



Differential Action of trans-10, cis-12 Conjugated Linoleic Acid on Adipocyte Differentiation of Ovine and 3T3-L1 Preadipocytes*

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ABSTRACT : Trans-10, cis-12 conjugated linoleic acid (CLA) has been reported to inhibit the adipocyte differentiation of preadipocytes in non-ruminant animals (mice, rat, and human). However, the effects of trans-10, cis-12 CLA have not been clear in ruminants. The objective of this study was to investigate the effects of trans-10, cis-12 CLA on adipocyte differentiation of ovine preadipocytes. Differentiation of these preadipocytes was facilitated by treatment with trans-10, cis-12 CLA. Trans-10, cis-12 CLA increased the number and size of oil red O-stainable lipid drops as well as the levels of GPDH activity. PPAR- γ 2 and adipophilin mRNA, adipogenic marker genes, were increased by treatment with trans-10, cis-12 CLA. This result was different from that observed with 3T3-L1 preadipocytes, a clonal cell line derived from rodents. Furthermore, trans-10, cis-12 CLA alone induced the adipocyte differentiation of ovine preadipocytes in differentiation-induction medium without troglitazone. These results suggest that CLA is an inducer and regulator in adipocyte differentiation of ovine preadipocytes, with species differences between ovine and rodent preadipocytes. (**Key Words** : trans-10, cis-12 CLA, Adipocyte, Sheep)

INTRODUCTION

Dietary products and meat from ruminant animals are important sources of energy, nutrients, and a variety of vitamins. Ruminant products such as milk, cheese, and beef contain some fat, with these fats being composed of many different types of fat and fatty acids. One of these fatty acids is conjugated linoleic acid (CLA) (Grinari et al., 2000; Daniel et al., 2004). CLA is produced as a result of bacterial biohydrogenation of lipids in the ruminant gut (Mir et al., 2004).

CLAs are a group of polyunsaturated fatty acids. The predominant isomers of CLA are cis-9, trans-11 and trans-10, cis-12 (McDonald, 2000; Mir et al., 2004). CLA has been studied because of its potentially beneficial effects on carcinogenesis (Ha et al., 1990; Liew et al., 1995), diabetes,

atherosclerosis (Lee et al., 1994), immune modulation (Cook et al., 1993; Miller et al., 1994), and body composition (McDonald, 2000; Wang and Jones, 2004). These effects appear to be mediated primarily by trans-10, cis-12 CLA and also cis-9, trans-11 CLA. Previous studies have demonstrated that CLA has anti-obesity effects in several species (Poirier et al., 2005). A great deal of attention has been created regarding oral administration of CLA due to its reported decreasing effect on fat deposition in animal models (Sisk et al., 2001; Dunshea et al., 2002; Wang and Jones, 2004; Takahashi et al., 2007; Zhou, 2008) and some humans (Brown and McIntosh, 2003). This fat deposition results from trans-10, cis-12 CLA and not from cis-9, trans-11 CLA. *In vitro* studies show that trans-10, cis-12 CLA inhibits preadipocyte differentiation and causes delipidation of newly differentiated adipocytes in 3T3-L1 cells (Brodie et al., 1999) and human preadipocytes (Brown et al., 2004; Chung et al., 2005a). This CLA-isomer specific effect is considered to largely be due to decreased glucose and fatty acid uptake and triglyceride synthesis as opposed to increased oxidation. The analysis of adipogenic gene expression in 3T3-L1 cells and human preadipocytes upon treatment of predominantly trans-10, cis-12 CLA showed a decrement of PPAR- γ 2 (peroxisome proliferator activated receptors- γ 2) gene expression (Granlund et al., 2003). In addition, CLA treatment decreased not only the

* This work was partly supported by Grant-in-Aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan.

