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Effects of Conjugated Linoleic Acid on Adipocyte Secreted Proteins *in vitro*

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Abstract

Conjugated linoleic acid (CLA) is a class of positional, geometric conjugated dienoic isomers of linoleic acid (LA). CLA activates the immune system, protects against tumorigenesis, and reduces the incidence of atherosclerosis. *Trans*-10, *cis*-12 CLA has specific effects on lipid metabolism, it has been shown to reduce body fat gain and regulates some adipocyte secreted proteins *in vivo* and *in vitro*. Here we report that a CLA mixture affects cytokine secretion from rat primary adipocytes. Rat primary adipocytes were treated with 1 mM, 100 μM, 1 μM or 100 nM CLA mixture doses; and leptin, tumor necrosis factor alpha (TNF α), interleukin-6 (IL-6) and glycerol levels in the medium were measured. Leptin secretion was lower, TNF α secretion higher and IL-6 secretion did not change in response to the CLA mixture. Leptin and TNF α secretions did not change with CLA mixture treatment in a dose-dependent manner. In addition, the CLA mixture did not appear to enhance lipolysis in rat primary adipocytes. In conclusion, our study demonstrates that the decrease in leptin and increase in TNF α secretion in adipocytes treated with CLA mixture may be due to the apoptotic effect and to a reduction in peroxisome proliferator-activated receptor gamma (PPAR γ) ligands.

Key words: conjugated linoleic acid, leptin, TNF α, IL-6, lipolysis

INTRODUCTION

CLA is a class of positional, geometric conjugated dienoic isomers of LA. CLA is found in dairy products, lamb, veal, beef, seafood, turkey and vegetable oils (1,2). LA is converted to CLA through bihydrogenation by fermentation bacteria, *Butyrovibrio fibrisolvens* (3-5). CLA has two primary isomer forms; *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. CLA has been shown to activate the immune system (6), protect against tumorigenesis (7), and reduce the incidence of atherosclerosis in rabbits (8) and hamsters (9). Both isomers have independent and synergistic actions. *Trans*-10, *cis*-12 CLA modulates lipid metabolism, body fat gain and regulates adipocyte secreted hormones *in vivo* (10-12) and *in vitro* (13). *Trans*-10, *cis*-12 CLA down-regulates leptin secretion in 3T3-L1 cells (13) and the reduction of leptin secretion induced by *trans*-10, *cis*-12 CLA appears to result from a decrease in PPAR γ expression (14). *Trans*-10, *cis*-12 CLA also induces apoptosis in preadipocytes (15-18). Several studies show CLA decreases adipocyte cellularity by decreasing proliferation (15,16) or adipocyte size (15,19, 20). The anti-adipogenic effect of *trans*-10, *cis*-12 CLA is mediated by inhibition of lipoprotein lipase (LPL) activity (21).

Leptin is a 16-kD protein, which is mainly secreted by adipocytes. It regulates appetite and energy metabolism in humans and rodents (22,23). Obese subjects have higher leptin levels than normal weight subjects (23-25). Circulating leptin concentrations have a strong correlation with body weight and are regulated by signaling to and from the brain (26,27). Leptin and its receptors show significant sequence and structural homology with the IL-6 family of cytokines (28) and their receptors (29).

Cytokines including TNF α also regulate leptin secretion (30-32). In rodent studies, TNF α treatment increases leptin production and mRNA expression (31-33).

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Obese mice lacking TNF α or TNF receptors exhibit significantly lower circulating leptin levels (33-35). In contrast, treatment with TNF α causes cultured adipocytes to decrease leptin secretion, with a chronic treatment inhibiting leptin gene expression and decreasing secreted protein (31,36). The TNF α gene is linked to the CCAAT/enhancer binding protein alpha (C/EBP α) gene and is located in close proximity to the *ob* gene promoter and its expression is closely linked to *ob* gene expression (37). C/EBP α and tumor necorsis factor receptor (TNFR) (30,33,35) are candidates for modulating leptin production by TNF α.

