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Structure–activity relationship of conjugated linoleic acid and its cognates in inhibiting heparin-releasable lipoprotein lipase and glycerol release from fully differentiated 3T3-L1 adipocytes

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Abstract

Conjugated linoleic acid (CLA) reduces body fat in part by inhibiting the activity of heparin-releasable lipoprotein lipase (HR-LPL) activity in adipocytes, an effect that is induced by the *trans*-10,*cis*-12 CLA isomer. In this study we used a series of compounds that are structurally related to CLA (i.e., CLA cognates) to investigate the structural basis for this phenomenon. None of the 18:1 CLA cognates that were tested, nor *trans*-9,*cis*-12 18:2, *cis*-12-octadecen-10-ynoic acid (10y,*cis*-12) or 11-(2′-(n-pentyl)phenyl)-10-undecylenic acid (designated P-*t*10), exhibited any significant effect on HR-LPL activity. Among the CLA derivatives (alcohol, amide, and chloride) that were tested, only the alcohol form inhibited HR-LPL activity, although to a lesser extent than CLA itself. In addition, intracellular TG was reduced only by *trans*-10,*cis*-12 CLA and the alcohol form of CLA. Hence it appears that the *trans*-10,*cis*-12 conjugated double bond in conjunction with a carboxyl group at C-1 is required for inhibition of HR-LPL activity, and that an alcohol group can partially substitute for the carboxyl group. We also studied glycerol release from the cells, observing that this was enhanced by *trans*-10 18:1, *trans*-13 18:1, *cis*-12 18:1, *cis*-13 18:1, P-*t*10 but was reduced by *cis*-9 18:1, the alcohol and amide forms of CLA or 10y,*cis*-12. Accordingly the structural feature or features involved in regulating lipolysis appear to be more complex. Despite enhancing lipolysis in cultured 3T3-L1 adipocytes, *trans*-10 18:1 did not reduce body fat gain when fed to mice.

Keywords: Conjugated linoleic acid; CLA; Lipoprotein lipase; 3T3-L1; *cis*-9; *trans*-11 CLA; *trans*-10; *cis*-12 CLA

1. Introduction

In the last decade, conjugated linoleic acid (CLA) has been studied intensively because of its unusual biological activities [1]. One of the most interesting aspects of CLA is its ability to reduce body fat while increasing lean body mass [2]. As part of the mechanism for these activities, it has been suggested that CLA reduces heparin-releasable lipoprotein lipase (HR-LPL) activity in adipocytes, and in fact this has been demonstrated in 3T3-L1 adipocyte cell culture model [2]. CLA also enhances fatty acid β-oxidation in muscle, which indicates increased use of fat as an energy source [2]. Since CLA is a mixture of isomers, it is of interest to determine which isomer is responsible for these biological activities. Previously we showed that the *trans*-10,*cis*-12 isomer of CLA (one of two major isomers present in synthetically prepared CLA) is responsible for its reduction in body fat and for inhibition of HR-LPL activity in 3T3-L1 adipocytes [3]. This isomer is also responsible for the inhibition of stearoyl-CoA desaturase (SCD) [4], and reducing apolipoprotein B (apo B) secretion in cultured HepG2 cells [5]. Thus it is of interest whether this CLA isomer may also be solely responsible for inhibition of HR-LPL activity. To determine the key structural feature of *trans*-10,*cis*-12 CLA, we tested fatty acids that are structurally related to *trans*-10,*cis*-12 CLA using 3T3-L1 adipocytes. We also tested these compounds on glycerol release and intracellular triacylglyceride (TG) to estimate lipolysis and total fat deposit, respectively. In addition, since previous findings indicate that a *trans*-10 double bond is a key structure for inhibiting apo B secretion in HepG2 cells [5], we tested *trans*-10 18:1 on body compositional changes compared to *trans*-10,*cis*-12 CLA.