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Received August 26, 2008; Accepted February 7, 2009

expression of PPAR- γ 2 mRNA but also the several mRNA levels that are involved in adipocyte differentiation, including CCAAT/enhancer-binding protein- α (C/EBP- α), sterol regulated element binding protein 1-c (SREBP-1c), and PPAR- γ 2 target genes such as fatty acid binding protein (aP2), perilipin-A (Chung et al., 2005b), lipoprotein lipase (LPL), and adiponectin (Brown and McIntosh, 2003; House et al., 2005).

However, the CLA effects on adipocyte differentiation of preadipocytes in ruminant are little understood. The objective of the present study was to clarify the isomer-specific effects of CLA on adipocyte differentiation of ovine preadipocytes. The study presented here shows that CLA stimulates the adipocyte differentiation of ovine preadipocytes.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Nutrient Mixture F-12 [HAM], bovine serum albumin (Fraction V, BSA), insulin (porcine insulin), dexamethasone (DEX), a lipid mixture (1,000 \times), and trypsin-EDTA solution were purchased from Sigma Chemicals Co., (St. Louis, MO). Fetal bovine serum (FBS) was from Equitech-Bio, Inc. (Kerrville, TX). Collagenase (Type I) was purchased from Worthington Biochemicals Corp. (Freehold, NJ). Triiodothyronine (T_3) and antibiotic-antimycotic mixed stock solution were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Troglitazone was from EMD Biocem, Inc. (San Diego, CA). CLA was from Cayman Chemicals (MI, USA).

Animals

Adult female Suffolk sheep weighing 45-82 kg were used. They were housed in individual cages, and fed with 800 g of hay cube and 200 g of concentrate once daily at 0800 h. The sheep were allowed free access to water. On the day of the experiment, animals were not fed until the sampling was completed. All experiments were conducted in accordance with the Shinshu University Guide for the Care and Use of Experimental Animals.

Isolation of ovine preadipocytes

Tissue was minced and subjected to collagenase digestion in Krebs-Ringer bicarbonate buffer containing 0.5 mg/ml collagenase, 1.1 mM glucose, 4% BSA, 10 mM HEPES (pH 7.4), and 2 mM sodium acetate for 1-1.5 h at 39.5°C with 100 cycle/min agitation. The digested tissue was filtered through a nylon mesh screen (250 μ m) to separate cells from undigested tissue fragments and blood vessels. The remaining suspension was centrifuged at 3,500 rpm for 5 min with KRB buffer containing 1% BSA at room

temperature. The pellet (stromal-vascular cells; S-V cells) was suspended with erythrocyte lysis buffer (154 mM NH_4Cl , 10 mM $KHCO_3$) containing 1% BSA and incubated for 5 min at 39.5°C. The cell suspension was centrifuged at 3,500 rpm for 5 min. The pellet was washed by centrifugation at 3,500 rpm for 5 min with DMEM. The final pellet was suspended with DMEM:Ham's F12 (1:1, vol/vol) medium containing 5% FBS, 2 mM sodium acetate, and 1% antibiotic mixture and seeded into 35-mm culture dishes at a density of 2,500 cells/cm². The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 24 h, culture medium was replaced with fresh medium to remove the unattached cells and then changed every other day.

Proliferation and adipocyte differentiation of ovine preadipocytes

Preadipocytes were seeded into 35-mm culture dishes at a density of 25,000 cells/cm². Ovine preadipocytes were grown to confluence and exposed to differentiation-induction medium; serum-free DMEM:Ham's F12 (1:1, vol/vol) medium with 1% antibiotic mixture, 2 mM sodium acetate, a 200 \times lipid mixture, 2 nM T_3 , 10 nM DEX, 100 nM insulin, and 5 μ M troglitazone. The medium was replaced every other day, and the cells were allowed to differentiate for 10 days. Both isomers of conjugated linoleic acid were added in differentiation-induction medium for 10 days.

Cell culture and adipocyte differentiation of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were grown to 2 days post-confluence in DMEM with 10% FBS. The cells were induced to differentiate by changing the differentiation-induction medium; DMEM with 10% FBS, 0.5 mM methyl-3-isobutylxanthine, 0.25 μ M DEX, and 1 μ g/ml insulin. After 2 days, this medium was replaced with DMEM containing 10% FBS and insulin (1 μ g/ml) for more 2 days. The medium was therefore replaced with DMEM containing 10% FBS. This medium was changed every other day. Both isomers of CLA were added in differentiation-induction medium for 10 days.

