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Effects of isomers of conjugated linoleic acid on porcine adipocyte growth and differentiation[1]

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Abstract

Conjugated linoleic acids (CLAs) decrease fat deposition in mammals, including pigs. To determine mechanisms for CLA effects on adipocyte growth, porcine stromal-vascular cells (preadipocytes) were isolated and plated in medium containing 10% fetal bovine serum. After 24 h, differentiation factors (insulin + hydrocortisone + transferrin) were added. Oleic acid (200 μM) was added to some plates as a positive control. One of two isomers of CLA (50 μM cis 9, trans 11 or 50 μM trans 10, cis 12), or a mixture of the two isomers (25 μM each) was added to other plates. The cell number increased 7+ times in 7 days after initiation of differentiation, and was not different among treatment groups. By 7 days, Oil Red O-stained material (OROSM), expressed per cell, increased 10+ times in control cells and 64 times in oleic acid-treated cells. Addition of either isomer of CLA or the mixture caused OROSM/cell to increase 10+ times at 2 days, with no further increase at later times. In CLA-treated cells there was no increase in peroxisome proliferator-activated receptor γ (PPARγ) or lipoprotein lipase mRNA concentrations. The increased OROSM/cell may represent triacylglycerol synthesis from medium CLA using existing biosynthetic capacity or provision of a limiting ligand for PPARγ already present. The results are different from those observed with rodent-derived clonal cells (3T3-L1 cells), wherein proliferation and differentiation are inhibited by CLAs, and the active isomer is trans 10, cis 12-CLA. The results suggest distinctions between clonal and primary preadipocytes, or species differences. Published by Elsevier Inc. All rights reserved.

Keywords: Adipocyte differentiation; Adipocyte proliferation; Conjugated linoleic acids; Pigs; Peroxisome proliferator-activated receptor γ; Lipoprotein lipase

1. Introduction

Conjugated linoleic acid (CLA) is a collective term for a group of octadecadienoic acids that are geometric and positional conjugated isomers of linoleic acid (C18:2) [1,2]. Oral administration of CLAs to mice [3-6], rats [7,8], hamsters [9], and pigs [10-14] causes decreased fat deposition. The body composition changes in mice result from the trans 10, cis 12-CLA isomer (10,12-CLA) and not from the cis 9, trans 11-CLA isomer (9,11-CLA) [2,15].

Mechanisms that have been proposed to explain the decreased fat deposition include decreased preadipocyte proliferation and differentiation, as demonstrated in the rodent-derived clonal cell line, 3T3-L1 [16-18]. Recent data indicates that 10,12-CLA, but not 9,11-CLA is a potent inhibitor of differentiation in these clonal cells [18–20]. Clonal cells may have properties distinct from cells isolated directly from an animal. Consequently, our objective was to determine the effects of the two main isomers of CLA (9,11-CLA and 10,12-CLA) on hyperplasia and differentiation of porcine stromal-vascular (**S-V**) cells, a cell fraction isolated from porcine adipose tissue that contains preadipocytes.

2. Materials and methods

2.1. Cell culture

Four- to 7-day-old crossbred pigs were purchased from the Texas Department of Criminal Justice, Huntsville, Texas. The pigs' weights ranged from 1.9 to 2.9 kg. Suckling pigs were transported to our facility, and killed ($\leq$ 3 h

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after removal from the sow) with a captive bolt pistol coupled with exsanguination. The Baylor College of Medicine Animal Care and Use Committee approved the animal protocol. Subcutaneous backfat was removed from the dorsal neck, shoulder, and back region under aseptic conditions. The adipose tissue was sliced and cells were isolated by collagenase digestion, as previously reported [21,22]. This digestion mixture was centrifuged at 800 × g for 10 min to obtain the S-V cell pellet containing preadipocytes. The pellet was washed three times with Dulbecco's modified Eagle medium: nutrient mixture F12 (Ham) in a 1:1 combination (**DMEM/F12**; Gibco-BRL #12400, Invitrogen-Life Technologies, Gaithersberg, MD), supplemented with penicillin and streptomycin plus an antifungal agent, Fungizone (Invitrogen-Life Technologies). The S-V cells were resuspended and plated in DMEM/F12 plus 10% fetal bovine serum (FBS) at a density of 6×10^4 cells/cm^2. After 24 hr incubation in a 37°C humidified atmosphere containing 5% CO_2, the medium was removed and the plates were washed with normal buffered saline to remove any unattached cells and cell debris. Differentiation medium was then added {DMEM/F12 plus 10% FBS, 100 nM bovine insulin, 140 nM hydrocortisone, 130 nM bovine holo-transferrin (all differentiation factors obtained from Sigma-Aldrich Inc., St. Louis, MO) ± a long-chain fatty acid (FA)}. The time of addition of differentiation medium was designated as 0 day. Cell culture medium was changed every 2 to 3 days. The cell culture methods are similar to those previously reported for serum-free culture of porcine S-V cells [22–24].