Like TNF α, IL-6 is expressed in adipose tissue (38), increases basal glucose uptake, and alters insulin sensitivity. Both IL-6 and TNF α inhibit LPL and stimulate lipolysis (39-41). IL-6 has affected with regard to cardiovascular disease by modulating VLDL and C-reactive protein (42,43).

Adipocyte secreted hormones including leptin, TNF α and IL-6 are altered by changing lipid metabolism and play a role as autocrine/paracrine hormones (38,44,45). CLA influences lipid metabolism, and this may influence leptin, TNF α and IL-6 secretions from adipocytes.

Therefore, in order to understand the effects of CLA on rat primary adipocyte secreted proteins, we used fully differentiated rat primary adipocytes that were treated with a range of dosages of CLA mixture. Leptin, TNF α, IL-6 and glycerol secretion levels were assayed from the adipocyte culture medium.

MATERIALS AND METHODS

Animals

Ten fourteen day old Sprague-Dawley (SD) rats were purchased from Hyochang Science, Taegu, South Korea and kept in a cage with the mother rat. The rats were housed in a controlled environment with a room temperature of 23 ± 3°C, relative humidity of 50 ± 5% and a 12 h light: dark cycle. Animals were provided free access to rodent chow (Daehan Animal Laboratory) and pathogen free water.

Cell culture

Fibroblastic preadipocytes were isolated from adipose tissue based on a previously reported method (46,47). The fat pads from 14 day-old male Sprague-Dawley rats (10 per group) were removed under sterile conditions and washed in a buffer of 135 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10 mM HEPES, 1.25 mM NaH_2PO_4, 1.25 mM Na_2HPO_4 and 1% (w/v) bovine serum albumin (Sigma). The fat pads were added to fresh buffer containing collagenase Type II (1 mg/mL; Sigma), 3.5% (w/v) bovine serum albumin and glucose (0.5 mg/mL; Sigma). Following incubation for 1 hour at 37°C in a shaking incubator the digested tissue was filtered through sterile 250 mm nylon mesh. The digested tissue was centrifuged at 200 g for 10 minutes and mature adipocytes removed by aspiration.

The pellet containing the stromal vascular fraction was resuspended in medium 199 (Bio Whittaker) supplemented with penicillin, streptomycin, and fungizone (100 units/mL, 100 mg/mL and 2.5 mg/mL, respectively; Bio Whittaker), and refiltered. Red blood cells were lysed by hypotonic shock. The suspension was centrifuged (200 g for 10 minutes) and the cells counted using a haemocytometer. The cell preparation (mainly fibroblastic preadipocytes) was adjusted to a density of 3.0×10^5 cells/mL in medium 199 with 10% fetal bovine serum (Bio Whittaker). 1 mL volumes were plated onto 12-well plates.

After 2 days in culture at 37°C in an atmosphere of 5% CO_2, differentiation was induced by the addition of medium 199 supplemented with isobutylmethylxanthine (0.5 mM; Sigma), dexamethasone (0.25 mM; Sigma) and insulin (10 mg/mL; Sigma). After 48 hours, the induction medium was removed and replaced by medium 199 containing 10% fetal bovine serum supplemented with insulin (10 mg/mL; Sigma) alone. This medium was changed every 2 days, and samples retained and centrifuged at 150 g for 10 minutes; the supernant was stored at -20°C.

CLA mixture treatment

The CLA mixture was obtained from Livemax Corporation (Korea). The CLA mixture was prepared from safflower oil using an alkali isomerization reaction. The composition of the 95% CLA mixture was: 36.5% *cis*-9, *trans*-11, 56.6% *trans*-10, *cis*-12, and 1.57% other isomers. The CLA mixture was dissolved in 0.25 mM free fatty acids (FFA) BSA (Calbiochem) containing medium 199 and cells treated with 1 mM, 100 μM, 1 μM and 100 nM CLA mixture doses. After 24 hours, the medium from treated cells was collected for measuring proteins and stored at -80°C.

