CLA differently regulates adipogenesis in stromal vascular cells from porcine subcutaneous adipose and skeletal muscle

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Abstract Conjugated linoleic acid (CLA), a mixture of isomers of linoleic acid, has previously been shown to be able to decrease porcine subcutaneous (SC) adipose tissue levels while increasing the count of intramuscular (IM) adipose tissue in vivo. However, the underlying mechanisms through which it acts are poorly understood. The objective of this study was to investigate the different effects of CLA on adipogenesis in cultured SC adipose tissue and IM stromal vascular cells obtained from neonatal pigs. As shown here, *trans*-10, *cis*-12 CLA decreased the expression of adipocyte-specific genes as well as adipose precursor cell numbers and the accumulation of lipid in cultured SC adipose tissue stromal vascular cells. However, the *cis*-9, *trans*-11 CLA did not alter adipogenesis in SC cultures. On the other hand, both CLA isomers increased the expression of adipocyte-specific genes in IM cultures, together with the increasing accumulation of lipid and Oil Red O-stained cells. Collectively, these data show that CLA decreases SC adipose tissue but increases IM adipose tissue by different regulation of adipocyte-specific gene expression. These results suggest that adipogenesis in IM adipocytes differs from that in SC adipocytes.—Zhou, X., D. Li, J. Yin, J. Ni, B. Dong, J. Zhang, and M. Du. **CLA differently regulates adipogenesis in stromal vascular cells from porcine subcutaneous adipose and skeletal muscle. *J. Lipid Res.* 2007.** 48: **1701–1709.**

Supplementary key words adipocyte-specific gene • adipose precursor cell • proliferation • differentiation • subcutaneous adipose tissue • intramuscular adipose tissue • conjugated linoleic acid

The total lipid content within the skeletal muscle has been shown to be highly associated with both meat quality in animal production (1, 2) and insulin resistance in humans (3–5). The intramuscular (IM) lipid consists of intramyocellular triglycerides within muscle cells and lipids stored in adipocytes interspersed between fiber fasciculi (IM adipocytes) (5–7). Previous studies suggested that total lipid content variability in skeletal muscle is caused mainly by changes in the lipid content of IM adipocytes (8, 9). Thus, it is necessary to investigate the potential factors that may influence the number and lipid accumulation of IM adipocytes.

Conjugated linoleic acid (CLA) is a mixture of isomers of linoleic acid. Both *cis*-9, *trans*-11 (c9,t11) and *trans*-10, *cis*-12 (t10,c12) CLA are isomers that occur naturally in food (10). Animal studies have demonstrated that dietary intake of CLA changes animal body composition by preventing obesity development (11–15). Furthermore, studies suggested that t10,c12-CLA but not c9,t11-CLA inhibits both the proliferation and the differentiation of 3T3-L1 preadipocytes and cultured stromal vascular cells from human subcutaneous (SC) adipose tissue, thus decreasing lipid accumulation (10, 16). Surprisingly, several experiments also demonstrated that CLA can increase IM fat deposition while decreasing SC fat (11, 17). Indirect evidence indicated that increased IM fat was attributable to the recruitment of more stromal vascular cells to IM adipocytes by CLA (18). Collectively, these studies suggested that the regulation of CLA in adipocyte development in SC and IM adipose tissue is in distinct mechanisms (19).

Adipocyte determination and differentiation factor-1 (ADD-1), peroxisome proliferator-activated receptor γ (PPARγ), and CAAT/enhancer binding protein α (C/EBPα) are three important adipogenic transcription factors. ADD-1 regulates lipid metabolism by modulating both the expression and activation of PPARγ (20). PPARγ is a master transcription factor of the adipocyte lineage, which is strictly required for adipose terminal differentiation and adipose cell formation (21–23). Chawla and Lazar (24) suggested that the sequential expression of

Abbreviations: ADD-1, adipocyte determination and differentiation factor-1; aP2, adipocyte fatty acid binding protein; C/EBPα, CAAT/enhancer binding protein α; CLA, conjugated linoleic acid; IM, intramuscular; IR, insulin receptor; OROSM, Oil Red O-stained material; PPARγ, peroxisome proliferator-activated receptor γ; SC, subcutaneous.

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PPARγ evoked the transactivation of adipocyte-specific genes involved in the terminal differentiation of preadipocytes and the metabolism of lipid. C/EBPα can transactivate adipocyte-specific gene expression to promote adipogenesis (25). LPL, FAS, and adipocyte fatty acid binding protein (aP2) play central roles in the control of lipid accumulation (26–29). LPL and FAS are key enzymes that are involved in the regulation of lipid metabolism. The target gene of PPARγ, aP2, is an indicator of adipocyte differentiation (16, 25). Collectively, these adipocyte-specific genes encode proteins that are involved in adipogenesis and/or adipocyte development, which are the candidate genes in the present study. As adipocytes located in SC adipose tissue, IM adipocytes also express these adipocyte-specific genes (30). However, the effect of CLA on the expression of these genes in muscle has not been investigated.

We hypothesized that the effect of CLA on IM adipogenesis differs from that on SC adipogenesis by different regulation of adipocyte-specific genes. Experiments were designed to detect the effect of CLA on adipocyte-specific gene expression, adipose precursor cell proliferation, and lipid accumulation in cultured SC adipose tissue and IM stromal vascular cells, which permits direct comparison of the response of SC adipose stromal vascular cells and IM stromal vascular cells to CLA.