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2. Methods and materials

2.1. Materials

Triolein, [9,10-^{3}H (N)] triolein (specific activity 12 Ci/mmol) was obtained from American Radiolabeled Chemicals Incorporation (St. Louis, MO) and [1-^{14}C] linoleic acid (specific activity 55 mCi/mmol) was obtained from Amersham Life Science (Arlington Heights, IL). 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Rockville, MD). *trans*-9,*cis*-12 octadecadienoic acid (31.7%), *cis*-12 octadecenoic acid (98.1%), *trans*-10 octadecenoic acid (89.9% for cell culture and 85.1% for animal feeding study), 11-(2′-(n-pentyl)phenyl)-10-undecylenic acid (P-t10, 95%, E:Z about 7:3), *trans*-10,*cis*-12/*cis*-9,*trans*-11-octadecadien-1-ol (CLA-Alc., 95%), *trans*-10,*cis*-12/*cis*-9,*trans*-11-octadecadienamide (CLA-amide, 95%), 1-chloro-*trans*-10,*cis*-12-octadecadiene (CLA-Cl, 95%), and *cis*-12-octadecen-10-ynoic acid (10y,*cis*-12, 94.5%) were prepared by chemical synthesis by Dr. Sih and colleagues, at the Department of Pharmacy, University of Wisconsin–Madison. CLA was prepared as described [6]; the composition of CLA was 45.7% *cis*-9,*trans*-11, 47.6% *trans*-10,*cis*-12, 1.71% *trans*,*trans*, 3.04% other isomer. In addition, *cis*-9,*trans*-11 (96.3%, 2.6% *trans*,*trans* isomer, and 1.08% others) and *trans*-9,*trans*-11 (100%) CLA were purchased from Matreya Inc. (Pleasant Gap, PA). Natural Lipids (Hovdebygda, Norway) kindly provided *trans*-10,*cis*-12 CLA (92.8% *trans*-10,*cis*-12, 1.61% *cis*-9,*trans*-11, 1.16% *trans*,*trans*, and 1.64% others). We purchased *cis*-10 (99%), and *trans*-10 (99%) heptadecenoic acid and *cis*-10 (99%), and *trans*-10 (99%) nonadecenoic acid from Nu-Chek Prep Corporation (Elysian, MN), and purchased *trans*-9 (100%), *trans*-11 (100%), *trans*-12 (100%), *trans*-13 (100%), *cis*-9 (99%), *cis*-11 (100%) and *cis*-13 (100%) octadecenoic acids from Sigma Chemical Co. (St. Louis, MO). Purity was checked by gas chromatography using a Hewlett-Packard 5890 series II chromatograph (Hewlett-Packard, Andover, MA) fitted with a flame ionization detector and 3396A integrator. A Supelcowax-10 fused silica capillary column (60 m × 0.32 mm, inner diameter film thickness 0.25 μm) was used and oven temperature was programmed from 50° to 190°C, increased 20°C/min, held for 40 minutes, increased 10°C/min to 220°C, and held for 20 minutes.

2.2. Cell culture

The 3T3-L1 preadipocytes were cultured as described [7]. Briefly, 3T3-L1 preadipocytes were grown to confluence at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). At 2 days postconfluence (designated day 0), cell differentiation was induced with a mixture of methylisobutylxanthin (0.5 mmol/L), dexamethasone (0.25 μmol/L), and insulin (1 μg/mL) in DMEM containing 10% FBS. On day 2 this medium was replaced with medium containing 10% FBS and insulin only. On day 4 and thereafter the medium consisted of DMEM plus 10% FBS only; this medium was subsequently replaced with fresh medium at 2-day intervals. Fatty acid–albumin complexes were prepared as described [2] and added to culture media for 48 hours at day 6, and cells were harvested at day 8. Final concentrations of fatty acids are indicated in each figure legend. All dishes including control had a final concentration of 100 μmol/L albumin. For the experiments with CLA derivatives, test compounds as well as CLA were dissolved in ethanol. In these experiments, control and treatment dishes all contained the same concentration of albumin (with no fatty acid) and ethanol (final concentration of 0.3%). At harvest, medium was collected, centrifuged to remove cell debris, and used for glycerol release analysis. Cells were then washed three times with phosphate buffered saline (PBS) and incubated with heparin containing DMEM (10 U/mL) for 1 hour, and the media was used for determining HR-LPL activity. Subsequently, cells were washed three times with PBS, scraped, and sonicated to give homogenious samples for TG determination. LPL activity (EC 3.1.1.34) was measured as described [8]. Briefly, concentrated substrate was prepared by combining 300 mg triolein, 18 mg lecithin, and 200 μL of [^{3}H] triolein in 5 mL glycerol and homogenizing. Fresh assay substrate was prepared by mixing 1 part concentrated substrate, 4 parts 0.2 mol/L Tris/HCl (pH 8.0) with 3% bovine serum albumin, and 1 part heat inactivated fasted rat serum. The reaction was started by adding 100 μL of samples to the same volume of assay substrate. After incubating at 37°C for 15 minutes, the assay was stopped by adding 3.25 mL methanol: chloroform:heptane solution (1.41:1.25:1). Subsequently, 1.05 mL 0.1 mol/L potassium carbonate/borate buffer (pH 10.5) was added, and the tubes were shaken vigorously and centrifuged at 3000 rpm for 20 minutes. A 200-μL quantity of the upper layer was used for radioactivity count. Nonreacted assay substrate was also used to get total count from each experiment. Recovery of free fatty acid was estimated at 71% using [^{14}C] linoleic acid.

Free and esterified glycerol were determined in the medium and cell sonicates using a Sigma Diagnostic Kit (GPO Trinder, St. Louis, MO). This kit used a two-step process; free glycerol was measured without lipase, then lipase was added to hydrolyse esterified glycerol. The difference between the amounts of free and total glycerol is esterified glycerol. Protein was determined in LPL and TG samples using Bio-Rad DC Protein assay kit (Hercules, CA).