Oil Red O staining

Before primary culture, 24-well plates with slide glasses coated with Poly-D-lysine were prepared. Cells were stained with Oil Red O. Slide glasses were fixed with 10% fresh Formalin (Sigma, St. Louis, MO, USA), rinsed in phosphate buffered saline (Biowhittaker), and then incubated in filtered Oil Red O stock solution at 4°C for at least 1 hour. They were washed with distilled water for 15 minutes twice. After Oil Red O staining, slide glasses were preserved by adding 2 μL mounting solution (Sigma) and then scanned.

Detection of leptin secretion

By using cultured rat adipocytes grown in 12 well plates leptin production measured. Measurement of leptin was performed with a sandwich enzyme-linked immunosorbent assay (ELISA). Anti-mouse leptin (R & D Systems, MN, USA) was used as a solid-phase antibody to leptin. This antibody was diluted (0.2 μg per each well) with 0.05 M Carbonate/Bicarbonate Coating buffer (Sigma), added to a 96-well plate and cooled overnight. Blocking was performed with 3% BSA (Sigma) at 37°C for at least 1 hour. Each well was then treated with cultured medium for 37°C for 1 hour and the plate treated with a diluted (100 ng per each well) biotinylated anti-mouse leptin antibody (R & D Systems, MN, USA) and shaken at room temperature for 1 hour. Streptavidin-conjugated horseradish peroxidase (R & D Systems, MN, USA) was diluted 1:200 and 100 μL was added to each well and shaken at room temperature for 1 hour. The plate was rinsed with phosphated buffered saline (Bio Whittaker) containing 0.05% Tween 20 (Calbiochem) between each step. 100 μL TMB (Amresco) was added to the plate and shaken in the dark for 30 minutes. Finally, 50 μL of 2 M sulfuric acid (Junsei) was added to stop the coloring reaction. After stopping the reaction, leptin concentration was measured at 450 nm absorbance.

Detection of TNF α and IL-6

TNF α and IL-6 levels were determined in the medium containing adipocytes using a commercially available ELISA kits for each specific cytokine (R & D Systems, MN, USA).

Glycerol analysis

The glycerol concentrations were determined in infranatant samples using the enzymatic reagent, GPO-TRINDER (Sigma). A 2.5 μL volume of each sample was added to 500 μL of pre-warmed 37°C reagent. Standards containing 12.5 and 25 μg amounts of glycerol were prepared for calibration using the same method. After incubation at 37°C for 5 minutes, 200 μL of each sample was transferred to a 96-well plate (Nunc) and optical densities assessed at 540 nm using a plate spectrophotometer.

Statistical analysis

The results are expressed as means ± SE. The significance of differences between groups was determined by carrying out Student's t-test.

RESULTS

Effect of CLA mixture on leptin secretion

To determine when the leptin secretion reaches the highest peak in primary adipocytes, the leptin concentrations in medium were assayed from the 2nd, 4th, 6th and 8th days after induction. The 8th day after induction was determined to be the most effective time point at which to assay the leptin concentration (data not shown). Oil Red O staining was performed to indicate the level of differentiation of the primary adipocytes (Fig. 1). By Oil Red O staining and leptin analysis, it was concluded that by the 8th day post induction primary adipocytes were fully differentiated and suitable for treatment with the CLA mixture.

Treatment of primary adipocytes with medium containing 1 mM, 100 μM, 1 μM and 100 nM concentrations of CLA mixture for 24 hours led to significant decreases in leptin secretion compared to the control group (Fig. 2). In the 0.25 mM dexamethasone treated group, leptin secretion was 100 ± 10.5% ($p < 0.01$) higher than in the control group (Fig. 2). The groups treated with 1 mM, 100 μM, 1 μM and 100 nM CLA mixtures had lower leptin secretion levels by 43.3 ± 9.1% ($p < 0.05$), 19.1 ± 4.5% ($p < 0.05$), 43.2 ± 5.5% ($p < 0.01$) and 38.2 ± 8.7% ($p < 0.05$), respectively, compared to the control group. However, leptin secretion did not change with CLA mixture treatment in a dose-dependent manner.