MATERIALS AND METHODS

Cell isolation and culture conditions

Isolation and culture of stromal vascular cells from SC adipose tissue and skeletal muscle tissue. Seven day old neonatal pigs (Dalland, Sino-Dutch Animal Husbandry Training and Demonstration Center, Beijing, China) were euthanized by a lethal injection of sodium pentobarbital. Both SC adipose tissue and the longissimus muscle were aseptically isolated, and visible connective tissue was removed. Tissues were then finely minced and incubated with 5 ml/g digestion buffer containing 250 U/ml Type II collagenase, 100 mmol/l HEPES, 1.5% (w/v) type V BSA (all from Invitrogen, Carlsbad, CA), 120 mmol/l NaCl, 50 mmol/l KCl, 5 mmol/l D-glucose, and 1 mmol/l $CaCl_2$ (all from Sigma, St. Louis, MO). The digestion period was 2 h, followed by centrifugation of the digestion mixture at 1,000 *g* for 5 min; in turn, this was filtered through 100 and 40 μm nylon cell strainers (Filcon; DAKO, Copenhagen, Denmark) and a 20 μm mesh filter (Millipore, Bedford, MA), then centrifuged for another 10 min at 1,000 *g* to obtain a stromal vascular cell pellet containing adipose precursor cells. The pellet was washed three times with DMEM. The SC adipocyte stromal vascular cells were plated in proliferation medium containing 90% DMEM, 100 ml/l FBS (both from Gibco, Grand Island, NY), 15 μmol/ml HEPES, 100 U/ml penicillin-streptomycin, and 5 μg/ml fungizone (all from Invitrogen) at a density of 1×10^4 cells/cm^2 in six-well cell culture clusters (Corning, Corning, NY). To maintain similar times required for cells to reach confluent densities, muscle stromal vascular cells were plated at a density of 5×10^4 cells/cm^2 as described previously (31). To perform immunohistochemistry and Oil Red O staining, cells were separated as described above but then seeded on glass cover slips (Electron Microscopy Science, Hatfield, PA) in 12-well cell culture clusters (Corning). After 24 h of incubation in a 37°C humidified atmosphere containing 5% CO_2, medium was removed and the plates were washed with DMEM to remove unattached cells and cell debris (32).

Induction of cell differentiation. Cells reached confluence in proliferation medium (day 0). Cells were washed three times with DMEM, then grown for another 3 days in differentiation medium [proliferation medium supplemented with 33 μmol/l biotin, 1 μmol/l dexamethasone, 0.25 mmol/l isobutylmethylxanthine, and 100 nmol/l insulin (all from Sigma)] to allow them to differentiate. Thereafter, cultures were exposed to adipocyte medium (proliferation medium supplemented with 33 μmol/l biotin, 1 μmol/l dexamethasone, and 100 nmol/l insulin) (10).

Fatty acid preparation

The CLA isomers, 9(*Z*),11(*E*)-octadecadienoic acid (c9,t11-CLA) and 10(*E*),12(*Z*)-octadecadienoic acid (t10,c12-CLA) were

TABLE 1. Primer sequences used for quantitative real-time PCR

Genes	Primers	Amplified Fragment Length	Annealed Temperature
		bp	°*C*
ADD1	Forward, 5′-GCGACGGTGCCTCTGGTAGT-3′ Reverse, 5′-CGCAAGACGGCGGATTTA-3′	262	56
aP2	Forward, 5′-GGCTTTGCTACCAGGAAAGT-3′ Reverse, 5′-CGCAGTGACACCATTCATGAC-3′	216	62
C/EBPα	Forward, 5′-GGTGGACAAGAACAGCAACG-3′ Reverse, 5′-GGTGGACAAGAACAGCAACG-3′	218	64
FAS	Forward, 5′-CTGCTGAAGCCTAACTCCTCG-3′ Reverse, 5′-CTGCTGAAGCCTAACTCCTCG-3′	242	62
IR	Forward, 5′-GGGCTTGAGACCACGCATCA-3′ Reverse, 5′-CTGAAGTGGGAGCCGTATTG-3′	216	62
LPL	Forward, 5′-GGGCAAGGATTGTTTTT-3′ Reverse, 5′-CCCAGTATCTTTTCTTTCTTT-3′	293	56
PPARγ	Forward, 5′-TGACCATGGTTGACACCG-3′ Reverse, 5′-AAGCATGAACTCCATAGTGG-3′	381	58
β-Actin	Forward, 5′-TGCGGGACATCAAGGAGAAG-3′ Reverse, 5′-AGTTGAAGGTGGTCTCGTGG-3′	216	64

ADD-1, adipocyte determination and differentiation factor-1; aP2, adipocyte fatty acid binding protein; C/EBPα, CAAT/enhancer binding protein α; IR, insulin receptor; PPARγ, peroxisome proliferator-activated receptor γ.

purchased from Matreya, Inc. (purity ≥ 98%; Pleasant Gap). Linoleic acid and 9(*Z*),12(*Z*)-octadecadienoic acid (C18:2) were purchased from Sigma (purity ≥ 99%; Sigma). All fatty acids were complexed to fatty acid-free BSA (Invitrogen) at a 4:1 molar ratio using 1 mmol/l BSA stock. The CLA mixture (CLA-Mix) consisted of c9,t11-CLA and t10,c12-CLA at a 1:1 ratio.

Experimental design

The objective of experiment 1 was to determine the dose response of CLA-Mix on the expression of adipocyte-specific genes. When confluence was reached (day 0), cultured SC adipose and muscle stromal vascular cells were induced to differentiate and treated continuously for 6 days with BSA (as a control) or 25 or 50 μmol/l CLA-Mix. The mRNA abundance of adipocyte-specific genes was determined by quantitative real-time PCR. Six replicates were used for the described experiments. Cells were isolated from a different pig for each replicate.

Experiment 2 was designed to determine the effect of CLA isomers on the mRNA transcript expression of adipocyte-specific genes and the protein expression of a key adipogenic transcription factor, PPARγ. At confluence (day 0), stromal vascular cultures were induced to differentiate and treated continuously for 6 days with BSA or 50 μmol/l CLA-Mix, c9,t11-CLA, t10,c12-CLA, or linoleic acid. The mRNA expression of adipocyte-specific genes and the protein expression of PPARγ were determined by quantitative real-time PCR and Western blot. Four to six replicates were used for the described experiments. Cells were isolated from a different pig for each replicate.