Fatty acid preparation

Both isomers of CLA were mixed to fatty acid-free BSA at a 4:1 molar ratio using 3 mM BSA stocks.

Oil Red O staining

To visualize the presence of intracellular lipids, the cells were stained with Oil Red O. Cells were washed three times with phosphate-buffered saline (PBS) and fixed for 1 h with 10% formalin in PBS. The cells were then stained with filtered Oil Red solution (0.12% Oil Red O in 60%

Table 1. Primers used for semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Gene			Length (bp)	Cycle	T _m ¹ (°C)
Sheep	PPAR- γ 2	forward	5'-ATGGGTGAAACTCTGGGAGA-3'	419	33
		reverse	5'-TGGAACCCCTGACGCTTTATC-3'		
	adipophilin	forward	5'-GCTGCCAATCATCCAAAAGT-3'	545	30
		reverse	5'-GGCCTTTTCGCTTAGCTTCT-3'		
	β -actin	forward	5'-AGGTCATCACCAATTGGCAAC-3'	363	35
		reverse	5'-ACTCGTCATACTCCTGCTTG-3'		
Mouse	PPAR- γ 2	forward	5'-TGGGTGAAACTCTGGGAGAT-3'	454	26
		reverse	5'-CCATAGTGGAAAGCCTGATGC-3'		
	adipophilin	forward	5'-AATATGCACATGGCCAACCA-3'	475	22
		reverse	5'-CCTCAGACTGCTGAACCTTC-3'		
	β -actin	forward	5'-AGGTCATCACTATTGGCAAC-3'	363	27
		reverse	5'-ACTCATCGTACTCCTGCTTG-3'		

¹T_m: Melting temperature.

isopropanol) for at least 10 min at room temperature. Finally, the cells were washed twice with 60% isopropanol to remove undissolved stain.

Glycerol-3-phosphate dehydrogenase (GPDH) enzymatic assays

To monitor the differentiation of preadipocytes, the activity of the marker enzyme GPDH was measured. GPDH activity was analyzed using a commercial kit (GPDH activity measuring test; Hokudo, Sapporo, Japan).

RNA isolation and semi-quantitative RT-PCR

Total RNA from ovine preadipocytes and 3T3-L1 preadipocytes was isolated using Trizol Reagent (Gibco BRL, Rockville, MD). Semi-quantitative RT-PCR was performed to measure the levels of PPAR- γ 2, adipophilin, and β -actin mRNA expression as previously described (Hong et al., 2005). β -Actin, the housekeeping gene, was used as an internal control. One microgram of total RNA was reverse-transcribed to cDNA in a 20- μ l RT reaction system containing oligo dT primers and AMV-RT. The RT reaction was carried out at 42°C. One microliter of the RT products was used for subsequent PCR amplification. Primers targeted to identified clones used for semi-quantitative RT-PCR were designed to contain 20 to 22 bases and to have a melting temperature of 55 to 60°C (Table 1). Products from PCR were resolved on a 1.2% agarose gel; the DNA was visualized by ethidium bromide staining and analyzed using NIH image software, where the band intensity is expressed in pixels. Relative gene expression was calculated as the ratio of band intensity of the cloned gene to that of β -actin. Amplified cDNAs were subcloned into pGEM-T easy vector, and the nucleotide sequences were confirmed using an ABI 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis

The data in Figure 2 and 3 are presented as means \pm SEM

of 3 experiments with the same protocol. Comparisons were tested by ANOVA, followed by Fisher's test for protected least significant difference as a posthoc analysis. Significance was set at $p < 0.05$.