CLA mixture effects on TNF α secretion

Treatment of primary adipocytes with medium containing 1 mM, 100 μM, 1 μM and 100 nM concentrations of CLA mixture for 24 hours led to significant increases in TNF α secretion compared to the control group (Fig. 3). The groups treated with 1 mM, 100 μM, 1 μM and 100 nM CLA mixtures had higher TNF α secretion by 253.6 ± 18.3% ($p < 0.01$), 193.6 ± 12.4% ($p < 0.01$), 248.0 ± 14.2% ($p < 0.01$), and 344.0 ± 18.4% ($p < 0.01$), respectively, compared to the control group. However, TNF α secretion did not change with CLA mixture treatment in a dose-dependent manner, either.

Effect of CLA mixture on IL-6 secretion

Treatment of primary adipocytes with medium containing 1 mM, 100 μM, 1 μM and 100 nM concentrations of CLA mixture did not significantly change IL-6 secretion compared to the control group (Fig. 4).

Effect of CLA mixture on lipolysis

CLA mixture decreased leptin secretion significantly in primary adipocytes. This suggests changes in lipid metabolism that might be related to lipolysis induced by the CLA mixture. Therefore, glycerol was measured in the medium after CLA mixture treatment. However, no significant changes in glycerol concentrations were found in comparison to the control group (Fig. 5).

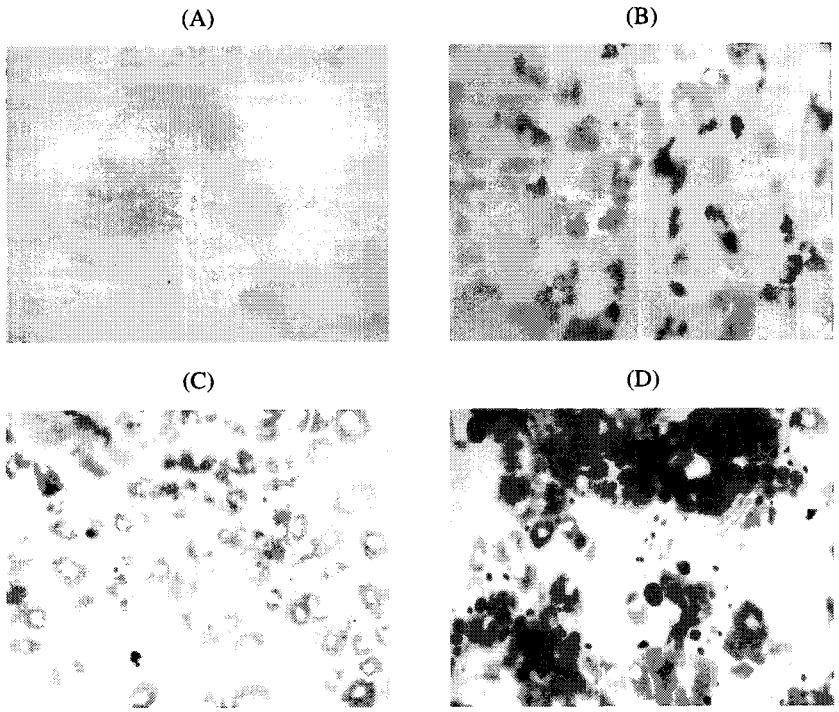

Fig. 1. Oil Red O staining of rat primary adipocytes after the (A) 2nd, (B) 4th, (C) 6th, (D) 8th days following induction of differentiation.