2.3. Animal studies and body composition analyses

Male retired breeder ICR mice and semipurified diet (TD94060, 99% basal mix) were purchased from Harlan Sprague-Dawley and Harlan Teklad (Madison, WI), respectively. The diet was composed as follows (ingredient, g/kg): sucrose, 476; casein, vitamin-free test, 210; corn starch, 150; DL-methionine, 3; corn oil 55; cellulose, 50; mineral mix, AIN-76, 35; vitamin mix, AIN-76A, 10; calcium car-

Table 1
Effects of *trans*-10 octadecenoic acid on body composition in mice

	ECW (g)	% Fat	% Water	% Protein	% Ash
Control	38.2 ± 1.3	12.71[a] ± 1.69	59.3[ab] ± 1.2	20.83[ab] ± 0.62	3.70 ± 0.06
t10,c12 CLA	37.8 ± 1.9	7.67[b] ± 1.11	63.0[a] ± 0.8	21.77[a] ± 0.27	3.88 ± 0.16
t10–18:1	40.4 ± 1.2	15.33[a] ± 1.50	57.9[b] ± 1.3	19.74[b] ± 0.41	3.88 ± 0.19

Twenty male retired breeder ICR mice were used. After a 5-day adaptation period mice were randomly separated into three groups and fed control diet, diet supplemented with 0.27% *trans*-10,*cis*-12 CLA (t10,c12 CLA), or diet supplemented with 0.27–0.29% *trans*-10 octadecenoic acid (t10–18:1) for 3 weeks. Numbers are mean ± SE ($n = 5$). Means with different superscript letters in each column are significantly different at $P < 0.05$. CLA = conjugated linoleic acid; ECW = empty carcass weight.

bonate, 4; choline bitartrate, 2; and ethoxyquin, 0.1. The mineral mix (AIN-76) was composed as follows (ingredient, g/kg): calcium phosphate (dibasic), 500; potassium citrate (monohydrate), 220; sodium chloride, 74; potassium sulfate, 52; magnesium oxide, 24; ferric citrate, 6.0; manganous carbonate, 3.5; zinc carbonate, 1.6; cupric carbonate, 0.3; chromium potassium sulfate, 0.55; potassium iodate, 0.01; sodium selenite, 0.01; and sucrose, 118. The vitamin mix (AIN-76A) was composed as follows (ingredient, g/kg): thiamin HCl, 0.6; riboflavin, 0.6; pyridoxine HCl, 0.7; niacin, 3.0; calcium pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; vitamin B_{12} (0.1% trituration in mannitol), 1.0; dry vitamin A palmitate (500,000 U/g), 0.8; dry vitamin E acetate (500 U/g), 10.0; vitamin D_3, trituration (400,000 U/g), 0.25; menadione sodium bisulfite complex, 0.15, and sucrose, 981.08.

Supplemental CLA (5 g/kg) was added to diets at the expense of corn oil. Diet was stored at −20°C until use. Mice were housed individually in wire-bottomed cages in a windowless room with a 12-h light-dark cycle in strict accordance with guidelines established by the Research Animal Resources Center of University of Wisconsin–Madison. Diet and water, available ad libitum, were freshly provided three times per week. After a 5-day adaptation period, mice were randomly separated into groups and fed control diet, diet supplemented with *trans*-10,*cis*-12 CLA (0.27%), or diet supplemented with *trans*-10 octadecenoic acid (0.29%) for 3 weeks.

For body composition analyses, animals were sacrificed, gut contents were removed (to obtain empty carcass weight [ECW]), and the carcasses frozen at −20°C. Frozen carcasses were chopped and freeze-dried to determine water content. Each dried carcass was ground to give a homogeneous sample before further analysis. Total nitrogen was analyzed by the Kjeldahl method [9] by the Department of Soil Science, University of Wisconsin-Madison. Carcass fat content was measured by extraction with diethyl ether overnight using a Soxhlet apparatus. Total ash content was determined by incineration (500–600°C, overnight).

2.4. Statistical analyses

One-way analysis of variance was performed on data presented in Table 1, and two-way analysis of variance (treatments and experiments) was performed on data depicted in the figures (individually cited later here). Of major interest here are the comparisons among the treatments; these were computed using the Statistical Analysis System (SAS Institute Inc., Cary, NC) with the general linear mean procedure and least square means options. If the interaction between treatment and experiment was significant, this interaction was then used as the error term in the least square means analysis.

3. Results

The concentrations used in this report are based on the finding that sera CLA levels of rats fed diet supplemented with 0.5% CLA for 28 days were 72 μmol/L (range 23–120μmol/L) [2]. Thus in this study, we used 50 μmol/L for single isomers and 100 μmol/L for mixed isomers.

Experiments were done in multiple sets of quadruplicates. Figures 1A–1D show inhibition of HR-LPL activity by various 18-carbon fatty acids. As previously reported, the *trans*-10,*cis*-12 isomer of CLA consistently and significantly reduced HR-LPL activity in 3T3-L1 adipocytes [3]. This effect is shown in all four sets of experiments and used as a negative control. In contrast the monounsaturated octadecenoic acids (18:1) with double bonds at *cis*-9, *cis*-13, *trans*-12, *trans*-13 (Fig. 1A), *cis*-11, *trans*-11, *trans*-9 (Fig. 1B), *cis*-12 (Fig. 1C), or *trans*-10 (Fig. 1D) exhibited no effects on HR-LPL activity. In addition the linoleic acid isomer *trans*-9,*cis*-12 18:2 (Fig. 1D), 10y,*cis*-12 (an 18-carbon structure similar to *trans*-10,*cis*-12 CLA but with a triple bond instead of a double bond at C:10) (Fig. 1C), and P-t10 (11-(2′-(n-pentyl)phenyl)-10-undecylenic acid) (a *trans*-10,*cis*-12 CLA related structure in which the *trans*-10 double bond is part of a benzene ring) did not exhibit any effect on HR-LPL activity (Fig. 1B). The effect of *cis*-9 18:1 (oleic acid) was marginal in that it enhanced HR-LPL activity in three experiments but induced no significant effect in three comparable experiments.