RESULTS

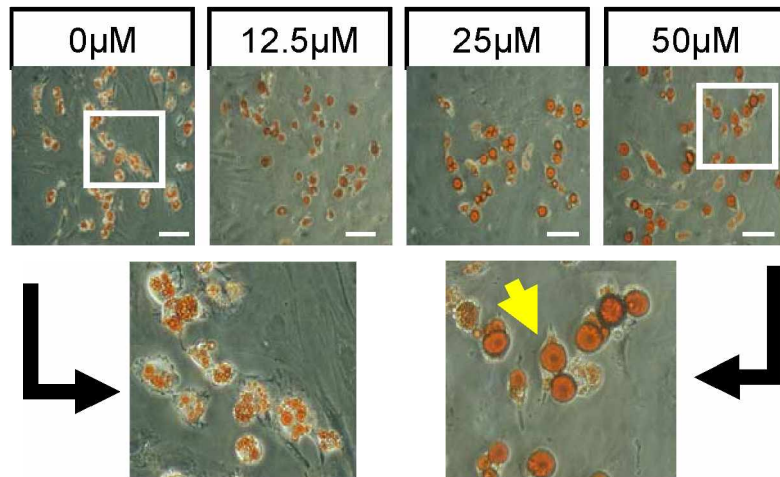
Effects of trans-10, cis-12 CLA on lipid accumulation during adipocyte differentiation of ovine preadipocytes and 3T3-L1 preadipocytes

To explore the potential effects of trans-10, cis-12 CLA during the process of adipocyte differentiation of two different preadipocytes, the cells were treated with 12.5, 25, 50 μ M trans-10, cis-12 CLA or vehicle (BSA) for 10 days in addition to standard differentiation medium. Lipid accumulation was confirmed and visualized with Oil Red O staining on day 10. Ovine preadipocytes treated with trans-10, cis-12 CLA had more differentiated adipocytes with large droplets compared with the control (Figure 1a). In addition, differentiated ovine adipocytes treated with 50 μ M trans-10, cis-12 CLA had more adipocytes with unilocular lipid droplets than the other treatments. Cis-9, trans-11 CLA had no observable effect on adipocyte differentiation of ovine preadipocytes (data not shown). However, a significant decrease in lipid drops in response to the treatments was seen in 3T3-L1 preadipocytes, as observed via microscope as depicted in Figure 1b.

Effects of trans-10, cis-12 CLA on GPDH activity during adipocyte differentiation of ovine preadipocytes and 3T3-L1 preadipocytes

GPDH activity was used as a marker of differentiation because GPDH activity is relatively high in terminally differentiated adipocytes, but is very low in undifferentiated preadipocytes. After induction of differentiation of preadipocytes, GPDH activity increased according to the degree of adipocyte differentiation. GPDH activity was analyzed at day 10 in differentiated ovine adipocytes and

(a) Ovine preadipocyte



(b) 3T3-L1 preadipocyte

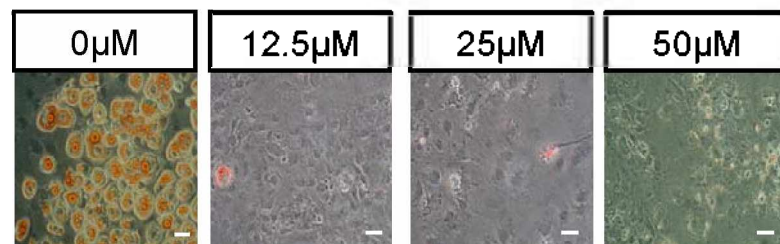


Figure 1. Effects of trans-10, cis-12 CLA on triacylglycerol accumulation during adipocyte differentiation of ovine preadipocytes and 3T3-L1 preadipocytes. Ovine preadipocytes (a) and 3T3-L1 preadipocytes (b) were differentiated with each differentiation-inducing medium, and then treated with 12.5, 25, and 50 μM trans-10, cis-12 CLA, or vehicle as control for 10 days. Differentiated ovine adipocytes and 3T3-L1 adipocytes were stained with Oil Red O to visualize lipid content on day 10 of differentiation. Scale bar = 50 μm .

