Antiadipogenic Effects of Different Molecular Forms of Conjugated Linoleic Acid on 3T3-L1 Cells: Comparison between Free Fatty Acid and Phosphatidylcholine Forms

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Abstract: The antiadipogenic activity of conjugated linoleic acids (CLA) in the form of phosphatidylcholines-bound (CLA-PC) or free fatty acids (FFA; CLA-FFA) was evaluated using 3T3-L1 adipocytes. Phosphatidylcholine from soya (soy-PC) was used as the comparison of PC form. Both the lipid accumulation and activity of glycerol-3-phosphate dehydrogenase were measured to determine lipogenesis, whereas the glycerol content was measured to evaluate lipolysis. The CLA uptake also measured to find out the utilization of CLA by the cells. As a results, lipid accumulation in 3T3-L1 adipocytes was inhibited in a dose-dependent manner following treatment with CLA-PC (50-400 μM). Both CLA-PC and soy-PC significantly suppressed lipid accumulation compared with CLA-FFA, even though the amount of CLA in CLA-PC was a half than CLA-FFA. The CLA uptake of PC form was superior to FFA form, however, no difference was noted between CLA-PC and soy-PC. These forms exerted their antiadipogenic activity via the suppression of lipogenesis, and not by increasing lipolysis. Short-term treatment, especially in the middle stage of differentiation, was more effective than long-term treatment; especially for CLA-FFA. The antiadipogenic effect of CLA-PC was partially attributed to the chemical structure of the PC molecule. These results provide important information for the utilization of physiologically functional fatty acids and particularly CLA in the food and medical fields.

Key words: antiobesity, conjugated linoleic acid, phosphatidylcholine

1 Introduction

Obesity, which is associated with increasing morbidity and mortality, has become a global epidemic. The World Health Organization reported that overweight and obesity are the fifth leading cause of death worldwide. Obesity is known to contribute to a number of health problems, such as certain types of cancer, cholesterol and cardiovascular disease, type 2 diabetes mellitus, hypertension, and sleep apnea⁴. Obesity can be prevented through the management of diet, intake of nutraceutical foods, exercise, also can be treated with drugs and surgery.

In 2007, the aggregate market value of dietary supplements reached approximately 20 billion dollars in sales in the United States, and was estimated to increase annually. Natural supplement products, primarily those targeting obesity, have been widely explored⁵. As a result, food components that have been shown to exert beneficial effects and assist weight loss have attracted increasing attention. Conjugated linoleic acids (CLA) constitute one of the groups of natural food components that have received increased attention.

Briefly, CLA refer to a mixture of positional and geometric isomers of linoleic acid (C18:2) with conjugated double bonds. They are naturally found in food, and the major dietary sources of CLA for humans are ruminant meat, such as beef and lamb, and dairy products, including milk and cheese. The isomer of CLA in natural foods is primarily cis-9, trans-11 CLA (c9,t11-CLA)⁶. Brodie et al. have previously studied the antiobesity properties of CLA using 3T3-L1 cells as adipocyte-like cells⁶. They found that
mature 3T3-L1 cells treated with CLA had reduced levels of intracellular triacylglycerol and smaller cell size than those treated with a similar linoleic acid.

Most studies on the antiobesity properties of CLA have been conducted using CLA in the form of free fatty acids (FFA)\(^{10}\). The CLA used in most studies were composed of CLA mixtures containing approximately equal amounts of trans-10, cis-12(t10,c12-CLA), and c9,t11-CLA isomers, or other specific isomers. It has been reported that t10,c12-CLA is superior as an antiadipogenic compound in CLA isomers\(^{10,13}\). Furthermore, Jeong et al. studied the effect of the molecular form of CLA\(^{10}\). They compared the antiobesity properties of CLA in the form of FFA, diglyceride (CLA-DG), and triglyceride (CLA-TG) at concentrations ranging from 10 to 1000 \(\mu\)g/mL. Their results showed that all treatments reduced the proliferation of preconfluent 3T3-L1 cells in a dose-dependent manner, with CLA-DG exerting the strongest effect compared with CLA-FFA and CLA-TG.