To determine the effects of these CLA cognates on lipolysis and fat deposition, respectively, we measured glycerol release into media and the amount of TG within cells. As previously reported [3], *trans*-10,*cis*-12 CLA increased lipolysis in this model. The 18:1 compounds with double

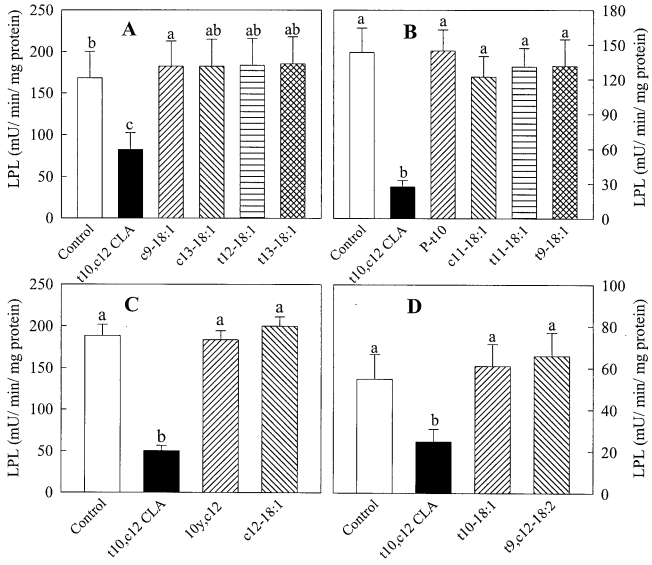

Fig. 1. Effects of 18-carbon fatty acids on heparin-releasable lipoprotein lipase (HR-LPL) activity in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds as an albumin complex for 48 hours. All dishes including control contained the same concentration of albumin (100 μmol/L). Numbers are mean ± SE, n = 23–24 for (*A*) from six independents experiments, n = 10–12 for (*B*) and (*C*) from three independent experiments, and n = 7–8 for (*D*) from two independent experiments. Means with different letters in each figure are significantly different at $P < 0.05$. Abbreviations and final concentrations used are as follows: (*A*) t10,c12 CLA, *trans*-10,*cis*-12 conjugated linoleic acid, 46 μmol/L; c9-18:1, *cis*-9 octadecenoic acid, 50 μmol/L; c13-18:1, *cis*-13 octadecenoic acid, 50 μmol/L; t12-18:1, *trans*-12 octadecenoic acid, 50 μmol/L; t13-18:1, *trans*-13 octadecenoic acid, 50 μmol/L; (*B*) P-t10, 11-(2′-(n-pentyl)phenyl)-10-undecylenic acid, 48 μmol/L; c11-18:1, *cis*-11 octadecenoic acid, 50 μmol/L; t11-18:1, *trans*-11 octadecenoic acid, 50 μmol/L; t9-18:1, *trans*-9 octadecenoic acid, 50 μmol/L; (*C*) 10y,c12, *cis*-12-octadecen-10-ynoic acid, 47 μmol/L; c12-18:1, *cis*-12 octadecenoic acid, 49 μmol/L; and (*D*) t10-18:1, *trans*-10 octadecenoic acid, 45 μmol/L; t9,c12-18:2, *trans*-9,*cis*-12 octadecadienoic acid, 32 μmol/L.

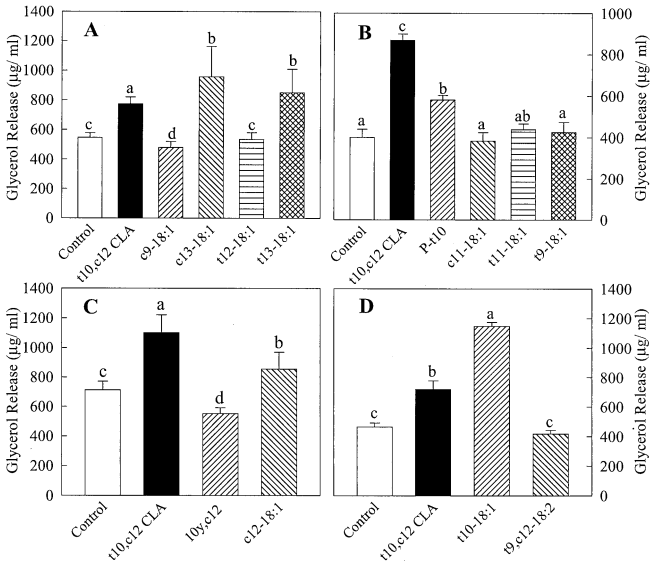

Fig. 2. Effects of 18-carbon fatty acids on glycerol release in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds as an albumin complex for 48 hours. At the end of incubation, culture medium was collected and analyzed for free glycerol after centrifugation. All dishes including control contained the same concentration of albumin (100 μmol/L). Numbers are mean ± SE, n = 23–24 for (*A*) from six independent experiments, n = 10–12 for (*B*) and (*C*) from three independent experiments, and n = 7–8 for (*D*) from two independent experiments. Means with different letters in each figure are significantly different at $P < 0.05$. For abbreviations and final concentrations used, see legend to Fig. 1.

