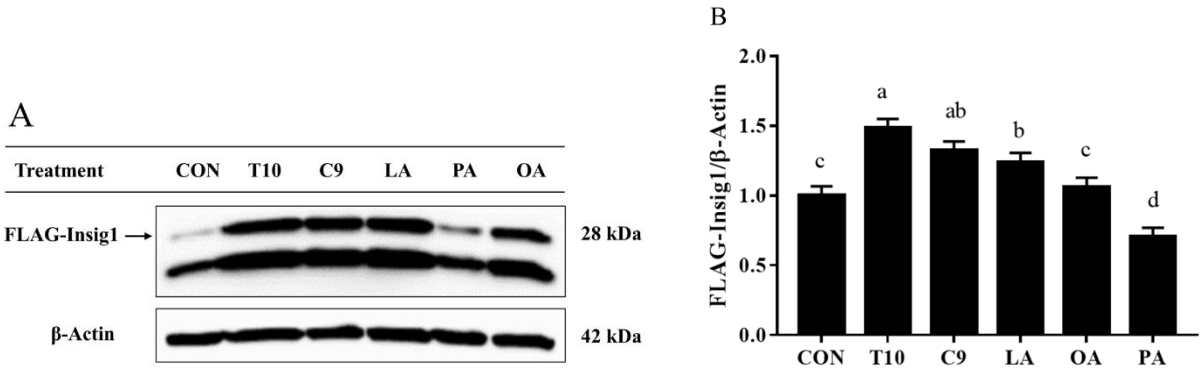

Figure 4. Polyunsaturated fatty acids increased Insig1 protein expression. Bovine epithelial cells (MAC-T) were transfected with pCMV-FLAG-Insig1 for 24 h, then incubated with 75 μM of the indicated fatty acids for 6 h. Immunoblots were visualized and quantified using Quality One software (Bio-Rad) (A) Representative of immunoblots of FLAG-Insig1 (28 kDa) of 3 independent experiments with similar results. (B) Data represent mean FLAG-Insig1 protein abundance normalized to β-Actin. Data are expressed as mean ± SEM, n=3. Differences were considered significant when $P < 0.05$ and denoted by means lacking a common superscript (a-d). **CON**: BSA, **T10**: t10, c12-CLA, **C9**: c9, t11-CLA, **LA**: linoleic acid, **OA**: oleic acid, **PA**: palmitic acid.

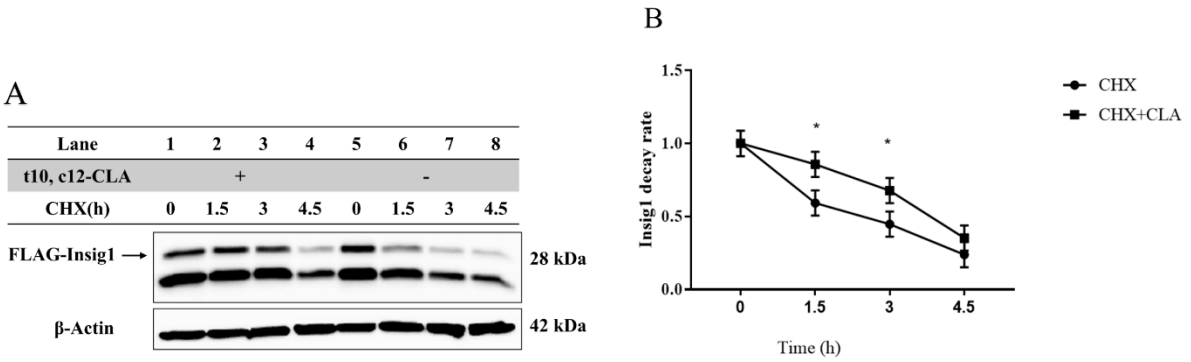

Figure 5. t10, c12-CLA reduced Insig1 degradation. Bovine epithelial cells (MAC-T) were transfected with pCMV-FLAG-Insig1 for 24 h. Cells were continued to incubate with 75 μM CLA for 6 h then treated with or without cycloheximide (CHX) for another 4.5 h. Cells were harvested at 0, 1.5, 3 and 4.5 h after CHX addition. Immunoblots were visualized and quantified using Quality One software (Bio-Rad) (A) Representative of immunoblots of FLAG-Insig1 (28 kDa) of 3 independent experiments with similar results. (B) Data represent mean FLAG-Insig1 protein abundance normalized to β-Actin. Data are expressed as mean ± SEM, n=3. Differences were considered significant when $P < 0.05$ and denoted with *.

