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Different effects of conjugated linoleic acid isomers on lipoprotein lipase activity in 3T3-L1 adipocytes

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Abstract

Conjugated linoleic acids (CLAs) are the positional and geometric isomers of linoleic acid. In the present study the effects of cis-9, trans-11 CLA (c9,t11 CLA) and trans-10, cis-12 CLA (t10,c12 CLA) on intracellular and heparin-releasable (HR-) lipoprotein lipase (LPL) activity in 3T3-L1 adipocytes were investigated. Cells were exposed to the two CLA isomers and linoleic acid, which were bound to bovine serum albumin (BSA). In the adipocytes insulin up-regulated and tumor necrosis factor alpha (TNFα) down-regulated HR-LPL activity, which corresponds with the findings in vivo. The experimental fatty acids at low concentrations (<30 μmol/L) moderately increased intracellular and HR-LPL activity. At a concentration of 100 μmol/L, c9,t11 CLA and t10,c12 CLA suppressed HR-LPL activity to 20 and 24% below the BSA control level, respectively, while linoleic acid had no effect unless its concentration was as high as 1000 μmol/L. Insulin abolished the inhibitory effect of c9,t11 CLA, but not of t10,c12 CLA. In the presence of insulin, t10,c12 CLA inhibited HR-LPL activity by 41% compared to BSA control. In contrast to TNFα, which suppressed both intracellular LPL and HR-LPL activity, CLAs suppressed HR-LPL activity without decreasing intracellular LPL activity. Additionally, t10,c12 CLA (100 μmol/L) partially prevented TNFα-induced decrease of intracellular LPL activity. These results indicate that CLAs differ from linoleic acid in regulating HR-LPL activity, and t10,c12 CLA appeared to be more effective than c9,t11 CLA.

Keywords: 3T3 L1 adipocytes; Conjugated linoleic acid; Lipoprotein lipase; Fatty acids

1. Introduction

A group of positional and geometric isomers of linoleic acid, which is named conjugated linoleic acid (CLA), has been demonstrated to have unique effects on lipid metabolism in vivo. For example, dietary supplementation of CLA has been shown to inhibit endogenous fatty acid synthesis and consequently decrease lipid secretion in the mammary gland in cows [1,2]. CLA decreased plasma lipid levels and reduced the development of atherosclerosis in rabbits [3] and hamsters [4]. It was initially thought that the potential anti-atherosclerosis property of CLA might be associated with its plasma lipid lowering effect. However, a recent study in our laboratory in hamsters showed that trans-10, cis-12 (t10,c12) CLA increased plasma VLDL-triglycerides [5]. Lipoprotein lipase (LPL) is the key enzyme, which hydrolyses VLDL-triglycerides. Decrease of LPL activity may lead to an increase of VLDL-triglycerides. This hypothesis is corroborated by the findings that dietary CLA rapidly reduced body fat and enhanced lean body mass in a number of experimental animals such as mice [6] and rats [7]. It is known that LPL plays an important role in fat storage in the body. LPL hydrolyses triglycerides in triglyceride-rich lipoproteins (such as VLDL and chylomicrons), generating free fatty acids that can serve either as a direct energy source in muscle tissue or can be stored in adipocytes in the form of triglycerides. Increased LPL activity is associated with the development of obesity [8]. In this context, both the body-fat reducing effect and plasma-VLDL increasing property of CLA might be mediated by suppressing lipoprotein lipase activity.

Physiologically, LPL is synthesized and secreted by adipocytes. After being secreted from the cells, LPL becomes

Abbreviations: BSA: bovine serum albumin; CLA: conjugated linoleic acid; HR-LPL: heparin-releasable lipoprotein lipase; LDL: low density lipoproteins; LPL: lipoprotein lipase; MDH: mitochondrial dehydrogenase; PBS: phosphate buffered saline; TNFα: tumor necrosis factor alpha; VLDL: very low density lipoproteins

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anchored to the luminal surface of the capillary endothelial cells. The LPL attached on endothelial cells can be released by heparin and is called heparin releasable LPL (HR-LPL). The aim of this study was to investigate the effects of cis-9,trans-11 (c9,t11) CLA and t10,c12 CLA on (HR-)LPL activity in cultured 3T3-L1 adipocytes. Although a number of CLA isomers have been found in meat and diary products [9], c9,t11 CLA and t10,c12 CLA are the principal two isomers contained in commercial CLA mixture, prepared by alkaline isomerization of sunflower oil [10].