The objective of experiment 3 was to evaluate the effect of CLA isomers on the proliferation and lipid accumulation of stromal vascular cells from SC adipose tissue and skeletal muscle. To study the effect of CLA on adipose precursor cell proliferation, a cell marker, Preadipocyte factor 1 (Pref-1), also known as delta-like 1 homolog (DLK1), was immunohistochemically stained on stromal vascular cultures after 2 days of BSA or 50 μmol/l fatty acid treatment. To determine the effect of CLA on lipid accumulation, stromal vascular cultures were induced to differentiate and treated continuously with BSA or 50 μmol/l fatty acids for 4 days. The cultures were then stained for Oil Red O and Pref-1 (DLK1) and lightly counterstained with hematoxylin to determine the effect of different fatty acids on the amount of lipid accumulation, Oil Red O-stained cells, and total cell number. Four replicates were used for the described experiments. Cells were isolated from a different pig for each replicate.

Total RNA isolation and reverse transcription

Total RNA was isolated from the cultured cells using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted RNA was dissolved in RNA-free water and quantified using ultraviolet-clear microplates (Corning) at an optical density of 260 nm. An RNA aliquot was verified for its integrity by electrophoresis on a 1% agarose gel stained with ethidium bromide. Then, 2 μg of total RNA was reverse-transcribed in a 25 μl reaction mixture using random primer Oligo-dT_{18} (Sangon, Shanghai, China) and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) as described by Lai et al. (33). The RT products (cDNA) were stored at −80°C until analysis of selected gene mRNA levels by quantitative real-time PCR.

Quantitative real-time PCR

Quantitative real-time PCR was performed using DNA Engine Opticon-2 (MJ Research, Waltham, MA) and DyNAmo SYBR Green qPCR commercial kits (Finnzymes, Finland). β-Actin was used as the reference gene. The primers of the selected genes are listed in **Table 1**. The PCR system consisted of 5.0 μl of SYBR Green qPCR mix, 1.0 μl of cDNA, 3.6 μl of double distilled water, and 0.4 μl of primer pairs (25 μmol/l forward and 25 μmol/l reverse) in a total volume of 10 μl. Cycling conditions were 50°C for 2 min followed by 95°C for 5 min, and by 35 cycles the

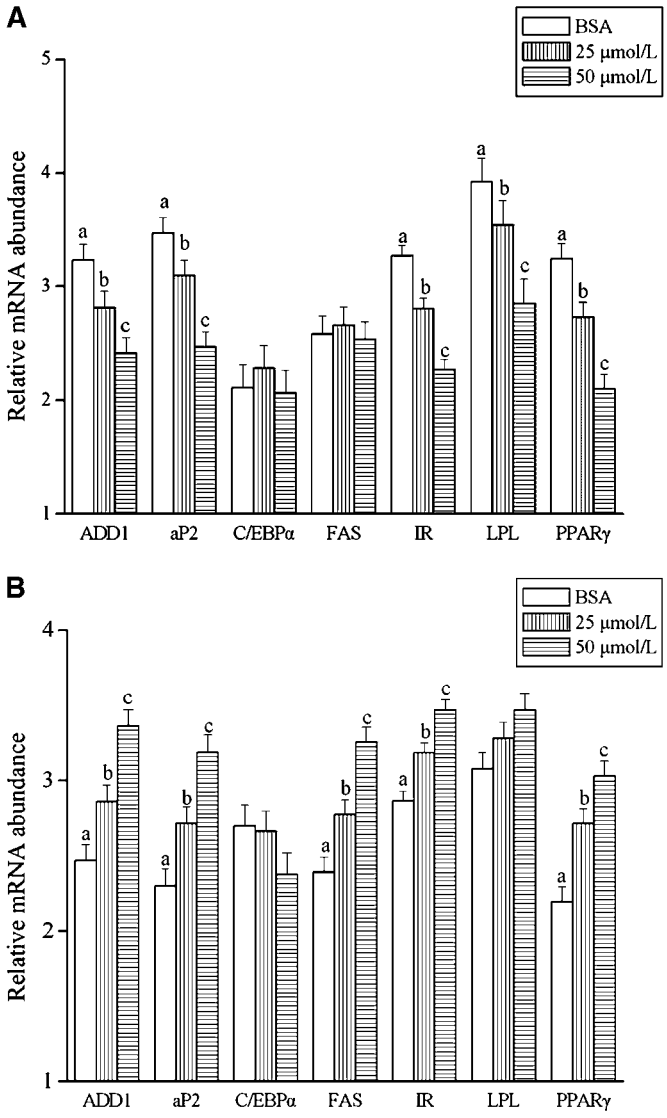

Fig. 1. Effect of the dose of conjugated linoleic acid (CLA)-mixture (CLA-Mix; c9,t11:t10,c12 = 1:1) on the expression of adipocyte-specific genes in porcine stromal vascular cell cultures. Porcine stromal vascular cells from subcutaneous (SC) adipose tissue (A) and longissimus muscle (B) reached confluence (day 0) in proliferation medium, then cells were grown in differentiation medium containing BSA (a vehicle control) or 25 or 50 μmol/l CLA-Mix for 6 days. Total RNA was harvested and used for first-strand cDNA synthesis. Quantitative real-time analyses were performed to analyze the expression of adipocyte determination and differentiation factor-1 (ADD-1), adipocyte fatty acid binding protein (aP2), CAAT/enhancer binding protein α (C/EBPα), FAS, insulin receptor (IR), LPL, and peroxisome proliferator-activated receptor γ (PPARγ). The relative expression abundance of a given gene was calculated after normalization to β-actin expression. Statistical differences reported are within tissue. Values are means ± SEM; n = 6. Values with different letters are significantly different ($P < 0.05$).

temperature was set at 95°C for 30 s, with the corresponding annealed temperatures listed in Table 1, and then extended for 30 s at 72°C. The melting curve program was 65–95°C, with a heating rate of 0.1°C/s and continuous fluorescence measurement. All samples were measured in triplicate. The relative mRNA levels of target genes were determined using the relative standard curve method as described previously (33).