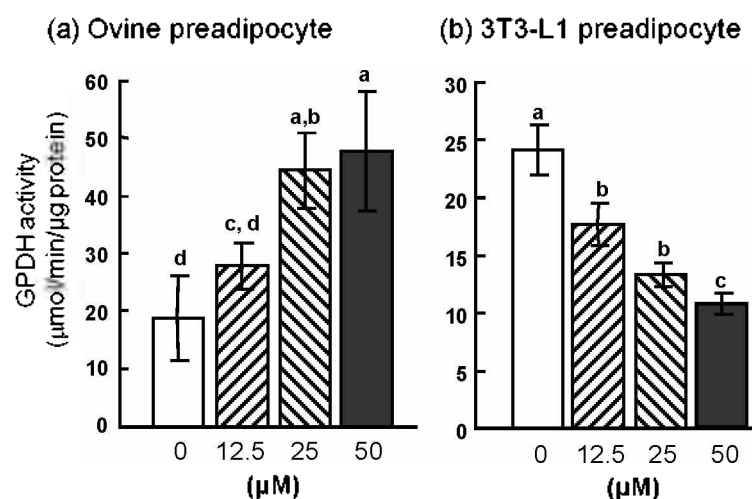


Figure 2. Effects of trans-10, cis-12 CLA on GPDH activity during adipocyte differentiation of ovine preadipocytes and 3T3-L1 preadipocytes. Ovine preadipocytes (a) and 3T3-L1 preadipocytes (b) were treated with 12.5, 25, and 50 μM trans-10, cis-12 CLA, or vehicle as control for 10 days. GPDH activity was measured on day 10 of adipocyte differentiation. The data present the mean \pm SEM of 3 separate experiments. Different letters within each treatment represent significant difference ($p < 0.05$).

3T3-L1 adipocytes treated with trans-10, cis-12 CLA. Trans-10, cis-12 CLA significantly increased GPDH activity during the adipocyte differentiation process of ovine preadipocytes ($p < 0.05$) (Figure 2a). Differentiated ovine adipocytes treated with 50 μM trans-10, cis-12 CLA increased GPDH activity by approximately 2.5 times the levels obtained with vehicle. However, GPDH activity was significantly decreased on adipocyte differentiation of 3T3-L1 preadipocytes by trans-10, cis-12 CLA treatment ($p < 0.05$) (Figure 2b).

Effects of trans-10, cis-12 CLA on the expression of adipogenic genes during adipocyte differentiation of ovine preadipocytes and 3T3-L1 preadipocytes

It is well known that the expression of several adipogenic genes is modulated depending on the degree of adipocyte differentiation. The expression of PPAR- γ 2 and adipophilin mRNA were analyzed with total RNA samples extracted from ovine and 3T3-L1 adipocytes treated with trans-10, cis-12 CLA for 7 days. Figure 3a shows that PPAR- γ 2 mRNA expression was up-regulated by trans-10, cis-12 CLA treatment in differentiated ovine adipocyte. Furthermore, the expression of adipophilin mRNA was increased in a dose-dependent manner in ovine adipocytes treated with trans-10, cis-12 CLA during differentiation. In contrast, 3T3-L1 adipocytes treated with trans-10, cis-12

CLA showed decreases in PPAR- γ 2 and adipophilin mRNA expression (Figure 3b).

Morphological changes and adipogenic gene expression in differentiated ovine adipocytes treated with trans-10, cis-12 CLA alone without troglitazone

Troglitazone, a PPAR- γ 2 agonist, is an inducer and is essential for adipocyte differentiation in primary ovine preadipocytes, as reported previously (Roh et al., 2005). As trans-10, cis-12 CLA promotes ovine adipocyte differentiation, we investigated the direct effects of trans-10, cis-12 CLA on lipid accumulation and adipogenic gene expression in ovine preadipocytes under the supplementation of differentiation medium without a PPAR- γ 2 agonist, troglitazone. Figure 4A shows that the differentiation medium with troglitazone induces adipocyte differentiation of ovine preadipocytes, and that trans-10, cis-12 CLA further stimulates this process. However, ovine preadipocytes did not show lipid accumulation with differentiation-induction medium without troglitazone and trans-10, cis-12 CLA. Ovine preadipocytes were differentiated to adipocytes in the presence of trans-10, cis-12 and showed lipid accumulation (Figure 4A).