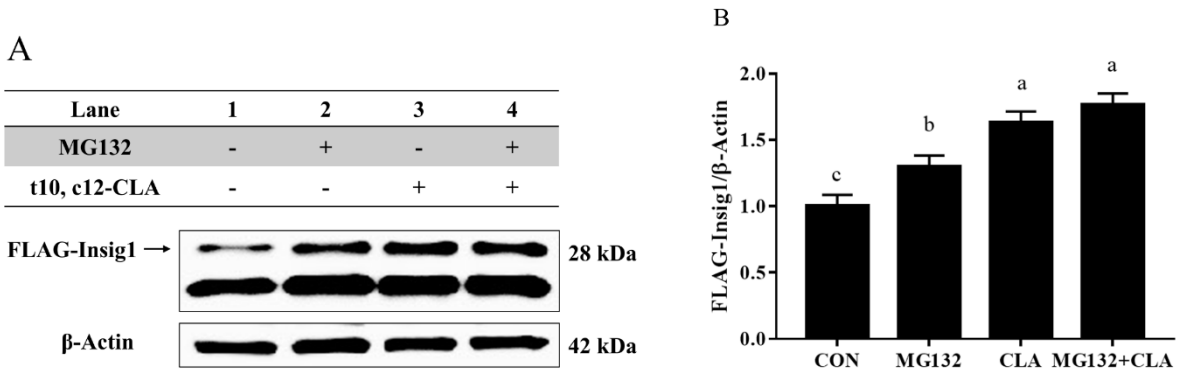

Figure 6. t10, c12-CLA blocked Insig1 degradation. Bovine epithelial cells (MAC-T) were transfected with pCMV-FLAG-Insig1 for 24 h. Cells were continued to incubate with either 10 μM MG132 or 75 μM t10, c12-CLA (CLA) or both for 6 h. Immunoblots were visualized and quantified using Quality One software (Bio-Rad) (A) Representative of immunoblots of FLAG-Insig1 (28 kDa) of 3 independent experiments with similar results. (B) Data represent mean FLAG-Insig1 protein abundance normalized to β-Actin. Data are expressed as mean ± SEM, n=3. Differences were considered significant when $P < 0.05$ and denoted by means lacking a common superscript (a-c).

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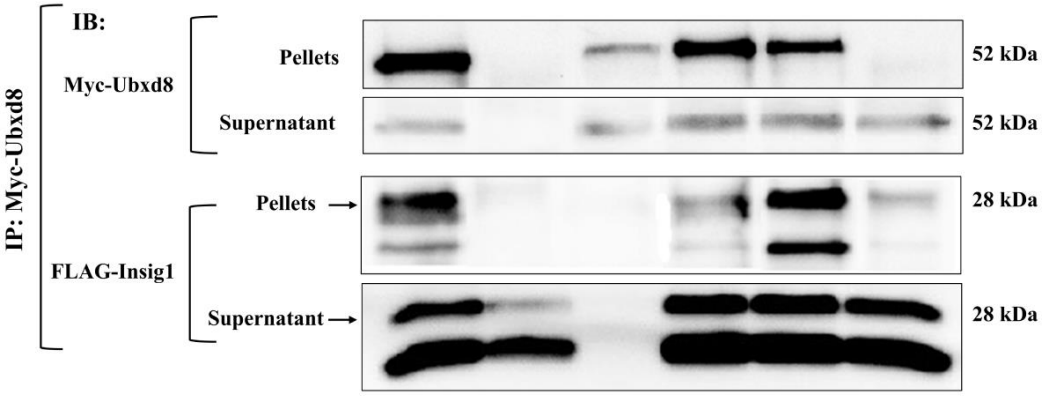
Lane	1	2	3	4	5	6
FLAG-Insig1	+	+	-	+	+	+
Myc-Ubxd8	+	-	+	+	+	+
CLA	-	-	-	+	-	-
PA	-	-	-	-	+	-
IgG	-	-	-	-	-	+



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600 **Figure 7.** t10, c12-CLA reduced interaction between Insig1 and Ubxd8. Bovine epithelial cells
601 (MAC-T) were transfected with either 5 µg of Myc-Ubxd8 or 5 µg of FLAG-Insig1 or both for
602 24 h. Cells were continued to incubate with either 75 µM t10, c12-CLA or 75 µM palmitic acid
603 for 6 h. Total protein lysates were subjected to immunoprecipitation with rabbit anti-Myc
604 antibody to precipitate transfected Myc-Ubxd8. The pellets along with 30 µg of supernatant
605 (input), were analyzed by immunoblotting with mouse anti-FLAG and mouse anti-Myc
606 antibodies. Lane 1: no fatty acid control, co-transfection with FLAG-Insig1 and Myc-Ubxd8.
607 Lane 2: single transfection control, only transfected with FLAG-Insig1. Lane 3: single
608 transfection control, only transfected with Myc-Ubxd8. Lane 4: t10, c12-CLA treatment, co-
609 transfection with FLAG-Insig1 and Myc-Ubxd8. Lane 5: Palmitic acid treatment, co-transfection
610 with FLAG-Insig1 and Myc-Ubxd8. Lane 6: IgG control, co-transfection with FLAG-Insig1 and
611 Myc-Ubxd8.

612