2.2. Fatty acid preparation

Oleic acid, 9(Z)-octadecenoic acid (C18:1), and the CLA isomers, 9(Z), 11(E)-octadecadienoic acid (cis-9, trans-11 CLA or 9,11-CLA) and 10(E), 12(Z)-octadecadienoic acid (trans-10, cis-12 CLA or 10,12-CLA) were purchased from Matreya, Inc. (Pleasant Gap, PA). The FAs were 98+% pure according to the manufacturer. Individual FAs were solubilized in chloroform:methanol (2:1, v/v) to facilitate handling and dilution, the solvent was removed by drying, and the FAs were dissolved in differentiation medium containing 5% fatty acid-free bovine serum albumin (**BSA**; Intergen Co., Purchase, NY) by stirring for at least 120 min. (The buffered neutral pH of the medium ionizes the FAs that then become soluble and bind to the BSA. The FAs are oils that float on the surface of the aqueous medium; after extensive stirring the FAs are solubilized to produce optically clear solutions.) The dissolved FAs were then diluted to the appropriate concentration with differentiation medium. All plates contained the same medium, i.e., DMEM/F12 + antibiotics + fungicide + differentiation factors + 10% FBS + 1% exogenous BSA ± a FA (control plates contained BSA, but no FA).

2.3. Cell number

Each replicate plate for cell count was treated with 0.5 ml of 0.25% trypsin-EDTA (Gibco BRL 25200-056, Invitrogen-Life Technologies) for 5 min at 37°C. After incubation, 0.5 ml of 1X phosphate buffered saline (Gibco BRL 20012-027, Invitrogen-Life Technologies) was added, the cells were rinsed to one end of the plate, mixed by gentle trituition, and an aliquot was counted using a hemocytometer. The resulting count was extrapolated to the total volume representing total cells on the plate.

2.4. Oil Red O staining

Oil Red O staining was performed using a minor modification of a previously described method [25]. The working solution of Oil Red O was prepared by dissolving 4.2 g of Oil Red O (Sigma-Aldrich Inc.) in 1200 ml of absolute isopropanol. This solution was allowed to sit overnight at room temperature without stirring, filtered through Whatman #4 qualitative filter paper (Whatman International Ltd., Maidstone, England), mixed with 900 ml of deionized water, and allowed to sit overnight at 4°C without stirring. The final working solution was filtered twice and stored at room temperature until use. Cell culture plates for staining were fixed for a minimum of 20 min with 10% neutral buffered formalin, drained, and covered with 100% propylene glycol for 3 min. Propylene glycol was removed and the plates were covered with the working solution of Oil Red O for 60 min. The Oil Red O was removed and the plates were subsequently destained with 60% propylene glycol using gentle agitation for 1 min. Finally, the plates were exhaustively rinsed with water and then dried in a 37°C oven for 1 h. Isopropanol was added twice to each dried culture plate to extract the dye from the cells using agitation. The extracted dye was immediately removed, brought to a constant volume with isopropanol, and the absorbance measured spectrophotometrically at 510 nm. The same batch of working Oil Red O solution was used for all determinations. The Oil Red O-stained material (OROSM) was expressed on a per cell basis using the cell numbers determined from similar plates.

2.5. Triacylglycerol and glycerol-3-phosphate dehydrogenase assays

Triacylglycerol (TG) was determined on total cell extracts using saponification coupled with enzymatic determination of glycerol (kit #320-A, Sigma-Aldrich Inc.). Briefly, 100 mm plates containing cell cultures were rinsed two times with phosphate-buffered saline, pH 7.4, and decanted completely. The plates were scraped with a Teflon policeman and the cells from multiple plates were pooled using 0.5 ml of extraction buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM mercaptoethanol at 4°C. The total volume was recorded, after which the extract was placed in

an ice bath and sonicated at 35% of maximum. A 200 μl sample was saponified with 0.5 ml of 95% ethanol plus 1 drop of 8 M KOH at 70°C for 20 min. One mL of 0.15 M $MgSO_4$ was added to the sample at room temperature. The samples were mixed, centrifuged at 21,000 × g for 3 min, and the supernate was decanted. A 200 μl sample of supernate was assayed according to the kit instructions using glycerokinase, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, and NADH. The oxidation of NADH was measured at 340 nm.

Glycerol-3-phosphate dehydrogenase (GPDH) was determined essentially as described [26]. The sonicated extract prepared for TG analysis was centrifuged at 7,000 × g for 5 min at 4°C, then the infranate was removed and centrifuged at 70,000 × g for 60 min at 4°C. An aliquot of the 70,000 × g infranate was assayed for GPDH activity in 100 mM triethanolamine/HCl, pH 7.5, 2.5 mM EDTA, 0.12 mM NADH, 0.2 mM dihydroxyacetone phosphate, and 0.1 mM mercaptoethanol. Reagents were obtained from Sigma-Aldrich Inc. The change in absorbance at 340 nm with time was used to calculate the rate.

Protein was measured on the 70,000 × g infranate, prepared for GPDH activity measurement, by a modified [27], Lowry method [28]; the protein was precipitated with trichloroacetic acid to eliminate interfering substances and BSA was the standard (P5656 kit, Sigma-Aldrich Inc.).