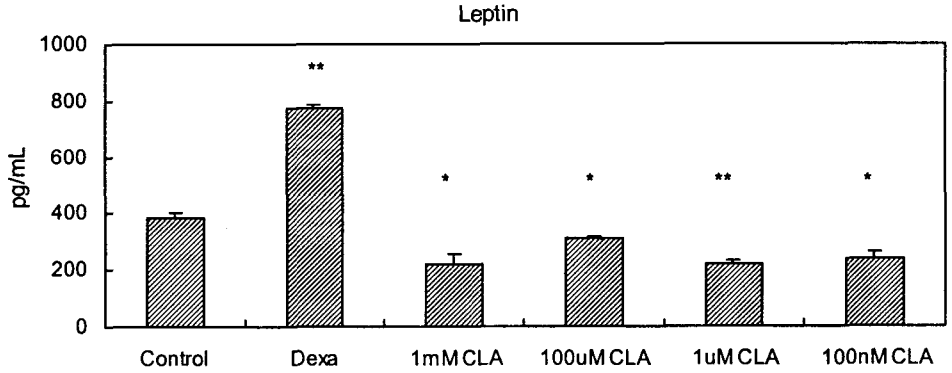

Fig. 2. Effects of CLA mixture on leptin secretion by rat primary adipocytes. Primary adipocytes were treated with medium containing 1 mM, 100 μM, 1 μM and 100 nM concentrations of CLA mixture. Values are mean±SE (n=2). Means with '*' indicates $p<0.05$. Means with '**' indicates $p<0.01$.

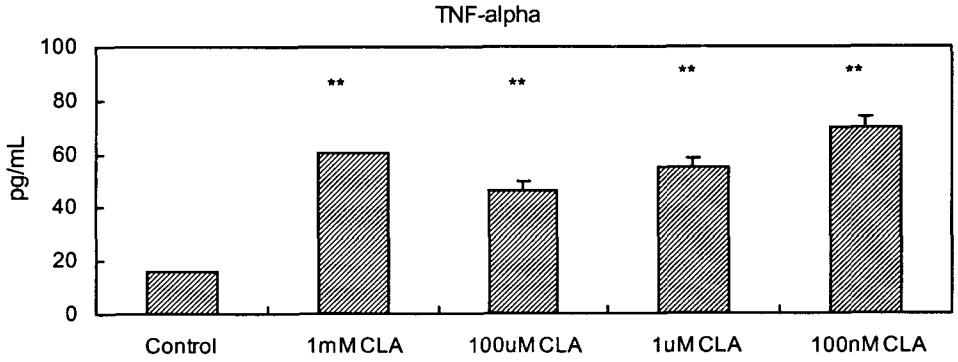

Fig. 3. Effects of CLA mixture on TNF α secretion from rat primary adipocytes. Primary adipocytes were treated with medium containing 1 mM, 100 μM, 1 μM and 100 nM concentrations of CLA mixture. Values are mean±SE (n=2). Means with '**' indicates $p<0.01$.

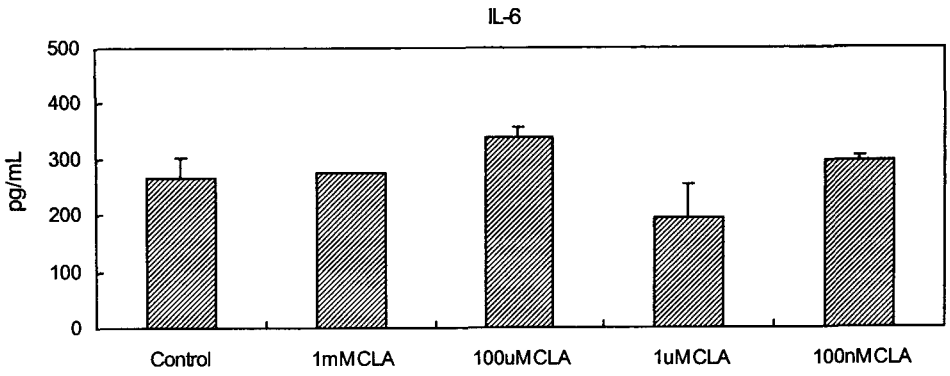

Fig. 4. Effects of CLA mixture on IL-6 secretion from rat primary adipocytes. Primary adipocytes were treated with medium containing 1 mM, 100 μM, 1 μM and 100 nM concentrations of CLA mixture. Values are mean±SE (n=2).