2. Materials and methods

2.1. Materials

3T3-L1 adipocytes were purchased from American Type Culture Collection, Rockville MD, USA. Dulbecco's modified Eagle's medium was obtained from Gibco BRL, Paisley, UK. Both fetal bovine serum and human serum were obtained from Bio Whittaker, Verviers, Belgium. 12-Well cell-culture plates were obtained from Costar, NY, USA. Methylisobutylxanthine, insulin, bovine serum albumin (BSA, essential fatty acid free), and linoleic acid were obtained from Sigma, St. Louis, MO, USA. Dexamethason was supplied by Alexis, CA, USA. The CLA isomers were provided by Unilever Research, Colworth laboratory, UK. The purity of t10,c12 CLA and c9,t11 CLA was 98.8% and 93.8%, respectively, as determined by gas-chromatography. EZ4U kits (for the determination of mitochondial dehydrogenase activity) were obtained from Sanvertech, Heerhugowaard, NL. Heparin (5000 U/ml) was from LEO Pharmaceutical Products, Weesp, NL. ^{3}H-triolein was obtained from Amersham, Bucks, UK. Picofluor scintillation solution was supplied by Packard, Groningen, NL. Tumor necrosis factor alpha (TNFα) (human origin, recombinant) was produced by Alexis Corp. San Diego, USA. All other chemicals were acquired from Sigma, St. Louis, MO, USA.

2.2. Cell culture

3T3-L1 adipocytes were cultured in basic medium, which consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at 56°C for 1 h), 4 mmol/L L-glutamine, 3.7 g/L natrium bicarbonate, 4.5 g/L glucose, 1 mmol/L natrium pyruvate, 20000 U/L penicillin, and 2 mg/L streptomycin. Cells were cultured in 12-well plates at 37°C in a humidified 10% CO_2 atmosphere. To initiate differentiation, the cells were allowed to reach 2 days post-confluence (counted as day one). These cells were then cultured for two days in differentiation-inducing medium, which consisted of basic medium supplemented with methyl-isobutylxanthin (0.5 mmol/L), dexamethason (0.25 μmol/L), and insulin (1 μg/L). Next, the cells were cultured in basic medium supplemented with insulin (1 μg/L) for another two days (the 3rd and 4th day), and subsequently cultured in basic medium. Experiments were conducted between day 7 and 14 when 60–100% of the cells were differentiated into adipocytes (containing fat in the cells). In experiments, cells were cultured in experimental medium, which consisted of basic medium supplemented with fatty acid-BSA complex.

2.3. Preparation of fatty acid-BSA complex

Fatty acids were bound to bovine serum albumin (BSA, essential fatty acid free) to form fatty acid-BSA complex, which was prepared according to the following steps: (1) BSA was dissolved (0.25 mmol/L at final concentration) in basic medium to form BSA-medium. (2) This BSA-medium was adjusted to pH10 with 1 mol/L NaOH. (3) Different fatty acid-BSA complexes were made by adding LA, c9,t11 CLA, t10,c12 CLA to BSA-medium to obtain 1 mmol/L concentration of the fatty acid. (4) The mixtures were sonicated until optically clear solutions were obtained. (5) The media were adjusted to pH 7.4 with 1 mol/L HCl. (6) Fatty acid-BSA complex (1 mmol/L) was diluted with BSA-medium to obtain the experimental media containing 10–1000 μmol/L of fatty acids. The homogeneity of the solution was determined by gas-chromatography. The analytical variation by measuring different fractions of the fatty acid-albumin complex medium was $<$5% of mean values. The experimental media were warmed to 37°C before use.

2.4. Evaluation of cell viability

The mitochondrial function of the cells was evaluated by measuring the mitochondrial dehydrogenase (MDH) activity by using the modified tetrazolium reduction assay (EZ4U test [11]). This tetrazolium salt (EZ4U) has higher solubility in water comparing to that used in the MTT test [12]. It is known that only living cells are able to reduce a colorless tetrazolium salt to an orange water-soluble derivative by mitochondrial dehydrogenases. This reduction is inactivated within a few minutes after cell death. In this test, 40 μl EZ4U solution was added to each well at thirty minutes before the termination of the experiment, and the color produced by the living cells was assayed at 450 nm. Fatty acids at the concentration up to 1000 μM did not lead to toxic effects as evaluated by this assay.