2.6. Transcript concentrations

Total RNA was extracted from the cells of replicate plates using the guanidinium-phenol-chloroform method [29], as previously described [30]. Details of riboprobe preparation and Northern analysis procedures were previously presented [22,24]. Briefly, radio-labeled riboprobes were generated by transcription of linearized plasmids containing the gene fragment of interest using the Strip-EZ kit (Ambion Inc., Austin, TX). Sequences and properties of the porcine gene fragments (GenBank accession number) for PPARγ (AF103946), LPL (AF102859), aP2 (AF102872), and 18S ribosomal RNA (AF102857) were previously reported [22,24]. Total RNA (25μg) from each sample was electrophoresed under denaturing conditions and transferred to nylon membranes. The membranes were prehybridized, hybridized, and washed as previously described [22,24]. The relative abundance of each transcript was then determined using phosphorimagery as previously described [24]. Each individual transcript concentration in a sample lane was normalized using the densitometric value for 18S ribosomal RNA transcript concentration in the same sample lane and presented in arbitrary units. The same radio-labeled riboprobe was used to hybridize all of the membranes at the same time. After evaluation with a single riboprobe, the membranes were stripped using Ambion's Strip-EZ RNA kit (Ambion Inc., Austin, TX) as per the manufacturer's instructions and reconstituted for reprobing with a different riboprobe. This system allowed the same membrane to be reprobed several times with different riboprobes. Potentially low copy transcripts were probed first to insure maximal hybridization signals from those transcripts.

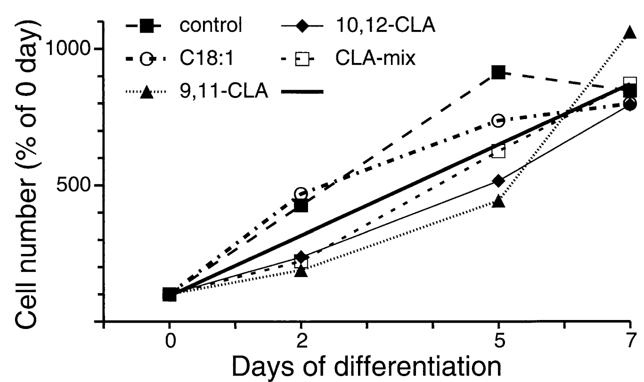

Fig. 1. Porcine preadipocyte proliferation. Stromal-vascular cells were isolated from porcine adipose tissue, plated in DMEM/F12 + antibiotics + fungicide + 10% fetal bovine serum and incubated for 24 h at 37°C. The medium was then changed to DMEM/F12 + antibiotics + fungicide + 10% fetal bovine serum + differentiation factors (insulin, hydrocortisone, and transferrin) + 1% bovine serum albumin ± a fatty acid. The concentration of added fatty acid was control = 0 μM, C18:1 = 200 μM, 9,11-CLA = 50 μM, 10,12-CLA = 50 μM, and CLA-mix = 25 μM 9,11-CLA + 25 μM 10,12-CLA. Cells were harvested at the times indicated using trypsin and were counted in a hemocytometer. The number of cells counted was extrapolated to the total number/plate; data are expressed relative to the 0 days cell number (= 100%). The data represent four replicates, each using cells isolated from a different animal. The 0 days cell number was 195,222 per plate with an SD = 51,268.The data in a replicate, obtained at different incubation times for an individual fatty acid or the control, were fit to a linear regression model. The linear model slopes were parallel (P = 0.82), the intercepts were equal (P = 0.27), and the lines were coincident (P = 0.56). The common line for all conditions is indicated by the solid line with no data points; the slope = 94 and the SD = 225.

2.7. Statistical analysis

A single batch of S-V cells, isolated from an individual pig, was used for each replicate. There were four replicates except for missing samples resulting from poor hybridization for individual transcripts from specific treatment groups at particular times, as indicated in the Fig. legends. A replicate included 0, 2, 5, and 7 days with control cells plus C18:1-, 9,11-CLA-, 10,12-CLA-, and CLA-mix-treated cells. The time course for cell proliferation was analyzed using a regression model. Because the data were not different for the experimental groups, a common linear regression was generated (Fig. 1). The data presented in Figs. 2, 4, and 5 were analyzed by analysis of variance with post hoc mean separation by the Tukey method. At a given time (either 2, 5, or 7 days), data for the experimental groups were compared. Also, the data at a given time (either 2, 5, or 7 days) were compared to 0 days data.