bonds at the *trans*-12 (Fig. 2A), *cis*-11, *trans*-11, or *trans*-9 (Fig. 2B) had no effect on glycerol release. However, the 18:1 compounds with double bonds at *cis*-9 (Fig. 2A) reduced glycerol release, whereas 18:1 with double bonds at *cis*-13, *trans*-13 (Fig. 2A) (Fig. 2C) or *trans*-10 positions (Fig. 2D) enhanced glycerol release. *Trans*-9, *cis*-12 18:2 had no effect on glycerol release (Fig. 2D). P-*t*10 increased (Fig. 2B) and 10y, *cis*-12 (Fig. 2C) decreased glycerol release in this system. Hence the structural feature or features involved in regulating lipolysis appear to be complex.

Total intracellular TG was reduced by *trans*-10,*cis*-12 CLA as reported previously [3]. All 18-carbon monounsaturated fatty acids tested had no effect on total TG (Fig. 3A–3D). No effect on TG was observed by treating with 10y, *cis*-12 (Fig. 3C), P-t10 (Fig. 3B), or *trans*-9, *cis*-12 18:2 (Fig. 3D).

We also tested CLA derivatives that have functional groups other than carboxylic acid. Compounds with alcohol, amide, and chloride groups were prepared and were tested for effects on HR-LPL activity, glycerol release, and intracellular TG. Since these compounds do not have the carboxylic group needed to complex with albumin, they were dissolved in ethanol before addition to the culture medium. The HR-LPL activity in cells treated with CLA dissolved in ethanol was not significantly different than when CLA was treated complexed to albumin (Fig. 4B). The alcohol form of CLA slightly reduced HR-LPL activity while the amide form of CLA (Fig. 4A), as well as the chloride form of CLA (Fig. 4B) had no effect on HR-LPL activity.

Glycerol release was increased by CLA and reduced by the alcohol and amide forms of CLA (Fig. 5A). The chloride form of CLA did not have any effect on glycerol release (Fig. 5B). Total TG was reduced by *trans*-10,*cis*-12 CLA as well as the alcohol form of CLA, but not by the amide and chloride forms of CLA (Figs. 6A and 6B).

Of the 17- and 19-carbon monounsaturated fatty acids with a double bond at the 10th position (either *cis* or *trans* configuration) that were studied, only *cis*-10 17:1 inhibited HR-LPL activity, but it was less active than *trans*-10,*cis*-12 CLA (Fig. 7A). In addition, *cis*-10 17:1 increased glycerol release whereas *trans*-10 17:1, *cis*-10 19:1 or *trans*-10 19:1, decreased glycerol release (Fig. 7B). *Cis*-10 17:1 and *cis*-10 19:1 slightly reduced cellular TG compared to control, but *trans*-10 17:1 and *trans*-10 19:1 were without significant effect (Fig. 7C).

Since *trans*-10 18:1 exhibited an effect on glycerol release, we also tested whether *trans*-10 octadecenoic acid

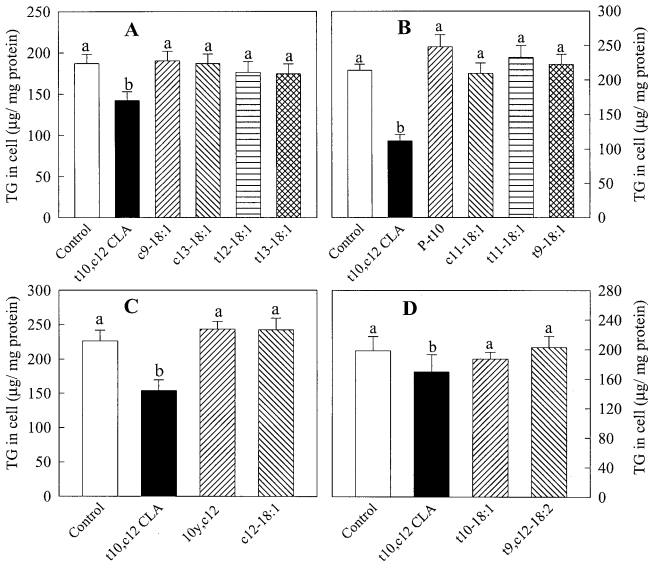

Fig. 3. Effects of 18-carbon fatty acids on esterified glycerol in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds as an albumin complex for 48 hours. All dishes including control contained the same concentration of albumin (100 μmol/L). Cells were scraped and sonicated to determine triacylglyceride (as estrified glycerol) and protein. Numbers are mean ± SE, n = 23–24 for (*A*) from 6 independent experiments, n = 10–12 for (*B*) and (*C*) from three independent experiments, and n = 7–8 for (*D*) from two independent experiments. Means with different letters in each figure are significantly different at $P <$ 0.05. For abbreviations and final concentrations used, see legend to Fig. 1.