Of note, CLA in the form of TG might have limited absorption in the intestine due to their polarity. Due to the lower polarity, TAG does not get through the cellular membrane easily. So, its absorption involves hydrolysis of TAG to release free fatty acids and monoacylglycerol (MAG) in the intestine lumen\(^{10}\). While PC having both hydrophilic and hydrophobic components, bile salts are able to facilitate micelle formation with it, which makes its absorption easily\(^{11}\). Hence, coupling a CLA with a polar molecule such as phosphatidylcholine (CLA-PC) should provide favorable bioavailability\(^{10}\). Currently, PC has been widely used for the dissolution of local fat deposits, with many studies reporting a reduction in fat deposits by a subcutaneous injection of PC. In particular, PC has been reported to cause adipocyte lipolysis and apoptosis of adipocytes\(^{12-15}\).

To date, no comparative studies have been performed on the antiobesity properties of CLA in the FFA (CLA-FFA) and PC (at the sn-2 position; CLA-PC) forms. The objective of this study is to reveal the antiadipogenic effect of the molecular form of CLA (FFA and PC) on 3T3-L1 adipocyte-like cells.

### 2 Experimental

#### 2.1 Materials

CLA-FFA is commercially available (CLA Jarrow Formula\TM, Los Angeles, USA). CLA-PC was prepared via phospholipase A\(_2\)-catalyzed esterification as previously described\(^{16}\). PC from soya (soy-PC) and glycerophosphocholine (GPC) were purchased from H. Holstein Co., Ltd. (Tokyo, Japan). Choline and linoleic acid (LA) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Since tocopherols did not give effect on cell growth and lipid accumulation in 3T3-L1 cells (data not shown), the tocopherols in samples were removed using activated charcoal powder, as previously reported\(^{16}\). The fatty acid compositions of CLA-FFA, CLA-PC, and soy-PC were analyzed by gas chromatography as previously reported\(^{16}\) and are summarized in Table 1. All solvents and other chemicals were of analytical grade.

| Table 1  | Fatty acid compositions of CLA-FFA, CLA-PC, and soy-PC used in this study (wt%) |
|----------|--------------------------------------------------------------------------------|
| Fatty acid | CLA-FFA | CLA-PC | soy-PC |
| C16:0     | 51 ± 0.6 | 36.1 ± 0.9 | 13.6 ± 0.1 |
| C18:0     | 1.5 ± 0.1 | 12.1 ± 0.4 | 3 ± 0.0 |
| C18:1     | 12.9 ± 0.6 | 8.1 ± 0.5 | 6.9 ± 0.0 |
| C18:2     | 1.2 ± 0.4 | – | 68.5 ± 0.1 |
| C10,c12-CLA | 36.6 ± 0.3 | 19.6 ± 0.8 | – |
| c9,t11-CLA | 36.9 ± 0.7 | 16.6 ± 0.6 | – |
| C18:3     | – | – | 7.9 ± 0.0 |
| n.i.      | 5.7 ± 0.9 | 7.5 ± 1.1 | – |

n.i.: not identified, –: not detected

Data shown is expressed as mean values ± s.d. (n=3)

#### 2.2 Cell culture

The 3T3-L1 preadipocyte cell line was obtained from the Japanese Collection of Research Bioresources (IF054016, Osaka, Japan). Cell culture and differentiation treatment followed the method described by Zebisch et al.\(^{17}\) with slight modifications. Cells were maintained in fresh Dulbecco’s modified Eagle’s medium (DMEM; Sigma Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Biosera, Nuaile, France), as well as 100 U/mL penicillin and 100 \(\mu\)g/mL streptomycin (maintenance media [MM]). Cells were cultured in 6 cm diameter culture dishes at 1.5 \(\times\) 10\(^4\) cells/mL at 37°C in a humidified atmosphere of 95% air and 5% CO\(_2\). After reaching 80% confluence, cells were passaged.

#### 2.3 Differentiation of 3T3-L1 cells

For adipocyte differentiation, cells were cultured in 12-well plates at 1.5 \(\times\) 10\(^4\) cells/mL/well (Scheme 1a). The medium was replaced every 2-3 d. At day 6 after cell seeding, the medium was replaced with MM containing 0.5 mM 3-isobutyl-1-methylxantine (IBMX) and 1 \(\mu\)M dexamethasone (initiation media [IM]), and cells were then incubated for another 2 d. After that, the medium was replaced with MM containing 10 \(\mu\)g/mL insulin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) (progression media [PM]) and cells were further incubated for 2 d, after which the medium was replaced with MM. Mature adipocytes were identified 8 d after changing the medium to IM. In this experiment, cells were treated with our experimental samples (CLA-FFA, CLA-PC, and soy-PC) at the same time of changing the medium (IM and PM) at concentration

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200 µM. All samples were diluted in dimethyl sulfoxide and added to the respective media at concentrations less than 0.1%. We employed two patterns of sample treatments in this study. In the first, treatment was carried out for 4 d through 2 additions (Scheme 1a), whereas in the second, treatment was performed for 2 d with a single addition at the same time of changing the medium (IM or PM) (Scheme 1b).