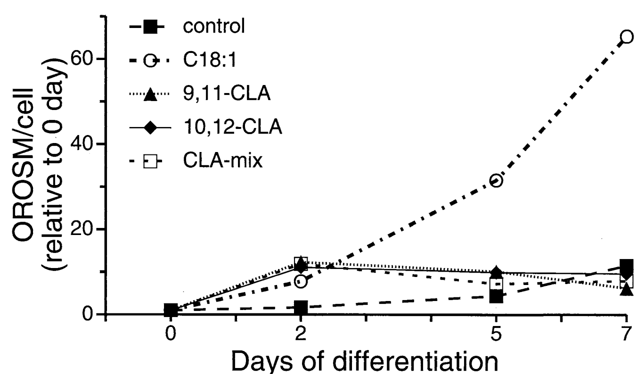

Fig. 2. Accumulation of Oil Red O-stained material in porcine preadipocytes. Porcine stromal-vascular cells were plated and grown exactly as indicated in Fig. 1. At the indicated times, plates were stained with Oil Red O and the extracted stain was quantified spectrophotometrically. The amount of stain extracted from the cells on a plate (OROSM) was expressed relative to the number of cells counted on comparable plates, as in Fig. 1. Data represent the same four replicates indicated in Fig. 1.The data for the control and each fatty acid at a given time (2, 5, and 7 days) were analyzed by analysis of variance. The pooled SDs were 2 days = 2.7, 5 days = 11.3, and 7 days = 16.6. The mean for a given variable (control or an individual fatty acid at a given time, i.e., 2, 5, or 7 days) was also compared to the 0 days value by analysis of variance. At 2 days, the OROSM/cell increased 5+ times ($P < 0.01$) in the C18:1-treated cells and 10+ times ($P < 0.01$) in the 9,11-CLA-, or 10,12-CLA-, or CLA-mix-treated cells relative to 0 days. At 7 days, the OROSM/cell increased 10.5 times ($P = 0.11$) in the control cells and 64 times ($P < 0.01$) in the C18:1-treated cells relative to 0 days. The OROSM/cell increased 6 times ($P < 0.01$) in the CLA-treated cells compared to the control cells at 2 days. There was no further increase in OROSM/cell in the various CLA-treated cells after 2 days. There was no distinction between the CLA isomers or the CLA mixture at any time point.

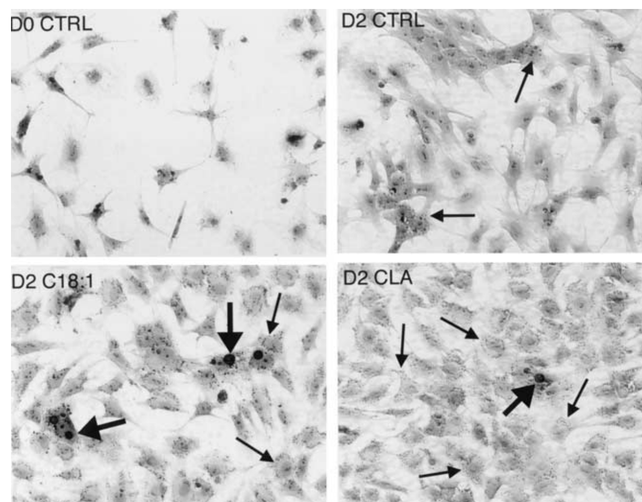

Fig. 3. Cell morphology. Cells on plates were fixed and stained with Oil Red O and counterstained with hematoxylin23 The microscopic magnification was 200X. Panels represent cells at 0 days, and differentiated cells at 2 days (Control, 200 μM C18:1-, and 50 μM 10,12-CLA-treated cells). Thin arrows indicate Oil Red O-stained lipid droplets in the cytoplasm. Thick arrows indicate large cytoplasmic Oil Red O-stained lipid droplets in the C18:1- and 10,12-CLA-treated cells.

3. Results

3.1. Cell proliferation (Figure 1)

Control porcine preadipocytes continuously increased in number over the 7-day period of the study. Cells treated with any of the FAs increased in number in a similar fashion to the controls. The linear regressions for each of the groups were parallel ($P = 0.82$), had equal intercepts ($P = 0.27$), and were coincident ($P = 0.56$). There was no effect of any FA on cell proliferation. At 2 and 5 days, there was a trend, i.e., $P < 0.15 > 0.05$, for the cell number to be decreased in the CLA-treated cells when the data for an individual day were analyzed by ANOVA. There was no difference in cell number at 7 days. The average regression line for all of the data is indicated as the solid line without data points; it increased 7+ times over the 7-day period.

3.2. Oil Red O staining (Figure 2)

The control cells gradually accumulated OROSM, expressed per cell, to reach 10.5 times the 0 days value at 7 days ($P = 0.11$). Addition of 200 μM C18:1 caused OROSM/cell to increase 5+ times between 0 and 2 days ($P < 0.01$) and 64 times ($P < 0.01$) between 0 and 7 days. Addition of 50 μM 9,11-CLA, 50 μM 10,12-CLA, or a mixture of the CLA isomers at 25 μM each, caused a 10+ times increase in OROSM/cell between 0 and 2 days ($P < 0.01$), and a 6 times increase at 2 days compared to the controls at 2 days ($P < 0.01$). There was no further increase in OROSM/cell in the CLA-treated cells after 2 days. There was no distinction between the CLA-treated groups at any time point.

3.3. Morphology (Figure 3)

The increase in cell number between 0 and 2 days is obvious in the Fig.. By 2 days, the control cells accumulated some OROSM as indicated by the small intracellular droplets. The C18:1- and 10,12-CLA-treated cells had large amounts of OROSM at 2 days as indicated by the small droplets, but also by larger lipid droplets. At 2 days, the cells tended to be more rounded in shape than at 0 days, suggesting differentiation.