Western blotting

Total cellular protein was lysed using a protein isolation kit (Kangchen, Shanghai, China) containing 50 mg/l soybean trypsin inhibitor and 0.1 mmol/l phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 14,000 *g* for 15 min, and the supernatant was used for Western blot analysis. Protein concentration was determined using a BCA protein assay reagent kit (Kangchen). The sample was boiled for 3 min, and cellular proteins were electrophoresed (Bio-Rad, Richmond, CA) on SDS-polyacrylamide gels and then electroblotted (Bio-Rad) onto polyvinylidene difluoride membranes (Millipore). Membranes were incubated for 1 h in a blocking buffer containing 5% nonfat dry milk in TBS (20 mmol/l Tris and 500 mmol/l NaCl, pH 7.6) (34). After blocking, the membrane was washed in TBS supplemented with 0.1% Tween 20 (TBST) and incubated for 1 h with mouse PPARγ monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was then rinsed in TBST and incubated for 1 h with horseradish peroxidase-labeled anti-mouse IgG (Kangchen). The membrane was then washed three times with TBST. GAPDH was used as the reference protein. Visualization of the reactive bands was accomplished by chemiluminescence and exposure of the blots to luminescence detection film (ECL Western Blotting detection reagents and Hyperfilm-ECL; Kangchen). The films were analyzed by a gel-imaging system (Tanon Science and Technology, Shanghai, China) with ImageJ analysis software (National Institutes of Health, Bethesda, MD).

Immunohistochemistry

In this study, a Pref-1 (DLK1) polyclonal antibody was used to identify or mark adipose precursor cells in both SC and IM stromal vascular cell cultures. Rabbit anti-human Pref-1 (DLK1) polyclonal antibodies were purchased from ProteinTech Group, Inc. (catalog No. 10636-1-AP). Western blot indicated that the Pref-1 (DLK1) antibodies also reacted with cellular proteins from porcine SC and IM stromal vascular cell cultures. Immunohistochemistry was performed as described by Hausman and Poulos (31). Briefly, cells were washed three times in PBS and then fixed with 4% paraformaldehyde (Sigma) for 30 min. Endogenous peroxides were quenched using 3% H_2O_2 solution for 10 min. Cells were then incubated in a humidified chamber for 30 min with Pref-1 (DLK1) polyclonal antibody (1:50). This was followed by incubation with biotinylated anti-rabbit IgG (1:200) for 25 min and extravidin peroxidase for 30 min. Reactivity was visualized using diaminobezidin, a colorimetric substrate (all from BSCX-Biotech, Wuhan, China). Lipid content was quantified using 60% Oil Red O solution for 10 min followed by hematoxylin (both from Sigma) staining for 2 min, as described previously (35). Total cell numbers were counted in every 100× magnified field, and quantification of adipose precursor cells (Pref-1+ cells) and Oil Red O-stained cells was completed using 200× magnified fields. We used computer-assisted image analysis (Image-Pro Plus; Media Cybernetics, Inc., Silver Spring, MD) to determine the integrated optical density of the Oil Red O-stained material (OROSM). Lipid content was expressed as the integrated optical density of OROSM per cell in each 200× magnified field. A minimum of five microscopic fields for each dish were quantified.

Statistical analysis

Data were analyzed using one-way ANOVA, followed by Duncan's multiple comparisons. All analyses were performed using SAS (version 8.1; SAS Institute, Cary, NC). Data are expressed as means and pooled SEM. $P < 0.05$ was taken to indicate statistical significance.

RESULTS

Gene expression

The dose response of the CLA-Mix on the expression of adipocyte-specific genes in muscle stromal vascular cells

TABLE 2. Effect of different fatty acids on the expression abundance of selected genes in adipose tissue and muscle stromal vascular cell cultures

Item	BSA	Linoleic Acid	CLA-Mix	c9,t11-CLA	t10,c12-CLA	SEM	*P*
Adipose tissue							
ADD1	3.03a	3.17a	2.60b	2.97a	2.39b	0.115	<0.01
aP2	3.48a	3.52a	2.67b	3.46a	2.53b	0.135	<0.01
C/EBPα	2.48	2.52	2.70	2.50	2.57	0.071	>0.05
FAS	2.59	2.83	2.78	2.76	2.53	0.133	>0.05
IR	3.07a	3.39a	2.55b	3.15a	2.49b	0.126	<0.01
LPL	3.92a	4.12a	3.28b	3.75a	3.10b	0.123	<0.01
PPARγ	3.15a	3.40a	2.51b	3.32a	2.12c	0.125	<0.01
Muscle tissue							
ADD1	2.47b	3.11a	2.96a	2.97a	3.29a	0.161	<0.05
aP2	2.40b	3.09a	2.90a	2.96a	3.20a	0.130	<0.01
C/EBPα	2.70	2.86	2.75	2.57	2.77	0.178	>0.05
FAS	2.49b	2.95a	3.19a	3.07a	3.00a	0.150	<0.05
IR	2.87b	3.39a	3.28a	3.57a	3.31a	0.139	<0.05
LPL	3.08	3.31	3.12	3.05	3.03	0.181	>0.05
PPARγ	2.20b	2.94a	3.14a	3.08a	2.83a	0.196	<0.05