Figure 4B shows the PPAR- γ 2 mRNA expression in differentiated ovine adipocytes treated with trans-10, cis-12 CLA and/or troglitazone. PPAR- γ 2 mRNA expression was

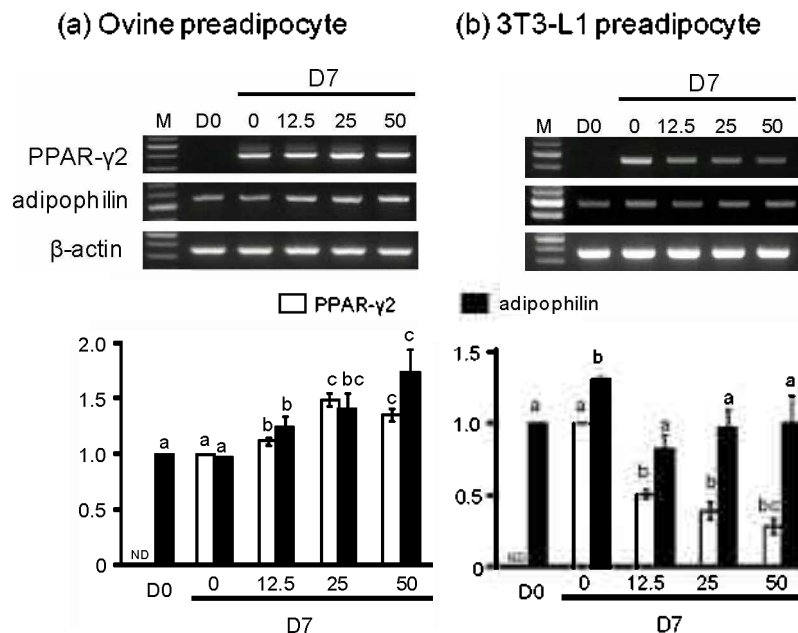


Figure 3. Effects of trans-10, cis-12 CLA on the expression of adipogenic gene expression during adipocyte differentiation of ovine preadipocytes and 3T3-L1 preadipocytes. Ovine preadipocytes (a) and 3T3-L1 preadipocytes (b) were proliferated to confluence and were then allowed to differentiate to adipocytes in differentiation medium with 12.5, 25, and 50 μM trans-10, cis-12 CLA, or vehicle as control for 7 days. After the differentiation medium was changed, total RNA was extracted from day 7 and subjected to RT-PCR analysis. Upper panel: representative ethidium bromide-stained agarose gel showing amplified PPAR- γ 2, adipophilin and β -actin with molecular marker. Lower panel: PPAR- γ 2 and adipophilin mRNA were normalized using β -actin mRNA. PPAR- γ 2 and adipophilin was expressed relative to vehicle at day 7 and at day 0, respectively. The data represent means \pm SEM of three independent experiments.