3.4. PPARγ transcript concentrations (Figure 4)

The PPARγ mRNA concentration doubled in the control cells between 0 and 2 days ($P = 0.15$); it increased 3+ times in the C18:1-treated cells ($P < 0.01$), and increased 35 to 60% in the CLA-treated cells ($P > 0.2$). After 2 days, the PPARγ mRNA concentrations decreased in all groups, including the controls. At 7 days, the control cells had 65% ($P > 0.2$), the C18:1-treated cells had 150% ($P > 0.2$), and the CLA-treated cells had 15 to 30% ($P > 0.15$) of the 0 days mRNA concentration. At 7 days, the mRNA concentration

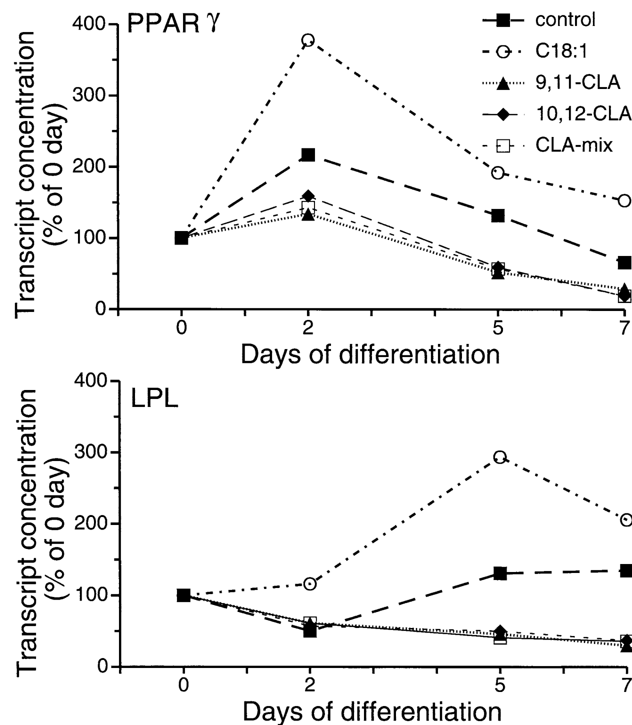

Fig. 4. Porcine preadipocyte transcript concentrations: PPARγ and LPL. Porcine, stromal-vascular cells were plated and incubated as indicated in Fig. 1. At the times indicated, total RNA was extracted from the cells, electrophoresed, transferred to membranes and probed with a radio- labeled porcine riboprobe. The density of the radio-labeled transcript was quantified by phosphor image analysis and normalized to the density of the hybridized 18S ribosomal RNA in the same lane. The data were expressed relative to the 0 days values (= 100%). Data represent the same four replicates indicated in Fig. 1. For PPARγ, N = 3 for the 10,12-CLA- and CLA-mix-treated cells at 7 days because of lost samples. For LPL, N = 3 for the control and CLA-mix-treated cells at 5 days, and N = 2 for the control at 7 days because of lost samples.The data for the control and each fatty acid, at each time point (2, 5, and 7 days), were analyzed by analysis of variance, as indicated in Fig. 2. The pooled SDs for PPARγ were 2 days = 67, 5 days = 51, and 7 days = 43. Compared to 0 days, the PPARγ mRNA concentration doubled at 2 days in control cells ($P = 0.15$), increased 3+ times in C18:1-treated cells ($P < 0.01$) and increased 35 to 60% in CLA-treated cells ($P > 0.2$). At 7 days, the PPARγ mRNA concentration was 65% of the 0 days value in control cells ($P > 0.2$), 150% of 0 days in C18:1-treated cells ($P > 0.2$) and 15 to 30% of 0 days in CLA-treated cells ($P > 0.15$). The PPARγ mRNA concentration was less in the CLA-treated cells than in the C18:1-treated cells at 7 days ($P < 0.01$). There was no difference between the response to the CLA isomers or the CLA-mix at any time point.The pooled SDs for LPL were 2 days = 20, 5 days = 28, and 7 days = 44. At 2 days, compared to 0 days, the LPL mRNA concentration decreased to 50% in control cells ($P < 0.05$), remained the same in C18:1-treated cells ($P > 0.2$), and decreased in each of the CLA-treated cell groups ($P < 0.1$). At 7 days, compared to 0 days, the control LPL mRNA was 135% in control cells ($P > 0.2$) and was tripled in C18:1-treated cells ($P = 0.06$). The mRNA was 30 to 40% of 0 days in the CLA-treated groups ($P > 0.2$). At 7 days, control and C18:1 values were not different ($P > 0.2$), and the CLA values were less than the controls ($P < 0.1$) and C18:1 ($P < 0.01$). There was no distinction between the CLA isomers or the CLA-mix at any time point.

in each CLA-treated group was less than that in the C18:1-treated group ($P < 0.01$). There was no distinction between the CLA isomers at any time point.

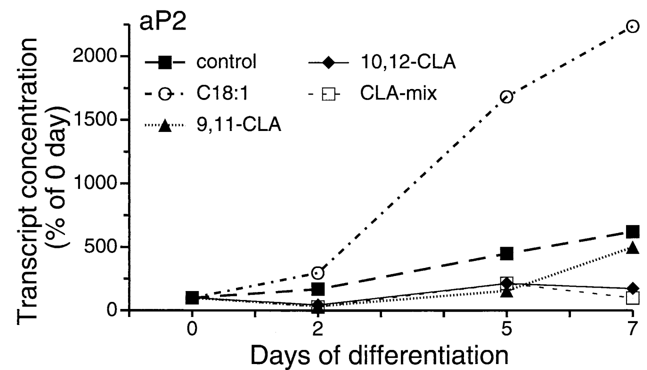

Fig. 5. Porcine preadipocyte aP2 mRNA concentrations. Cells were plated and incubated as indicated in Fig. 1. Total RNA was extracted at the indicated times. The membranes for hybridization were the same as used for Fig. 4. Data were analyzed as for Fig. 4. There were four replicates, each with cells from a different animal, but N = 3 for most data points and N = 2 for a few data points because of loss of samples. The pooled SDs were 2 days = 39, 5 days = 420, and 7 days = 540. The aP2 mRNA concentration in the control cells increased gradually during differentiation to 5 times the 0 days value at 7 days ($P > 0.2$). In C18:1-treated cells, the mRNA was 21 times the 0 days value at 7 days ($P < 0.01$); the mRNA concentration was greater in C18:1-treated cells than in control cells at 7 days ($P < 0.05$). The aP2 mRNA did not differ from the 0 days value for any of the CLA-treated groups at any time point (2, 5, or 7 days). The mRNA concentration was numerically less in the CLA-treated groups than in the control cells or the C18:1-treated cells ($P < 0.05$) at each time point (2, 5, and 7 days).