CLA, conjugated linoleic acid. Porcine stromal vascular cells from subcutaneous adipose tissue and longissimus muscle reached confluence (day 0) in proliferation medium, then cells were grown in differentiation medium containing BSA (a vehicle control), 50 μmol/l CLA-mixture (Mix), c9,t11-CLA, t10,c12-CLA, or linoleic acid for 6 days. Total RNA was harvested and used for first-strand cDNA synthesis. Quantitative real-time analyses were performed to analyze the expression of ADD-1, aP2, C/EBPα, IR, LPL, and PPARγ. The relative expression abundance of a given gene was calculated after normalization to β-actin expression. Statistical differences reported are within tissue. Values are means and pooled SEM (n = 6). Values with different letters are significantly different ($P < 0.05$).

differed from that in SC adipose tissue stromal vascular cells. After a 6 day treatment, the mRNA expression of ADD-1, PPARγ, aP2, LPL, and insulin receptor (IR) decreased ($P < 0.05$) as the level of CLA-Mix increased in a dose-dependent manner. No difference was found in the mRNA expression of C/EBPα and FAS. In muscle stromal vascular cultures, however, as the level of CLA-Mix increased from 0 to 50 μmol/l, the mRNA expression of ADD-1, PPARγ, aP2, FAS, and IR increased. No difference was found in the mRNA expression of C/EBPα and LPL (**Fig. 1**).

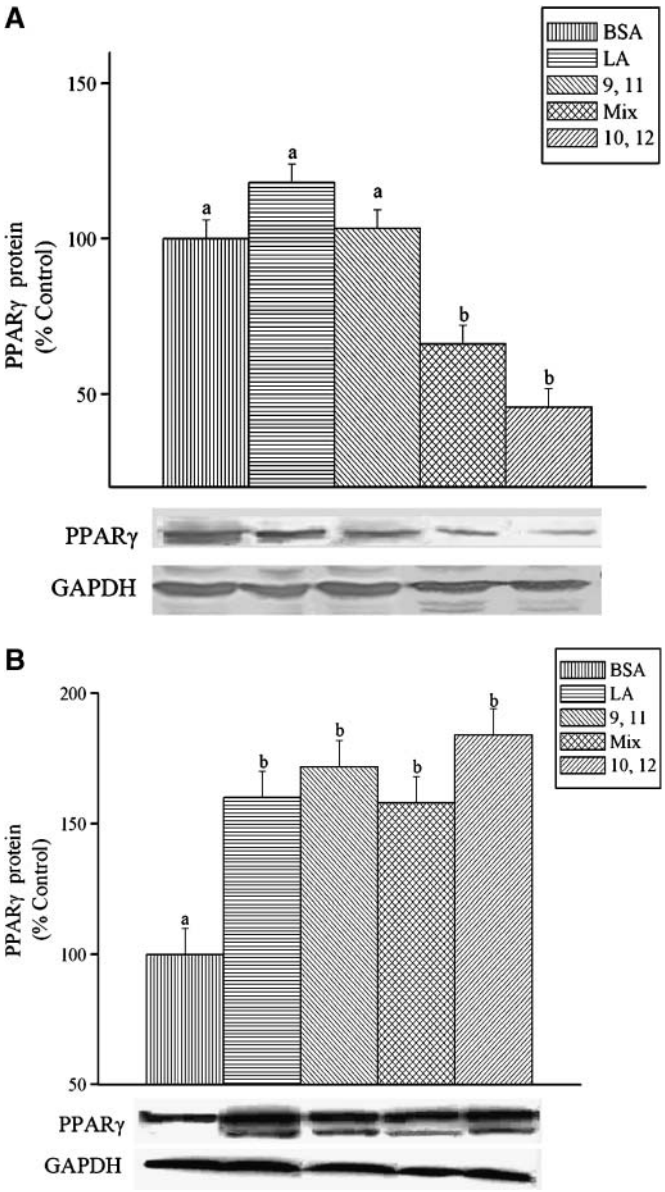

Fig. 2. Effect of CLA on the protein expression of PPARγ in porcine stromal vascular cell cultures. Porcine stromal vascular cells from SC adipose tissue (A) and longissimus muscle (B) reached confluence (day 0) in proliferation medium, then cells were grown in differentiation medium containing BSA (a vehicle control), 50 μmol/l CLA-Mix (Mix), c9,t11-CLA (9, 11), t10,c12-CLA (10, 12), or linoleic acid (LA) for 6 days. Total cellular protein was harvested, and Western blot analyses of PPARγ were conducted. GAPDH was used as the reference protein. The blots for PPARγ and GAPDH were quantified by densitometry, and data are expressed as the amount of PPARγ relative to GAPDH in each fatty acid group as a percentage of the BSA-treated control group. Statistical differences reported are within tissue. Values are means ± SEM; n = 4. Values with different letters are significantly different ($P < 0.05$).