2.4 Analysis

2.4.1 Oil red O staining

Oil red O staining and quantification of relative lipid accumulation in adipocytes were performed (Scheme 1). Briefly, on day 8 of the differentiation stage, mature adipocytes in 12-well plates were rinsed twice with Dulbecco’s phosphate-buffered saline (D-PBS). Cells were treated and fixed with 1 mL 10% formaldehyde for 20 min and then rinsed twice with D-PBS. Cells were then rinsed with 60% isopropyl alcohol (IPA) for 1 min. Subsequently, cells were treated with 3 mg/mL Oil red O solution in 60% IPA and fixed for 10-20 min. Cells were then washed with 60% IPA followed by washing with D-PBS. Oil droplet-stained adipocytes were observed under a microscope and lipids stained with Oil Red O pigment were extracted using 100% IPA (0.5 mL). The absorbance of the extract at 490 nm was measured using a microplate reader (Varioskan, Flash, Thermo Fisher Scientific, USA). Relative lipid accumulation was determined as the percentage of extracted intracellular lipid content (%) by comparing the treatment with the control.

2.4.2 CLA uptake

CLA uptake analysis was performed to observe CLA in the samples were consumed by the cells during the culture. The CLA content in the media treated with CLA-FFA or CLA-PC was measured before and after the culture. The media was recovered and the CLA in the media was extracted using mixture of chloroform-methanol-water (3:10:5, v/v). Then the chloroform layer was collected and evaporated. The extracted CLA was dissolved by ethanol and the absorbance was read at 233 nm using a microplate reader (Varioskan, Flash, Thermo Fisher Scientific, USA). A standard curve of CLA-FFA and CLA-PC were prepared, respectively, and the equation functioned with the concentration of samples were determined (CLA-FFA: $y = 0.0103x + 0.2106$ ($R^2 = 0.9993$); CLA-PC: $y = 0.0046x + 0.2052$ ($R^2 = 0.9997$)). The CLA uptake was determined as $A_0 - A_1$, where $A_0$ was CLA concentration detected before culture, and $A_1$ was that after culture. Sample treatment was carried out during two days of differentiation stage, Early stage or Middle stage (Scheme 1).

2.4.3 Glycerol-3-phosphate dehydrogenase (GPDH) activity

On day 8 of the differentiation stage, the GPDH activity of cells was measured (Scheme 1). Cells were washed twice with 1 mL D-PBS, and then the collected cells were dissolved in 1 mL enzyme extraction solution followed by sonication. After sonication, the mixture was centrifuged at 12,800 × g for 5 min at a temperature maintained at 4°C. The supernatant was collected and used for analysis. The GPDH activity in cells was measured using a GPDH assay kit (Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer’s protocol. The protein content was deter-
determined as described by Lowry et al.\textsuperscript{18} for the quantification of GPDH activity per mg of protein. Results were expressed as % of the control.

2.4.4 Glycerol content

At day 8 of the differentiation stage, the glycerol content was measured in the media to determine the degree of lipolysis (Scheme 1a). The glycerol released in media was measured using a glycerol colorimetric assay kit (Cayman Chemical, Michigan, USA) according to the manufacturer’s protocol. Results were expressed as % of the control.

2.4.5 Cell viability

Cell viability was assessed using the trypan blue exclusion assay (Scheme 1b). On day 8 of differentiation, cells were rinsed twice with D-PBS, trypsinized, and centrifuged at 1,000 \( \times \) g for 5 min. The supernatant was discarded, and cells were dissolved in 2 mL D-PBS. The cell solution was mixed with trypan blue solution at a ratio of 1:1, and cells were counted using a hemocytometer chamber.