3.5. LPL transcript concentrations (Figure 4)

Between 0 and 2 days, the LPL mRNA concentrations decreased to 50% in the control ($P < 0.05$) and CLA–treated cells ($P < 0.1$), whereas the mRNA in the C18:1-treated cells did not change. By 7 days, the control cell LPL mRNA concentration was 35% greater than at 0 days ($P > 0.2$), whereas the C18:1-treated cell LPL mRNA was doubled compared to 0 days ($P = 0.06$). The 7-day control and C18:1-treated cell LPL mRNA concentrations were not different ($P > 0.2$). On the other hand, the LPL mRNA concentration in the CLA-treated cells had decreased to 30 to 40% of the 0 days values by 7 days ($P > 0.2$). At 7 days, the LPL mRNA concentrations for the CLA-treated groups were less than in the controls ($P < 0.1$) or the C18:1-treated cells ($P < 0.01$). There was no distinction between CLA isomers at any time point.

3.6. Transcripts for aP2 (Figure 5)

The aP2 mRNA concentration increased gradually in the control cells between 0 and 7 days to reach 5 times the 0 days value ($P = 0.05$). The aP2 mRNA concentration increased 21 times between 0 and 7 days ($P < 0.01$) in the C18:1-treated cells and was greater than in control cells at 7 days ($P < 0.05$). In the CLA-treated cells, the aP2 mRNA concentration was not different from the 0 days values ($P > 0.2$) at any time point. At 2 days, the aP2 mRNA concentration was lower in the CLA-treated porcine cells than in

the controls ($P < 0.1$) or in the C18:1-treated cells ($P < 0.01$). The aP2 mRNA concentration was less in the CLA-treated cells than in the C18:1-treated cells at 5 days ($P < 0.1$) and at 7 days ($P < 0.05$). There was no distinction between the CLA-treated groups at any time point.

Although aP2 mRNA concentration has been used as a marker for adipocyte differentiation, including differentiation of porcine S-V cells [22], we found that aP2 was not a differentiation marker for porcine cells when FAs were present in the medium. The aP2 mRNA concentration was increased in the presence of several FAs when differentiation was not increased (S-T Ding and HJ Mersmann, unpublished data).

4. Discussion

4.1. Culture system

Conditions used to differentiate preadipocytes vary with the requirements of the particular preadipocyte preparation, but also with the laboratory. For example, with porcine S-V cells, a glucocorticoid is necessary for differentiation [31–33]. However, it is removed after a time by some laboratories [34,35], but kept in the medium by others [23,33]. Porcine S-V cells continuously exposed to a glucocorticoid undergo considerable differentiation in both serum-free medium [22,23,33,36] and in serum-containing medium (e.g., C18:1-treated cells in Figs. 2 and 3).

Serum stimulates proliferation, but retards differentiation [23,34]. Regardless, porcine S-V cells were differentiated in 10% FBS (Fig. 2) because it prevented the major cell losses encountered upon addition of a polyunsaturated fatty acid (PUFA) to serum-free medium [36]. Lower concentrations of FBS did not prevent PUFA-induced cell loss (RL McNeel, unpublished data). Concomitant with cell proliferation (Fig. 1), OROSM, expressed per cell, increased (Figs. 2 and 3) in porcine S-V cells cultured with FBS and differentiation factors. In the C18:1-treated cells, visual appraisal indicated >90% of the cells had OROSM at 7 days; consequently, C18:1 treatment was used as a positive control. The culture system with FBS plus differentiation factors is somewhat like the situation in vivo (particularly obvious in tissue from young animals), wherein there is concomitant hyperplasia and differentiation (i.e., accumulation of OROSM) in adipose tissue. We are not suggesting that differentiated cells divide.

The accumulation of OROSM is presumed to represent an increase in TG. Firstly, the staining method detects TG and cholesterol esters, but not unesterified FA or phospholipids [25]. Secondly, the OROSM accumulates in discrete, visible droplets, as does lipid (the majority of which is TG) in adipocytes in vivo [37]. Thirdly, an additional two replicates measured OROSM, and TG in control, C18:1-treated, and 10,12-CLA-treated porcine S-V cells at 0, 2, 5, and 7 days of differentiation. The correlation of OROSM/plate with TG/plate was 0.81. Finally, it is unlikely that OROSM represents accumulation of unesterified FAs provided in the serum or medium, because the detergent properties of the FAs would lead to cytotoxicity.