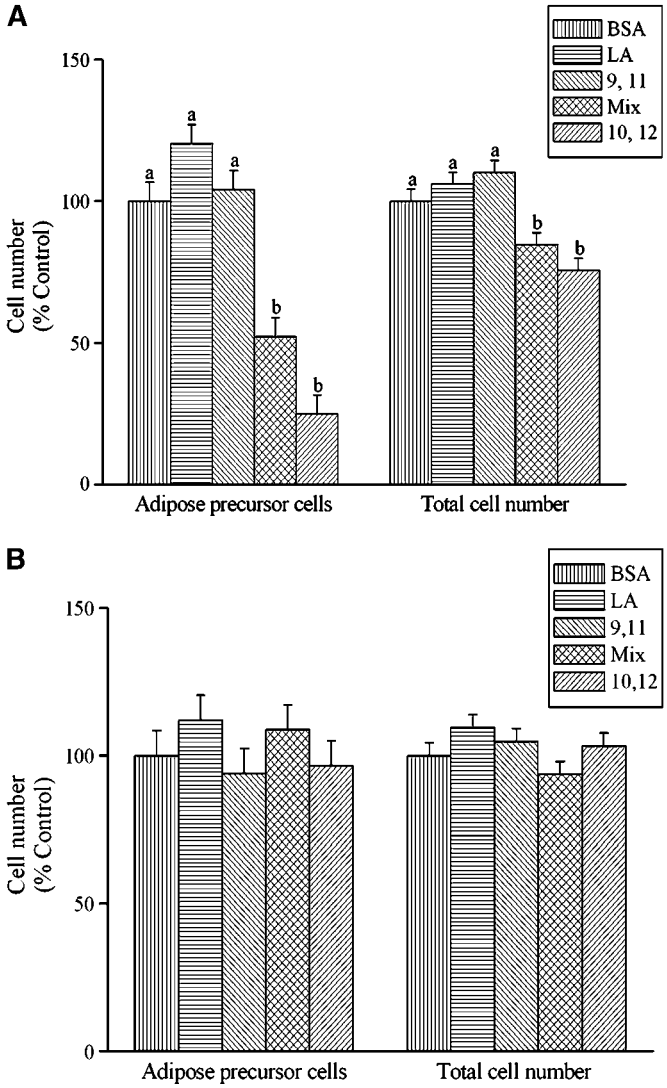

Fig. 3. Effect of CLA on numbers of adipose precursor cells and total cells during proliferation of cultured porcine stromal vascular cells. After 24 h of plating, stromal vascular cells from SC adipose tissue (A) and longissimus muscle (B) were exposed to proliferation medium containing BSA (a vehicle control), 50 μmol/l CLA-Mix (Mix), c9,t11-CLA (9, 11), t10,c12-CLA (10, 12), or linoleic acid (LA) for 2 days. Cells were then harvested and fixed in 4% paraformaldehyde, and cultures were stained for Pref-1 to identify adipose precursor cells and lightly counterstained with hematoxylin to determine total cell numbers. The data are expressed as adipose precursor cell numbers (200× microscopic field) and total cell numbers (100× microscopic field) per microscopic field in each fatty acid group as a percentage of that in the control group. Statistical differences reported are within tissue. Values are means ± SEM; n = 4. Values with different letters are significantly different ($P < 0.05$).

Different effects of CLA isomers on the expression of adipocyte-specific genes of preadipocytes from SC adipose tissue and IM stromal vascular cells are shown in **Table 2** and **Fig. 2**. In SC adipose tissue stromal vascular cell cultures, the mRNA expression of ADD-1, PPARγ, aP2, LPL, and IR was decreased by t10,c12-CLA and CLA-Mix, and the protein abundance of PPARγ was also lower in these two treatments ($P < 0.05$). c9,t11-CLA, however, had no effect on the expression of these genes. In muscle stromal vascular cell cultures, the mRNA abundance of PPARγ and ADD-1 together with aP2, FAS, and IR in each fatty acid-treated group was more than that in the control group ($P < 0.05$). PPARγ protein expression was also increased by CLA in IM cultures ($P < 0.05$). No difference was found in the mRNA expression of LPL and C/EBPα.

Adipose precursor cell proliferation

Adipose tissue stromal vascular cells treated with CLA-Mix and t10,c12-CLA showed lower adipose precursor cell numbers and total cell numbers ($P < 0.01$), whereas c9,t11-CLA and linoleic acid had no effect on them (**Fig. 3A**). In IM stromal vascular cell cultures, no fatty acids affected adipose precursor cell numbers and total cell numbers (Fig. 3B).

Cell morphology and lipid accumulation

t10,c12-CLA and CLA-Mix decreased lipid accumulation in cultured SC adipose stromal vascular cells ($P < 0.01$), and cells in these two groups tended to be less rounded and had fewer lipid droplets compared with the control group. c9,t11-CLA and linoleic acid had no significant effect on lipid accumulation and cell shape (**Figs. 4A, 5**). In contrast to SC cultures, IM stromal vascular cells in CLA-treated groups had higher lipid accumulation ($P < 0.01$). Larger lipid droplets and more Oil Red O-stained cells were also found in the CLA-treated cultures (Figs. 4B, 5). Total cell numbers did not differ among control and fatty acid-treated groups in either SC adipose tissue or IM stromal vascular cell culture (Fig. 4).

DISCUSSION

To our knowledge, this study is the first to show the different effects of CLA on the proliferation and differentiation of adipose precursor cells in SC adipose tissue and skeletal muscle in vitro. This provides new insight into the effect of CLA on muscle fat accumulation and body fat distribution.

Several studies have demonstrated adipocyte-specific genes that play important roles in the regulation of lipid metabolism and/or cell development, such as ADD-1, PPARγ, and aP2 mRNA, which were highly expressed at day 6 of preadipocyte differentiation (10, 32, 36, 37). Our data clearly show that t10,c12-CLA but not c9,t11-CLA reduced the mRNA expression of these genes in SC adipose tissue stromal vascular cultures with a 6 d treatment. In contrast, the expression of such genes in muscle stromal vascular cell cultures was increased by both CLA isomers under the same treatment. This suggests that the effect of CLA on the expression of adipocyte-specific genes in SC adipose tissue differs from that in IM adipose tissue.