2.4.6 Statistical analysis

All experiments were performed in triplicate. Data were expressed as the mean value with standard deviation (s.d.). A significant difference in each treatment was determined using the Scheffe’s test \((p < 0.01)\).

3 Results and Discussion

3.1 Dose dependent inhibition of lipid accumulation in 3T3-L1 adipocytes by CLA-PC

Figure 1 shows the relative lipid accumulation of 3T3-L1 adipocytes after treatment with various concentrations (50, 100, 200, and 400 \( \mu M \)) of CLA-PC. We conducted this experiment to determine the dose dependency of CLA-PC on lipid accumulation and to determine the concentration of samples in the next experiment. The timeline for treatment of samples to the differentiation of 3T3-L1 cells was in accordance with Scheme 1a.

As a result, we noticed that CLA-PC suppressed lipid accumulation in a dose-dependent manner. This finding was in line with some studies showing that CLA-FFA inhibited lipid accumulation in 3T3-L1 adipocytes in a dose-dependent manner\textsuperscript{4, 8}. We did not observe any improvement in the suppression of lipid accumulation at concentrations of CLA-PC > 200 \( \mu M \). Therefore, we used a concentration of 200 \( \mu M \) for all samples in the following experiments.

3.2 Lipid accumulation in 3T3-L1 adipocytes treated with CLA-FFA, CLA-PC, or soy-PC

To observe the effect of the different molecular forms of CLA, 3T3-L1 preadipocytes were treated with CLA-FFA or CLA-PC at the time of replacing PM and IM during cell culture (Scheme 1a). We used soy-PC as a comparison of PC form for CLA-PC.

We noticed that treatment with CLA-PC significantly suppressed lipid accumulation in cells to a greater extent than treatment with CLA-FFA at the same concentration in molecular weight. As shown in Table 1, the amount of CLA in PC form was a half of FFA form. From this result, it was shown that the ability of PC form on suppressing lipid accumulation in 3T3-L1 adipocytes was superior to FFA form, even though the amount of CLA in CLA-PC was lower than CLA-FFA. However, we did not observe any significant difference between the CLA-PC and soy-PC treatments (Fig. 2). These observations were also supported by the morphological appearance of cells (Fig. 3). From these results, it was suggested that PC form could suppress lipid accumulation better than FFA form in 3T3-L1 adipocytes. Jung et al., reported that PC could promote lipolysis and induce apoptosis through TNFα-mediated pathway\textsuperscript{15}. In addition, even though the concentration of CLA in CLA-PC is half of CLA-FFA, the uptake of CLA of PC form into 3T3-L1 cells was ~20% higher than that of FFA form in both early and middle stage of treatment (Fig. 4). This difference of the bioavailability between FFA and PC form also might be a
3.3 Effect of lipogenesis and lipolysis on 3T3-L1 adipocytes

It is well known that the GPDH activity increases in adipocytes when they start to differentiate and accumulate lipids. Besides, the amount of glycerol released in the culture medium is a marker of lipolysis.

As shown in Fig. 5, the GPDH activity of adipocytes after treatment with CLA-PC and soy-PC was significantly lower than that after treatment with CLA-FFA; however, we did not observe any significant differences between the treatments with CLA-PC and soy-PC. These results suggested that the GPDH activity in 3T3-L1 cells was effectively suppressed by treatment with any of the PC forms. Furthermore, we found that the amount of glycerol in the culture medium did not differ among treatments (Fig. 6). A similar reason that PC form shows superior activity.

Fig. 3 Morphology of 3T3-L1 adipocytes. Lipid accumulation was observed using Oil Red O staining; magnification 400 ×. A. Control; B. CLA-FFA (200 µM); C. CLA-PC (200 µM); D. soy-PC (200 µM).

Fig. 4 CLA uptake of 3T3-L1 adipocytes treated with CLA-FFA and CLA-PC at 200 µM. * indicate significant difference (p<0.01, Scheffe’s test).

Fig. 5 GPDH activity (U/mg protein) of 3T3-L1 adipocytes treated with 200 µM CLA-FFA, CLA-PC, or soy-PC. Values are presented as % of control. Different letters indicate significant difference (p<0.01, Scheffe’s test).