Much of the increase in OROSM/cell probably represents increased differentiation, particularly in the C18:1-treated cells wherein the PPARγ and LPL mRNA concentrations were increased (Fig. 4). Although LPL is a standard marker for adipocyte differentiation, the enzyme activity is not necessary for TG accumulation when unesterified FAs are present in the medium. Previous studies indicated C18:1 treatment increased porcine S-V cell OROSM and transcript concentrations in a dose-dependent and time-dependent manner in serum-free medium [36].

4.2. Effects of CLAs on preadipocyte hyperplasia

A potential mechanism for the dietary CLA-induced decrease in fat deposition is a decrease in preadipocyte hyperplasia, as demonstrated in CLA-treated rodent-derived clonal preadipocytes, i.e., 3T3-L1 cells, by three different laboratories [16–18]. The extent of hyperplasia in porcine preadipocytes treated with CLA under our experimental conditions was not different from that observed in the control preadipocytes. Potential explanations for the divergence of results with porcine and clonal preadipocytes are: 1. The method for detecting hyperplasia, i.e., cell counting was defective. This is unlikely because cell counting was used for some of the studies with 3T3-L1 cells, and the correlation of cell counts with the viable cell number determined by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI) was $r^2 = 0.99$ (RL McNeel, unpublished data). 2. Perhaps the CLA concentration was too low. However, 50 μM CLA was equal to or even greater than the CLA concentrations used with 3T3-L1 cells. 3. The 3T3-L1 cell experiments studied cell proliferation in serum-containing medium, but there were no exogenous differentiation factors in the medium as was the case for the porcine preadipocyte studies. 4. There may be species differences. Human preadipocytes had decreased hyperplasia when cultured in the presence of 9,11-CLA or 10,12-CLA under conditions almost identical to those used for the porcine preadipocytes (RL McNeel and HJ Mersmann, unpublished data). Thus, the divergent results between 3T3-L1 cells and porcine S-V cells may result from different culture conditions, distinctions between clonal and primary preadipocytes, or species-specific responses. It should be noted that CLA-treated rats do not have a decreased adipocyte number [7,38].

4.3. Effects of CLAs on adipocyte differentiation

A dose-dependent decrease in 3T3-L1 preadipocyte differentiation was observed in 3T3-L1 cells treated with 9,11-CLA [17], or 10,12-CLA or a mixture of CLA isomers [18]. However, a third laboratory observed a dose-dependent

increase in lipid synthesis and accumulation of OROSM when 3T3-L1 preadipocytes were treated with a mixture of CLA isomers [16]. There are many subtle differences between these experiments in different laboratories, but an explanation for the divergent results is not apparent. Although not a clear explanation for the divergence in outcomes, it was recently reported that 9,11-CLA-treated 3T3-L1 cells have increased TG, whereas 10,12-CLA-treated cells have decreased TG [19]. Treatment of human preadipocytes with 10,12-CLA also decreased differentiation [39]. In CLA-treated porcine preadipocytes, in serum-free medium, there was an acute increase in Oil Red O-stained cells at 1 and 2 days [40,41], as well as an increase in OROSM/cell at 2 days with no further increase at 5 or 7 days in serum-containing medium (Fig. 2). The effects of CLA on preadipocyte differentiation are not clearly defined; they probably depend on the exact experimental conditions, the species studied, and perhaps the CLA isomers used.

As preadipocytes differentiate, the mRNA concentration for key transcription factors, such as PPARγ and CCAAT/enhancer binding protein α (C/EBPα), and adipocyte characteristic proteins, such as LPL and aP2 is expected to increase [42,43]. In 3T3-L1 cells treated with 9,11-CLA, the decreased differentiation was accompanied by decreased mRNA concentrations for PPARγ, C/EBPα, and aP2 [17]. Also, the LPL activity was decreased in CLA-treated 3T3-L1 adipocytes [3,44]. In contrast to these results, 10,12-CLA inhibited TG deposition in differentiating 3T3-L1 preadipocytes, but increased the PPARγ protein concentration, whereas 9,11-CLA did not modify the TG and tended to increase the PPARγ protein concentration; the aP2 mRNA concentration was not changed by either CLA isomer [19]. In porcine S-V cells differentiated in serum-containing medium containing CLAs, there was no increase in PPARγ mRNA concentration at 2 days or LPL mRNA concentration at 5 and 7 days (Fig. 4). Like the effects on differentiation, the effects of CLAs on transcription factor transcripts are also mixed. Perhaps when transcript concentrations do not change in concert with changes in OROSM or TG, these changes do not represent modulation of differentiation.

4.4. Acute effects of CLAs

Does the increase in OROSM (presumably TG) in CLA-treated cells between 0 and 2 days represent increased differentiation? For a cell to accumulate TG, the TG biosynthesis pathway must be active. The GPDH activity in undifferentiated porcine preadipocytes is low and increases as differentiation progresses and the cells accumulate OROSM [33]. We measured the OROSM/cell, TG/cell, and GPDH activity/cell in porcine S-V cells differentiated in serum-containing medium (two replicates). These variables were not increased in control cells after 2 days of differentiation. In cells treated with 200 μM C18:1, at 2 days, these variables were 250, 230, and 180% of the 0 days values (expressed as 100%), respectively. In cells treated with 50 μM 10,12-CLA, at 2 days, these variables were 210, 157, and 132%, respectively. Expressed per mg protein, the GPDH activity at 2 days was 175% of 0 days in both the C18:1- and CLA-treated cells, but only 80% of 0 days in the control cells. The data suggest that the CLA-treatment increased differentiation of porcine preadipocytes at 2 days.