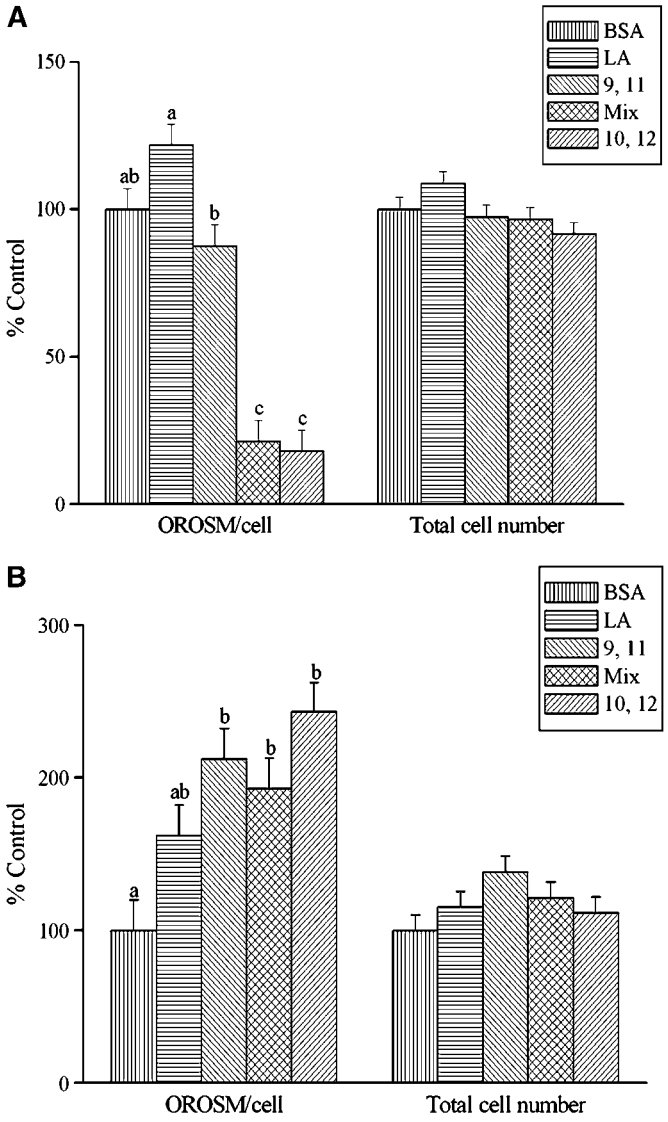

Fig. 4. Effect of CLA on lipid accumulation and total cell number during early differentiation of cultured porcine stromal vascular cells. Porcine stromal vascular cells from SC adipose tissue (A) and longissimus muscle (B) reached confluence (day 0) in proliferation medium, then cells were induced to differentiate and treated with BSA (a vehicle control), 50 μmol/l CLA-Mix (Mix), c9,t11-CLA (9, 11), t10,c12-CLA (10, 12), or linoleic acid (LA) for 4 days. Cells were then harvested and fixed with 4% paraformaldehyde, and cultures were stained for Oil Red O and lightly counterstained with hematoxylin. Total cell number was quantified using image analysis (100× microscopic field). Lipid was quantified by determining the integrated optical density of the Oil Red O-stained material (OROSM) in each 200× microscopic field. The amount of lipid was adjusted for total cell number. The data are expressed as the amount of OROSM per cell and total cell number in each fatty acid group as a percentage of that in the control group. Statistical differences reported are within tissue. Values are means ± SEM; n = 4. Values with different letters are significantly different ($P < 0.05$).

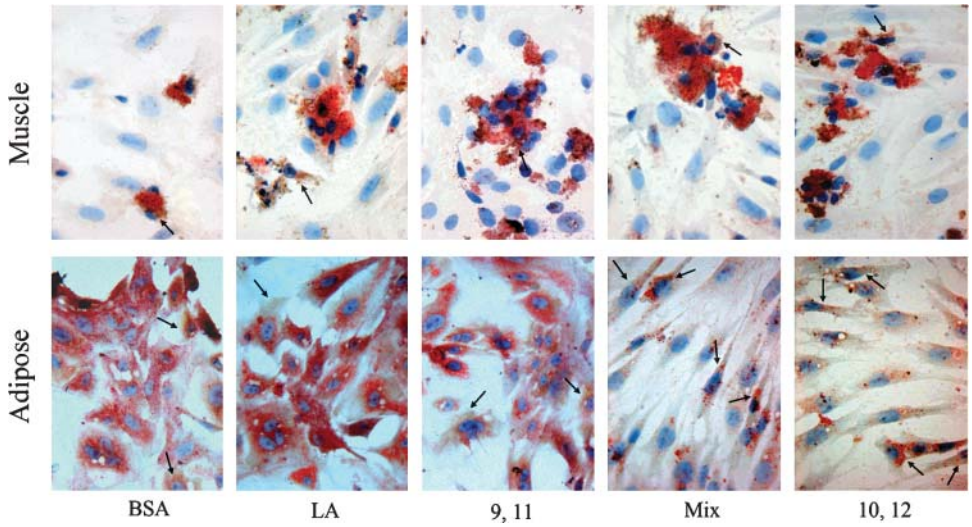

Fig. 5. Cell morphology and lipid droplet in cultured porcine stromal vascular cells. Porcine stromal vascular cells from SC adipose tissue (A) and longissimus muscle (B) reached confluence (day 0) in proliferation medium, then cells were induced to differentiate and treated with BSA (a vehicle control), 50 μmol/l CLA-Mix (Mix), c9,t11-CLA (9, 11), t10,c12-CLA (10, 12), or linoleic acid (LA) for 4 days. Cells were then harvested and fixed with 4% paraformaldehyde, and cultures were double-stained for Pref-1 and Oil Red O and lightly counterstained with hematoxylin. Intracellular lipid droplets were stained in red, and nuclei were stained in blue. Arrows indicate Pref-1+ cells (400× microscopic field).

It was reported that insulin can modulate the transcription of ADD-1 through IR in its responsive cells (38). In our study, we found that the expression of IR mRNA was reduced by t10,c12-CLA, which may reduce the insulin sensitivity of preadipocytes in SC adipose tissue. The expression of ADD-1 mRNA was downregulated by the low insulin sensitivity, which may result in the reduction of PPARγ mRNA. However, we found that the effect of CLA isomers on the expression of adipocyte-specific genes in IM stromal vascular cell cultures differs dramatically from that in SC adipose stromal vascular cell cultures. We first found that CLA increased the expression of PPARγ mRNA together with ADD-1 and IR in vitro.