2.5. Determination of LPL activity

2.5.1. Heparin-releasable (HR) LPL

After incubation of the cells with experimental medium, the medium was discarded. The cells were rinsed with 0.5 ml phosphate buffered saline (PBS, pH 7.4) and then cultured in heparin-PBS (0.3 ml/well, 10 U/ml heparin) for 1 h. The experiment was terminated by putting the culture plates on ice, and the conditioned heparin-PBS was collected from each well for the assay of HR-LPL activity.

2.5.2. Intracellular LPL

After the collection of the conditioned heparin-PBS, cells were rinsed with PBS and were then scraped from the wells with 0.3 ml PBS. The cell suspensions were sonicated to get homogeneous solutions in which the intracellular LPL activity was determined within 1 h after termination of the experiments.

2.5.3. Assay of (HR-)LPL activity

The (HR-)LPL activity was measured as described by Iverius et al. [13]. In brief, an emulsion of ^{3}H-triolein stock solution was prepared by mixing ^{3}H-triolein (45 μCi, 600 mg triolein) with phosphatidylcholine (36 mg) in glycerol (10 ml), and sonicated until an optically clear solution was obtained. The substrate solution was obtained by mixing the ^{3}H-triolein stock solution with Tris-HCl buffer (pH 8.2 at 37°C), 3% (w/v) BSA solution, 0.78 M NaCl, and inactivated human serum at ratio 1:4:1:1 (v/v/v/v). This substrate solution consisted of ^{3}H-triolein (10 nmol/ml, 1.5×10^6 dpm/ml), lecithin (0.7 mg/ml), glycerol (170 mg/ml), BSA (170 mg/ml), inactivated human serum (170 mg/ml, as source of apoC-II), Tris-HCl (159 mmol/L), and NaCl (110 mmol/L). The LPL activity was measured by adding 100 μl substrate to 100 μl conditioned PBS- or cell suspensions and incubate at 37°C in a shaking waterbath for 1 h. The reaction was stopped by adding 3 ml of methanol-dichloromethane-heptane (1.41:1.25:1, v/v/v) to the mixture. Free (^{3}H-)fatty acids released from the substrate were extracted by adding 1 ml 50 mmol/L carbonate-borate buffer (pH 10.5). One ml of the upper aqueous phase was transferred to a scintillation vial containing 10 ml of scintillation liquid (Picofluor). The radioactivity of the samples was counted. The LPL activity is expressed in milli-units, which corresponds to one nanomole of fatty acid released from triglycerides per minute at 37°C.

The assay conditions (pH 8.2, glycerol/phospholipid stabilized emulsion of triolein and in the presence of apoC-II) employed are optimal for LPL but poor for other lipases [14–16]. Park et al. reported that LPL activity accounted for >88% of the total lipolytic activity under this assay conditions in adipocytes [17]. Similar results have also been observed by Fried et al. [18]. Therefore, the intracellular total lipolytic activity was used as indication of intracellular LPL activity in the present study.

2.6. Data analysis

The values of intracellular LPL, HR-LPL and MDH activity were normalized for the cellular protein content. Data were presented as percentage of the corresponding BSA control, which was essential fatty acid free. Data were analyzed with two-way (treatment and experiments) analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