might induce body compositional changes similar to those induced by the *trans*-10,*cis*-12 CLA isomer (Table 1). At the end of the experimental period the group fed diet supplemented with *trans*-10,*cis*-12 CLA exhibited significantly reduced body fat (40% reduction compared to control), whereas whole body water was significantly enhanced and whole body protein, and ash were apparently enhanced. By contrast, the group fed *tran*-10 octadecenoic acid exhibited no significant change in any of these measurements (Table 1). There were no significant difference in mean body weights (control group, 40.5±1.2 g; *trans*-10,*cis*-12 CLA-fed group, 40.3±2.2 g; and t10 18:1-fed group, 42.6±1.3 g) or mean total feed intake (control group, 91.2±4.1 g; *trans*-10,*cis*-12 CLA-fed group, 83.7±2.4 g; and t10 18:1-fed group, 89.8±4.2 g).

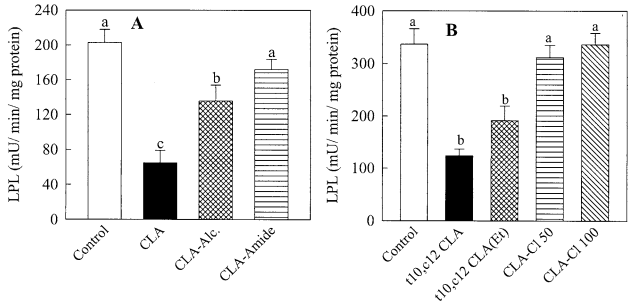

Fig. 4. Effects of CLA derivatives on heparin releasable lipoprotein lipase (HR-LPL) activity in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds for 48 hours. Except for t10,c12-CLA, all treatment compounds were dissolved in ethanol. All dishes including t10,c12-CLA and control contained the same concentration of albumin (100 μmol/L) and ethanol (0.3% final concentration). Numbers are mean ± SE, n = 6–8 from two independent experiments. Means with different letters in each figure are significantly different at $P < 0.05$. Abbreviations and final concentrations used are as follows: (*A*) CLA, *trans*-10,*cis*-12 (48%) and *cis*-9,*trans*-11 (46%) isomers, 98 μmol/L; CLA-Alc., *trans*-10,*cis*-12/*cis*-9,*trans*-11-octadecadien-1-ol, 95 μmol/L; CLA-amide, *trans*-10,*cis*-12/*cis*-9,*trans*-11-octadecadienamide, 95 μmol/L; and (*B*) t10,c12-CLA, *trans*-10,*cis*-12 conjugated linoleic acid, 46 μmol/L, Et indicates dissolved in ethanol before treating to cells; CLA-Cl, 1-chloro-*trans*-10,*cis*-12-octadecadiene, 48 or 96 μmol/L.

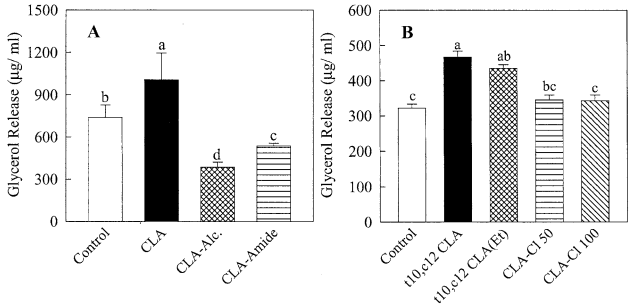

Fig. 5. Effects of CLA derivatives on glycerol release in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds as an albumin complex for 48 hours. Except for t10,c12-CLA, all treatment compounds were dissolved in ethanol. All dishes including t10,c12-CLA and control contained the same concentration of albumin (100 μmol/L) and ethanol (0.3% final concentration). At the end of incubation culture medium was collected and analyzed for free glycerol after centrifugation. Numbers are mean ± SE, n = 6–8 from two independent experiments. Means with different letters in each figure are significantly different at $P <$ 0.05. For abbreviations and final concentrations used, see legend to Fig. 4.

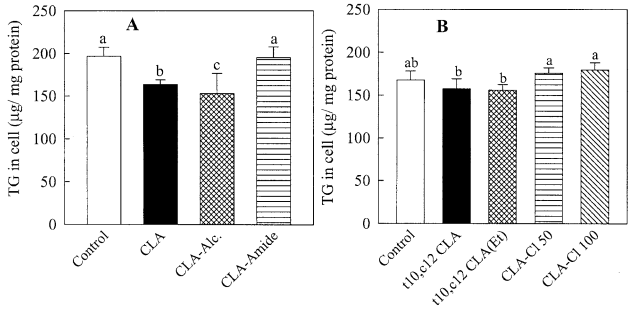

Fig. 6. Effects of CLA derivatives on esterified glycerol in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with these compounds for 48 hours. Except for t10,c12-CLA, all treatment compounds were dissolved in ethanol. All dishes including t10,c12-CLA and control contained the same concentration of albumin (100 μmol/L) and ethanol (0.3% final concentration). Cells were scraped and sonicated to determine triacylglyceride (as estrified glycerol) and protein. Numbers are mean ± SE, n = 6–8 from two independent experiments. Means with different letters in each figure are significantly different at $P < 0.05$. For abbreviations and final concentrations used, see legend to Fig. 4.