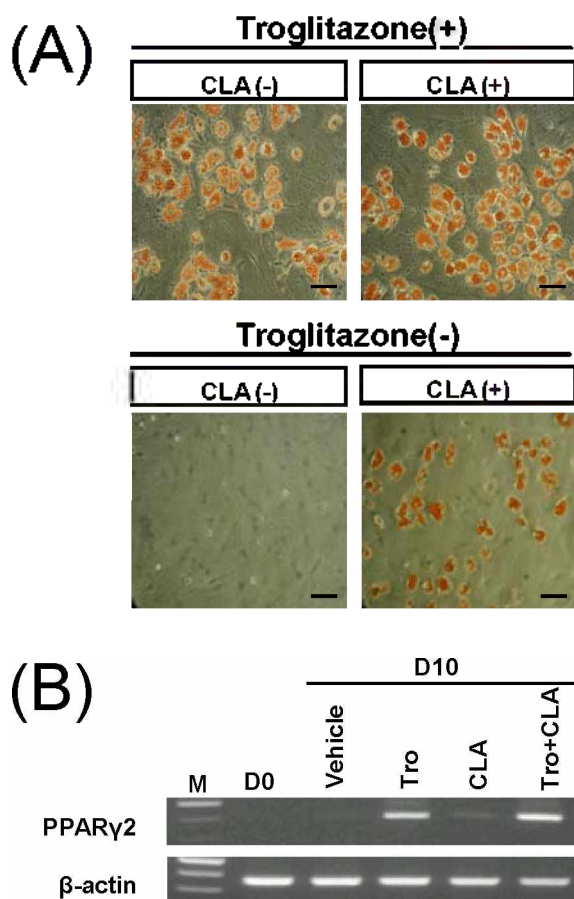


Figure 4. (A) Morphological changes in differentiated ovine adipocytes treated with trans-10, cis-12 CLA and/or troglitazone. Ovine preadipocytes were proliferated to confluence and were then allowed to differentiate to adipocytes in differentiation medium with trans-10, cis-12 CLA (50 μ M) and troglitazone (5 μ M) for 10 days. Differentiated ovine adipocytes and 3T3-L1 adipocytes were stained with Oil Red O to visualize lipid content on day 10 of differentiation. Scale bar = 50 μ m. (B) Effects of trans-10, cis-12 CLA on PPAR- γ 2 mRNA expression during the adipocyte differentiation of ovine preadipocytes. Ovine preadipocytes were proliferated to confluence and were then allowed to differentiate to adipocytes in differentiation medium with/without trans-10, cis-12 CLA (50 μ M) and troglitazone (5 μ M) for 10 days. Representative ethidium bromide-stained agarose gel showing amplified PPAR- γ 2 and β -actin with molecular markers.

induced by trans-10, cis-12 CLA alone at day 10. However, troglitazone alone elevated the expression of PPAR- γ 2 genes more than trans-10, cis-12 CLA. Moreover, treatment with both troglitazone and trans-10, cis-12 CLA further increased the PPAR- γ 2 gene expression, which was not detected with treatment of vehicle.

DISCUSSION

This study first demonstrated that trans-10, cis-12 CLA,

not cis-9, trans-11 CLA, stimulates the adipogenic process and increases the expression of PPAR- γ 2 mRNA in ovine adipocytes. In contrast, trans-10, cis-12 CLA inhibited the adipocyte differentiation of 3T3-L1 cells. The results regarding the expression of some adipogenic genes from ovine preadipocytes reported herein were in contrast to those obtained with 3T3-L1 cells. The divergent results between ovine preadipocytes and 3T3-L1 cells may result from a species-specific response. CLA inhibited leptin gene expression and secretion and adiponectin secretion *in vitro* in primary rat adipocytes as non-ruminant animals (Perez-Matute et al., 2007). There are great differences between ruminant and non-ruminant animals with regard to lipid metabolism in adipocytes (Sasaki, 2002; Roh et al., 2006). A typical example is the energy sources of these animals: VFA is a ruminant animal energy source, while glucose is a non-ruminant animal energy source. In the progression of adipogenesis, ruminants synthesize lipids primarily from acetic acid and in part from lactate, or from hydrolyzed plasma triacylglycerols (TG) by lipoprotein lipase (LPL) (Bonnet et al., 2000; Chilliard et al., 2000). Ovine adipocytes exhibit insulin resistance due to the lower activity of insulin signal transduction. The reduction of glucose transporter 4 (GLUT4) translocation that is stimulated by insulin has been observed in ovine adipocytes (Sasaki, 2002).