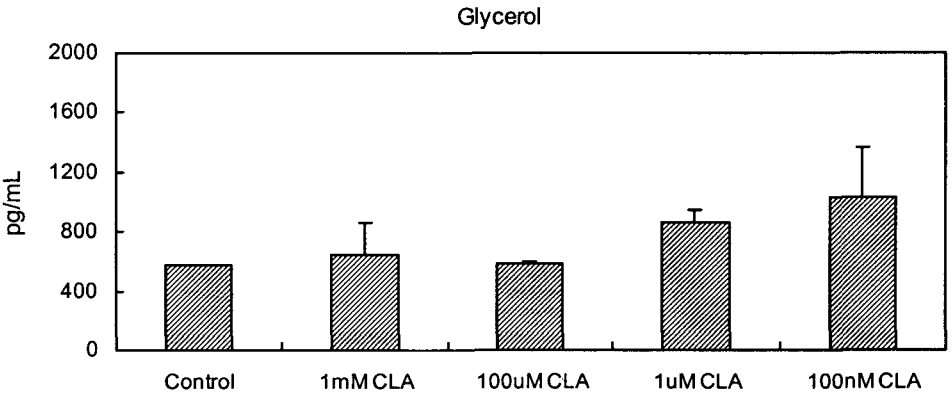

Fig. 5. Effects of CLA mixture on lipolysis from rat primary adipocytes. Primary adipocytes were treated with medium containing 1 mM, 100 μM, 1 μM and 100 nM concentrations of CLA mixture. Values are mean±SE (n=2).

DISCUSSION

In this study, we demonstrated that CLA mixture reduces leptin secretion from cultured primary adipocytes (Fig. 2) and increases TNF α secretion (Fig. 3). However, the secretion level of leptin and TNF α were not altered by CLA mixture in a dose dependent manner. CLA mixture did not alter the IL-6 secretion levels (Fig. 4) and neither did it change glycerol secretion levels (Fig. 5).

The decrease in leptin secretion by the CLA mixture is similar to findings in a previous report (13). It has been proposed that the reduction of leptin secretion resulting from treatment with *trans*-10, *cis*-12 CLA may be due to the reduction of adipogenic factors, adipocyte transcriptional factor PPAR γ, adipocyte gene fatty acid synthase (FAS) and aP2 expression (48).

It is unclear whether the decrease of leptin secretion from adipocytes is only due to a direct effect of adding the CLA mixture to the adipocytes, transcriptional factors or to an unknown indirect mechanism. In this study, the CLA mixture was shown to increase TNF α secretion from rat primary adipocytes. There was also a report that dietary CLA supplementation increases TNF α levels *in vivo* (18). Leptin secretion is decreased by apoptosis or lipolysis. However, CLA did not induce lipolysis (Fig. 5), which was consistent with a previous report (11). These previous studies imply that the decrease in leptin secretion may be due to the increased TNF α levels. In obesity, the elevated levels of TNF α contribute to lipolysis (49). TNF α also decreases leptin secretion, with a chronic treatment inhibiting leptin gene expression and decreasing secreted protein *in vitro* (33,36). Therefore, CLA-mediated increases in TNF α secretion (18) and decreases in leptin secretion may induce an apoptotic effect. TNF α can reduce PPAR γ mRNA expression *in vitro*, as well (50). The decrease in PPAR γ expression decreases leptin secretion by reducing the efficacy of PPAR γ ligands (50). The CLA mixture may induce an apoptotic effect by increasing TNF α secretion (18). Therefore increasing TNF α secretion and decreasing leptin secretion may induce apoptosis by reduction of PPAR γ ligands.

However, secretion of the inflammatory cytokine IL-6 did not change significantly with CLA mixture treatment (Fig. 4). We can hypothesize that the CLA mixture may

stimulate different transcriptional factor sites of TNF α and IL-6. There were no significant changes in either IL-6 secretions or in glycerol concentrations. These results indicate that CLA modulates lipid metabolism, but does not do so by inducing lipolysis.

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