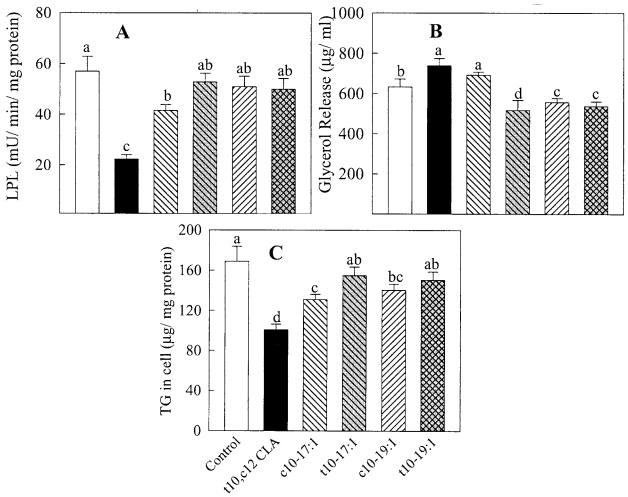

Fig. 7. Effects of odd-numbered fatty acids on heparin-releasable lipoprotein lipase (HR-LPL) activity (*A*), glycerol release (*B*), and cellular esterified glycerol (*C*) in 3T3-L1 adipocytes. At day 6 of differentiation, cells were treated with 50 μmol/L of fatty acid for 48 hours. All treatments had 100 μmol/L of albumin. Reported values are mean ± SE ($n = 12$, collected from three independent experiments). Means with different letters in each figure are significantly different at $P < 0.05$. CLA, conjugated linoleic acid; c, *cis*; t, *trans*; 17:1, heptadecenoic acid; 19:1, nonadecenoic acid.

4. Discussion

Table 2 summarizes the results of this study along with those of previously published investigations [2,3,10–12]. Among octadecadienoic acids only *trans*-10,*cis*-12 CLA reduced the activity of HR-LPL, whereas *cis*-9,*trans*-11, *trans*-9,*trans*-11, linoleic acid, or *trans*-9,*cis*-12 octadecadienoic acid did not reduce HR-LPL activity. Various 18-carbon monounsaturated fatty acids with double bonds located between carbons 9 and 13, in either *trans* or *cis* configurations, did not inhibit HR-LPL activity. CLA amide and chloride derivatives were without effect, whereas CLA alcohol exhibited a modest inhibitory effect that was less than that seen with *trans*-10,*cis*-12 CLA. We propose that this is due to the conversion of the alcohol form to free acid form; hence the effect of the alcohol form of CLA is less active than CLA itself. The data support the conclusion that the *trans*-10,*cis*-12 conjugated double bond in conjunction with a carboxyl group at C-1 is required for inhibition of HR-LPL activity.

The TG content in fat cells reflects the balance between fat uptake by HR-LPL, lipogenesis, and lipolysis. Our data consistently show that any compound that reduced HR-LPL activity in adipocytes also reduced body fat in animals. Since the amount of protein secreted into heparin containing medium was not different between treatment groups, it is unlikely that the effect of CLA on HR-LPL inhibition results from less protein secretion. Therefore the effects of CLA on HR-LPL can be due to regulation of HR-LPL expression, modification of location of HR-LPL, or interaction with HR-LPL directly.

Unlike its consistent inhibition of HR-LPL activity, the effects of CLA on intracellular LPL activity vary, either inhibiting [13] or having no effect [12]. We were able to observe inhibitory effects of CLA (as a mixture of *trans*-10,*cis*-12 and *cis*-9,*trans*-11 isomers) on HR-LPL activity in as short a time as 20 minutes (unpublished observations, [14]). This appears to be too rapid for a transcriptional effect; the effect of CLA on HR-LPL activity may result in part from direct interaction between *trans*-10,*cis*-12 CLA and LPL, similar to the inhibition mechanism of CLA on stearoyl CoA desaturase (SCD, Δ-9 desaturase) [4]. Alternatively, CLA might inhibit LPL enzyme relocation to membrane, making less LPL available for the activity. In addition, we cannot rule out the possibility that CLA may further interact at the transcriptional level, which has been reported by others, for example reduced LPL mRNA in 3T3-L1 adipocytes or adipose tissues by *trans*-10,*cis*-12 CLA [14–16].

Previously, Lin et al. [12] reported that both *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA isomers reduced HR-LPL activity from 3T3-L1 adipocytes, although the concentrations were much higher than those we used in this study. When similar concentrations were compared there was no effect by *cis*-9,*trans*-11 CLA,whereas *trans*-10,*cis*-12 CLA effectively reduced HR-LPL activity.

CLA may also affect lipogenesis in adipocytes. Previously our group reported that CLA did not affect activities of acetyl-CoA carboxylase and fatty acid synthetase (key enzymes involved in lipogenesis), both in vivo and during 1 week of feeding [13] and in 3T3-L1 adipocytes [17]. This is consistent with observations by Bee [18]. However, others report that CLA reduced activities and/or mRNA level of these two enzymes in adipocytes [15,16,19–21]. Thus CLA may result in less TG in adipocytes by reducing lipogenesis; however, we did not measure lipogenesis in these experiments.