Facilitation of lipid accumulation and GPDH activity are related to the up-regulation of PPAR- γ 2 mRNA expression in ovine preadipocytes in the dose-dependent manner of trans-10, cis-12 CLA. The adipogenesis and induction of the metabolic pathway related to lipid metabolism are led by the sequential expression of C/EBP β , PPAR- γ 2 and C/EBP α , resulting in transactivation of adipogenic genes (Rosen and Spiegelman, 2000; Valet et al., 2002; Feve, 2005). PPAR- γ 2 is known as a master regulator of adipogenesis because of its ability to transactivate adipogenic target genes such as aP2, GLUT4, fatty acid synthase, LPL, acetyl-CoA carboxylase, and steroyl-CoA desaturase (van Bilsen et al., 2002; Blanquart et al., 2003). Additionally, SREBP-1c, a member of the SREBP family (SREBP-1a, -1c, and -2) plays an important role associated with fatty acid synthesis in adipose tissue. SREBP-1c activates transcription of more than 30 genes involved in cholesterol, fatty acid, TG and phospholipid synthesis in adipose tissue (Feve, 2005). Our results showed that the differentiated ovine preadipocytes treated with trans-10, cis-12 CLA had bigger droplets than the control at day 10. This lipid-filling effect on ovine preadipocytes during adipogenesis might be due to this SREBP-1c activation effect on fatty acid synthesis. In addition, the expression of adipophilin mRNA was increased by treatment of trans-10, cis-12 CLA during the differentiation of ovine preadipocytes. Adipophilin, a member of the PAT protein

family (perillipin, adipophilin, PAT47), localizes on the surface of intracellular lipid droplets (Wolins et al., 2005). Increases in the number of lipid drops may be involved with the up-regulation of adipophilin genes in the progression of ovine adipogenesis.

Interestingly, trans-10, cis-12 CLA alone without troglitazone in differentiation medium induced adipocyte differentiation. In our culture system, troglitazone was found to be an essential factor in adipocyte differentiation. Adipogenic stimulation by trans-10, cis-12 CLA alone indicated that CLA is one of the factors that activate the transcription of PPAR- γ 2. Some reports have described that CLA can be bound with several types of PPARs. There is great evidence that trans-9, cis-10 CLA and trans-10, cis-12 CLA are ligands and activators of PPAR- α (Moya-Camarena et al., 1999). In addition, CLA may act as PPAR- γ ligand on human vascular smooth muscle cells (Ringseis et al., 2006). However, it is not clear whether CLA directly acts on the ligand of PPAR- γ 2 or in ovine adipocytes. Further study is necessary to investigate the ligand-binding assay in the promoter of PPAR- γ 2 gene.

Until now, 3T3-L1 preadipocytes derived from mouse and human preadipocytes have shown inhibitory effects on differentiation upon chronic treatments with CLA (Brodie et al., 1999). This mechanism of this inhibitory effect, however, has not been clarified. One of the presumptive mechanisms by which CLA impairs adipogenesis in human adipocytes is by increasing secretion of cytokines (Brown et al., 2004). *In vitro* studies have shown that decreasing glucose uptake and insulin resistance through promoting NF κ B and ERK1/2 activation induces cytokine production in human adipocytes (Chung et al., 2005a). In addition, trans-10, cis-12 CLA activates the integrated stress response pathway in adipocytes and stimulated the release of proinflammatory cytokines via NF κ B pathway *in vivo* and *in vitro* (LaRosa et al., 2007). These results suggest that stimulation of adipocyte differentiation by trans-10, cis-12 CLA in ovine adipocytes may be induced by decrement of cytokines through the promotion of NF κ B and ERK1/2 activation.

In conclusion, our data clearly demonstrate that CLA differentially acts on the adipogenic process between ovine preadipocytes and mouse 3T3-L1 cells. Therefore, trans-10, cis-12 CLA may be mediated by a different mechanism in adipogenesis between ovine preadipocytes and 3T3-L1 cells. However, the mechanism of trans-10, cis-12 CLA on ovine adipogenesis remains to be further explored.

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