The results of this study are consistent with the finding that, in vivo, the expression of PPARγ mRNA in longissimus muscle was increased by dietary CLA treatment, which supported the hypothesis that differences exist in the regulation of CLA on the SC and IM adipocytes (18). Our data also demonstrated that FAS and aP2 mRNA increased in IM cultures treated with CLA. However, the expression of LPL was not altered by any fatty acid in this study, which may be attributable to the expression of LPL that occurs spontaneously at confluence and is independent of the additional agents required for adipocyte differentiation (25, 39).

The inhibition of adipocyte markers of differentiation by the two CLA isomers in SC adipose stromal vascular cells in our study contrasts with the results of two previous studies that also detected the effect of CLA isomers in differentiating cultures of primary porcine preadipocytes from SC adipocytes. In these two studies, no isomer-specific effects of CLA isomers on the mRNA expression of PPARγ, aP2, or LPL was found (32, 40). In the first study (40), cultured SC adipose stromal vascular cells were treated with 300 μM CLA isomers in serum-free medium for 24 h, which is markedly different from being treated with 25 or 50 μM CLA isomers in medium containing 10% FBS for 6 days, as in the present study. Brandebourg and Hu (41) suggested that the ability to detect the inhibition of CLA on differentiation was possibly confounded by a low differentiation response in control cells in the second study.

Conditions used to differentiate preadipocytes vary not only between preadipocyte types but also between laboratory conditions (32). Novakofski (42) indicated that disparities in culture conditions may markedly affect results. In the present study, porcine preadipocytes were induced to differentiate in culture that contained 10% FBS and differentiation factors including insulin, dexamethasone, and isobutylmethylxanthine. The culture together with FBS plus differentiation factors is to some extent similar to the situation in vivo (32).

Primary SC adipose stromal vascular cell culture contains a variety of cell types, including endothelial cells, pericytes, blood cells, and adipose precursor cells, among them preadipocytes, adipoblasts, and poor differentiated mesenchymal stem cells (43, 44). Primary IM stromal vascular cell culture contains myogenic cells, satellite cells, mesenchymal stem cells, and preadipocytes (31, 45, 46). The transmembrane protein Pref-1 (DLK1) was used as a preadipocyte marker in several studies in 3T3-L1 cell lines and SC adipose, for it is only expressed in preadipocytes but extinguished during preadipocyte differentiation (47–49). Huff et al. (50) demonstrated that Pref-1 may be used as an immunohistochemical marker for preadipocytes in bovine muscle tissue. Frühbeck and Gómez-

Ambrosi (44) suggested that adipoblasts and mesenchymal stem cells also express Pref-1 protein. Collectively, these studies suggested that Pref-1 can be used to identify precursor cells that are able to develop to adipocytes in SC and IM stromal vascular cell cultures. The commercial antibody used in this study was specifically designed for humans; by alignment of pig Pref-1 partial coding domain sequences (GenBank accession number AY007208) and human Pref-1 (GenBank accession number BC007741), which was designed by a commercial manufacturer as described in the instructions, pig partial coding domain sequences showed 86% identity to human Pref-1. Furthermore, our Western blot showed that the Pref-1 antibodies demonstrated reactive recognition of the cellular proteins of porcine SC and IM stromal vascular cell cultures. In the present study, therefore, the Pref-1 antibody was used as an adipose precursor cell marker in both SC and IM cultures.

Our results showed that t10,c12-CLA decreased the adipose precursor cell numbers in SC cultures during proliferation. The total cell numbers were also decreased by t10,c12-CLA before differentiation induction, possibly as a result of the reduction of adipose precursor cells caused by t10,c12-CLA isomer. These results are consistent with another study (41). The findings in our study suggest that the CLA-induced SC fat reduction in pigs could be partly attributable to its effect on adipose hyperplasia. On the other hand, changes caused by t10,c12-CLA in adiopocyte-related gene expression altered the morphology of certain cells and lipid accumulation in primary SC cultures. In concert with the downregulation of PPARγ expression, we found that cells treated with t10,c12-CLA tended to be less rounded and remained fibroblast-like, which suggests an inhibition effect of the differentiation of t10,c12-CLA. Our data also demonstrated that the t10,c12-CLA isomer decreased OROSM per cell, which represents accumulation in triglyceride. LPL and aP2 are genes closely associated with the synthesis of triglyceride (51, 52). Low expression of LPL and aP2 mRNA in t10,c12-CLA-treated cultures may be the main reason for the reduction of triglyceride. Collectively, our data suggest that t10,c12-CLA decreased fat accumulation in SC adipose tissue by inhibiting both adipose precursor cell proliferation and differentiation.

It has been demonstrated in several experiments that satellite cells and muscle stem cells can be induced to differentiate to adipocyte lineage by PPARγ activators such as thiazolidinedione, together with the inhibition of differentiation of myoblasts (22, 53, 54). On the other hand, some studies suggested that CLA has structural and physiological characteristics similar to those of the PPAR ligand peroxisome proliferators (55). Furthermore, Houseknecht et al. (56) found that CLA was able to activate PPARγ in vitro. These studies suggested that CLA may have the ability to transdifferentiate myoblasts to adipocytes. Although our data indicated that IM adipose precursor cell numbers were not affected by CLA during proliferation, CLA increased the OROSM per cell together with the upregulation of PPARγ expression. We also found that there were more Oil Red O-stained cells in the CLA-treated group during differentiation. This confirms the view that CLA has the ability to transdifferentiate myoblasts to adipocytes by inducing the expression of PPARγ in muscle tissue.

In conclusion, this study provides new views on the effect of CLA, showing the dramatic difference in the regulation of CLA on proliferation and differentiation in cultured IM adipose precursor cells and SC adipose precursor cells. This novel finding may explain the contrasting results of CLA on SC and IM fat content observed in previous feeding trials. These findings suggest that different mechanisms exist in adipogenesis in IM and SC adipocytes.

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