Glycerol release represents lipolysis in adipocytes. Our results here indicate that glycerol release did not correlate well with TG content in cells or with reduced body fat in vivo. This may be due to the sensitivity of the method used in our experiments. However, nordihydroguaiaretic acid (NDGA) and AA861, both well known inhibitors of lipoxygenase and hormone-sensitive lipase, decreased lipolysis in our experiments, as expected, compared to control (as μg glycerol/mL: control 181 ± 8, NDGA (1×10^{-5} mol/L) 131 ± 9, AA861 (1×10^{-6} mol/L) 150 ± 22, and CLA 237 ± 11, $n = 7–8$ from two independent experiments). This suggests that this method represents the effects of treatment in this model. This was accompanied by reduced TG (as μg/mg protein: control 153 ± 13, NDGA (1×10^{-5} mol/L) 93 ± 10, AA861(1×10^{-6} mol/L) 95 ± 7, and CLA 94 ± 6, $n = 7–8$ from two independent experiments) and reduced body fat in mice for NDGA (for experimental conditions, see [22]). However, compounds that increased glycerol release only did not result in decreased TG, for example, *trans*-10 18:1 effectively increased glycerol release without affecting HR-LPL activity in 3T3-L1 adipocytes and had no

Table 2
Summary of compounds used

Compound	Inhibition of HR-LPL	Glycerol release	Total TG
Octadecadienoic acid			
trans-10,*cis*-12 CLA	+++	↑	↓
cis-9,*trans*-11 CLA [3,12]	− [3]/++ [12]	↑	−
trans-9,*trans*-11 CLA [3]	−	↑	−
cis-9,*cis*-12 octadecadienoic acid (linoleic acid) [2,3]	−	↑	↑
trans-9,*cis*-12 octadecadienoic acid	−	−	−
Octadecenoic acid			
cis-9 octadecenoic acid (oleic acid)	−	↓	−
cis-11 octadecenoic acid	−	−	−
cis-12 octadecenoic acid	−	↑	−
cis-13 octadecenoic acid	−	↑	−
trans-9 octadecenoic acid	−	−	−
trans-10 octadecenoic acid	−	↑ ↑	−
trans-11 octadecenoic acid (vaccenic acid)	−	−	−
trans-12 octadecenoic acid	−	−	−
trans-13 octadecenoic acid	−	↑	−
Eicosadienoic acid			
Cis-11,*cis*-14 eicosadietnoic acid [10]	−	−	−
Cis-11,*trans*-13 and *trans*-12,*cis*-14 CEA [10]	++	↑	↓
CLA derivatives			
trans-10,*cis*-12/*cis*-9,*trans*-11-octadecadien-1-ol (CLA-Alc.)	+	↓	↓
trans-10,*cis*-12/*cis*-9,*trans*-11-octadecadienamide (CLA-Amide)	−	↓	−
1-chloro-*trans*-10,*cis*-12-octadecadiene (CLA-CI)	−	−	−
Odd-numbered fatty acids			
cis-10 heptadecenoic acid	+	↑	↓
trans-10 heptadecenoic acid	−	↓	−
cis-10 nonadecenoic acid	−	↓	↓
trans-10 nonadecenoic acid	−	↓	−
Cis-10,*cis*-13 nonadecadienoic acid [11]	−	↑	−
Cis-10,*trans*-12 and *trans*- 11-,*cis*-13 CNA [11]	++++	↑	↓
Others			
cis-12-octadecen-10-ynoic acid (10y,*cis*-12)	−	↓	−
11-(2′-(n-pentyl)phenyl)-10-undecylenic acid (*P-t10*)	−	↑	−

− = effect; + = inhibition; ↑ = increase; ↓ = decrease.
No numbers in square brackets indicate references from which data are collected.

effect on total TG or body fat in mice. This suggests that this lipolysis assay method may not be sensitive enough to enable us to observe the difference in TG; thus further studies are need to clarify the correlation between lipolysis and TG in this cell culture model.

Previously we reported that the *trans*-10,*cis*-12 CLA isomer effectively inhibited hepatic SCD [4]. In addition, *trans*-10,*cis*-12 CLA is also responsible for decreased apo B secretion in HepG2 cells, although a *trans*-10 double bond is a key factor for this activity [5]. It was of interest that odd-numbered fatty acids such as CNA [11] can inhibit HR-LPL activity as well as reduce the TG in the cell. Since CNA and CLA cannot share common metabolites because of the difference of one carbon, we proposed that CLA itself was directly effective. In this study, we observed that *cis*-10 17:1 inhibits HR-LPL activity, although less so than *trans*-10,*cis*-12 CLA, indicating that a double bond at the 10th position may play an important role in these activities.

It appears, then, that *trans*-10,*cis*-12 CLA is directly responsible for reduction of HR-LPL activity, and that the *trans*-10 double bond may be the key. In contrast, either a *trans*-10 or *cis*-12 bond may induce glycerol release from adipocytes. This study also suggests that lipolysis may not be a good indicator of predicting TG content in the 3T3-L1 adipocyte model; rather, inhibition of HR-LPL activity may provide a better prediction of TG content in this cell line and fat level in animal models.

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