Porcine S-V cells readily differentiate in DMEM/F12 serum-free medium [22–24,33], but because this medium contains only 0.15 μM linoleic acid, medium FAs probably are not used for esterification. Differentiation probably is dependent on the development of FA biosynthesis because undifferentiated porcine cells have limited capacity to synthesize FAs de novo [45] and minimal FA synthase mRNA [24]; both increase during differentiation. The 10% FBS in our serum-containing medium added 32 μM FAs with the major FAs being 9 μM palmitic acid, 2 μM palmitoleic acid, 4 μM stearic acid, 7 μM oleic acid, 2 μM linoleic acid, and 4 μM arachidonic acid. Control cells incubated in FBS-containing medium gradually accumulated OROSM/cell, whereas additions of 200 μM C18:1 or 50 μM CLAs substantially increased accumulation of OROSM/cell (Fig. 2 and 3). The data are not incompatible with the possibility that high concentrations of medium FAs might be used for TG synthesis.

Although the entire effect of exogenous FAs (C18:1 or CLAs) to increase OROSM/cell may be to provide a source of FA for esterification, an alternative or perhaps concomitant mechanism is that the increase in OROSM at 2 days in C18:1- or CLA-treated cells results from provision of ligand for PPARγ at a time when de novo FA synthetic capacity is low and much of the FA available is utilized for membrane synthesis. In porcine S-V cells, there is considerable PPARγ mRNA at 0 days, the time of initiation of differentiation [22]. The PPARγ1 protein is detectable in undifferentiated porcine S-V cells, and the PPARγ2 protein is detectable after 1 day of incubation in 10% FBS (comparable to our 0 days) [46]. Both 9,11-CLA and 10,12-CLA bind to PPARγ [47,48], and 9,11-CLA activates PPARγ [49]. Thus, addition of a limiting PPARγ ligand would be expected to stimulate porcine preadipocyte differentiation without a change in PPARγ transcript or protein concentration.

Experiments with differentiating human S-V cells strengthen the concept that CLAs act as PPARγ ligands to acutely stimulate differentiation. Human adipocytes have minimal capacity to synthesize FAs [50]. Human preadipocytes do not differentiate unless a PPARγ ligand is added to the medium. Although a thiazolidinedione compound is the usual ligand, addition of C18:1, or 9,11-CLA or 10,12-CLA acutely increased the OROSM/cell (RL McNeel, unpublished observations).

4.5. Do CLAs inhibit porcine S-V cell differentiation?

Culture of porcine cells in FBS plus differentiation factors is not an optimal system to measure inhibition of

differentiation because the control cells differentiate at a slow rate. In the CLA-treated cells, there was an initial increase in OROSM/cell between 0 and 2 days suggesting an increase in differentiation, but the OROSM/cell did not continue to increase after 2 days (Fig. 2). The lack of continued accumulation of OROSM/cell in the CLA-treated cells compared to the control or C18:1-treated cells suggests CLA inhibited differentiation after the acute increase between 0 and 2 days. Furthermore, CLA decreased the transcripts for adipocyte determination and differentiation-dependent factor 1 in differentiating porcine preadipocytes, suggesting a potential mechanism to reduce adipocyte growth by inhibiting anabolic lipid metabolism [41].

4.6. Isomers of CLA

Most animal feeding trials use synthetic preparations of CLA containing approximately 40 to 80% CLA, with 9,11-CLA and 10,12-CLA being present in approximately equal proportions and together accounting for 60 to 85% of the CLA present. Evidence is accumulating that 9,11-CLA and 10,12-CLA produce different biological effects [2]. Studies in mice suggest that 10,12-CLA is responsible for the decreased body fat and increased body protein, whereas 9,11-CLA has little effect on body composition [6,15]. Mice fed 10,12-CLA, but not 9,11-CLA, have decreased mRNA expression for hepatic stearoyl-CoA desaturase mRNA, an enzyme involved in the synthesis of monounsaturated fatty acids [51]. The decrease in bovine milk fat synthesis is caused by 10,12-CLA and not by 9,11-CLA [52]. The decrease in LPL activity and accumulation of TG in 3T3-L1 adipocytes results from 10,12-CLA and not 9,11-CLA [15,19,20]. However, in differentiating porcine preadipocytes, i.e., S-V cells, we found no distinction between 50 μM 9,11-CLA and 50 μM 10,12-CLA.

4.7. Conclusions

In contrast to the results in rodent-derived 3T3-L1 preadipocytes, CLAs did not inhibit proliferation in porcine preadipocytes. As in 3T3-L1 cells, chronic exposure of porcine preadipocytes to CLAs decreased OROSM/cell, at least compared to that in the presence of C18:1. However, acute exposure of porcine cells to CLAs increased OROSM/cell in both serum-free [40] and serum-containing medium (Fig. 2). Finally, in contrast to some experiments with rodents or rodent-derived cells, we did not observe any difference between the 9,11-CLA and 10,12-CLA isomers regarding effects on proliferation, differentiation, or PPARγ, LPL, or aP2 mRNA concentrations in porcine S-V cells. The divergent results between rodent cells and porcine cells may result from different culture conditions, distinctions between clonal and primary preadipocytes, or species-specific responses.

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