Fig. 6 Glycerol content of 3T3-L1 adipocytes treated with 200 µM CLA-FFA, CLA-PC, or soy-PC. Values are presented as % of control. No significant difference was observed among treatments.
finding was reported by Kim et al.\textsuperscript{19} who found that there was no significant difference on the amount of glycerol released between CLA-FFA-treated and control 3T3-L1 adipocytes. Our results suggested that the inhibition of lipid accumulation in 3T3-L1 cells was not mediated by the lipolysis pathway but through the inhibition of lipogenesis via suppression of the GPDH activity.

3.4 Effect of different treatment timing on lipid accumulation and cell viability at of 3T3-L1 cells

To study the effect of the treatment on lipid accumulation at different stages of the differentiation of 3T3-L1 cells, cells were treated with CLA samples as indicated in Scheme 1b. Treatment of cells with each sample was performed only once at the early or middle stage of differentiation, and the lipid accumulation and cell viability of 3T3-L1 adipocytes were monitored. As shown in Fig. 7, at the early stage of differentiation, the lipid accumulation in the CLA-FFA-treated cells was lower than that in the CLA-PC and soy-PC groups (Fig. 7a), and the same tendency was observed for cell viability (Fig. 7b). At the middle stage of differentiation, there were no significant difference between the samples treated (Fig. 7a). We found that the cell viability of 3T3-L1 adipocytes at the middle stage of differentiation was lower after treatment with CLA-FFA compared with CLA-PC and soy-PC treatments (Fig. 7b).

This result suggested that the suppression of lipid accumulation by CLA in both forms was higher in the short compared with the long treatment period (Figs. 2 and 7a). In addition, we noticed that treatment in the middle stage of differentiation was more effective than that in the early stage (Fig. 7a). We also observed that CLA-PC was effective in reducing lipid accumulation in adipocytes, whereas CLA-FFA induced lipid accumulation but decreased the cell number (Fig. 7b). This finding was in line with a previous report by Satory and Smith\textsuperscript{5} that CLA could inhibit proliferation by decreasing the cell number and promoting lipogenesis and lipid filling in 3T3-L1 adipocytes. In contrast, the effect of CLA-PC on suppressing lipid accumulation in 3T3-L1 adipocytes was shown to be between that of CLA-FFA and soy-PC or almost the same as that of soy-PC. Therefore, CLA-PC might be an effective form of CLA in terms of daily intake compared with the FFA form.

3.5 Effect of PC components on lipid accumulation in 3T3-L1 adipocytes

It was reported that PC could induce lipolysis and apoptosis in 3T3-L1 adipocytes through the TNFα-mediated pathway\textsuperscript{13, 15}. To gain insight into the mechanism by which the PC form could suppress lipid accumulation more effectively than the FFA form, we investigated the effect of treatment with the PC component on lipid accumulation in 3T3-L1 adipocytes. In this experiment, we used choline, GPC, and LA (as a major fatty acid in soy-PC) as samples.

We accordingly found that GPC and LA showed significantly higher lipid suppression in cells than the control (Fig. 8). This result was in line with a previous report that
LA could reduce lipid accumulation in 3T3-L1 adipocytes, even though the effect was lower than that of CLA\(^{7}\). Based on this, we assumed that the suppression of lipid accumulation was affected by the chemical components of PC, although the mechanisms remain unclear.

4 Conclusion
In conclusion, CLA-PC was shown to be superior to CLA-FFA in decreasing lipid accumulation in 3T3-L1 adipocytes. This effect was attributed to the suppression of lipogenesis but not lipolysis in adipocytes. Therefore, considering a daily intake routine, the PC form of CLA is promising for the reduction of lipid accumulation in the body compared with the FFA form.

Author Contributions
Designed research; Y.Y., T.Y., and S.O., Performed research; R.R.F. and R.C., Contributed analytic tools; Y.Y. and R.C., Analyzed data or wrote the manuscript; R.R.F. and R.C., Contributed analytic tools; Y.Y. and T.Y., Performed research; Y.Y., T.Y., and S.O., Contributed analytic tools; Y.Y., T.Y., and S.O., Wrote the manuscript; R.R.F. and T.Y., Contributed analytic tools. Y.Y. and T.Y., Designed research; Y.Y., T.Y., and S.O., Performed research; Y.Y., T.Y., and S.O., Contributed analytic tools; Y.Y., T.Y., and S.O., Wrote the manuscript; R.R.F. and T.Y., Contributed analytic tools.

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