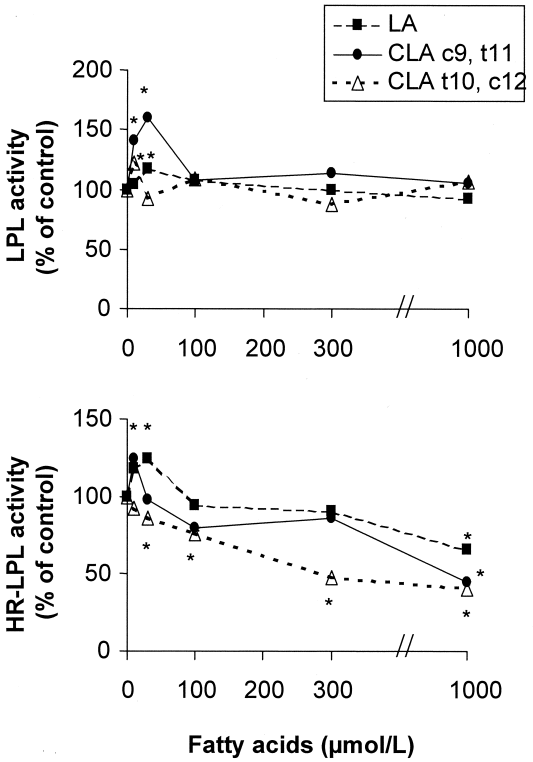

Fig. 1. Effects of fatty acid dose on intracellular LPL activity (upper panel) and HR-LPL activity (lower panel). 3T3-L1 adipocytes were cultured for 16 h in the media containing various concentrations of fatty acids, with BSA as control. After this incubation, cells were rinsed with PBS and then exposed to heparin-PBS (10 Unit/ml) for 1 h. The conditioned heparin-PBS was harvested and HR-LPL activity was measured. The cells, after rinsed with PBS, were used for the determination of intracellular LPL activity. Data are presented as percentage of the respective values of BSA controls. For the BSA control, the LPL activities varied from 26 to 62 milli-units and HR-LPL activities from 11 to 20 milli-units, depending on the independent experiments. For clarity only mean values, which were collected from three independent experiments, are shown. The SE is within 12% of the mean. * Indicates the value is significantly different from that of BSA control (as 100%) ($P < 0.05$).

3. Results

3.1. The dosage of CLA on LPL activity

Cells were cultured in the presence of different concentrations of indicated fatty acids for 16 h. Figure 1 shows that intracellular LPL activity increased by 22–59% when cells were exposed to 10–30 μM fatty acids. Upon increasing the fatty acid concentrations, the intracellular LPL activity returns to the initial level. The HR-LPL activity was suppressed by t10,c12 CLA in a dose dependent manner. The maximal inhibitory effect (-52%) was reached at a concentration of 300 μmol/L t10,c12 CLA . In contrast to t10,c12 CLA, both c9,t11 CLA and LA at low concentration (10 μmol/L) remarkably stimulated HR-LPL activity to the level of 18–24% above the BSA control. This phenomenon was also observed by Park et al. [19]. At higher concentration (>100 μmol/L) c9,t11 CLA began to suppress HR-

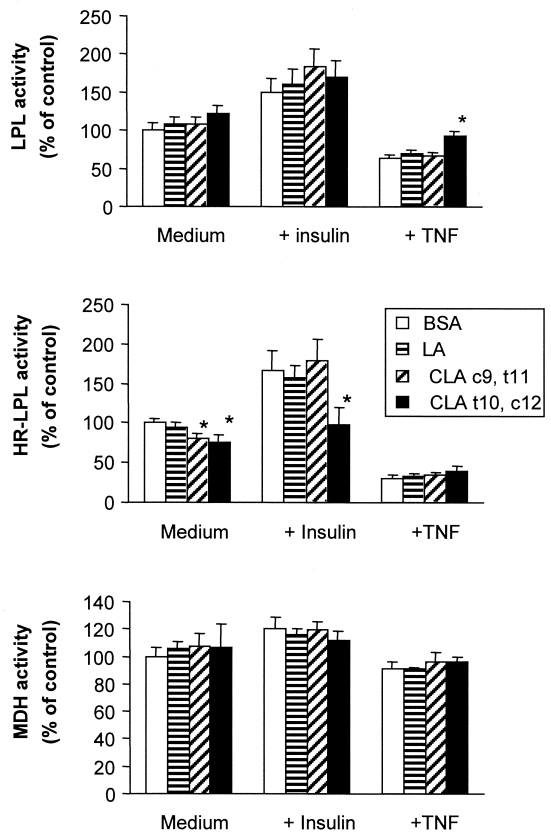

Fig. 2. Effects of fatty acids on intracellular LPL (upper panel), HR-LPL (middle panel), and MDH (lower panel) activity in the presence or absence of insulin (6 μg/ml) or TNFα (20 ng/ml). 3T3-L1 adipocytes were cultured for 16 h in the media containing 100 μmol/L of indicated fatty acids, with BSA as control. The (HR-)LPL activity was determined as described in Figure 1. For the BSA control, the LPL activities varied from 26 to 62 milli-units and HR-LPL activities from 11 to 20 milli-units, depending on the independent experiments. MDH activity was determined at 450 nm for the production of color derivatives of tetrazolium salt. Data are presented as percentage of the values of BSA in the medium without insulin or TNFα. The means ± SE, collected from three independent experiments in triplicate, are shown. * Indicates that the value is significantly different ($P < 0.05$) from that of BSA within the correspond group. The (HR-) LPL activities are significant difference between the treatments of medium, insulin or TNFα ($P < 0.05$).

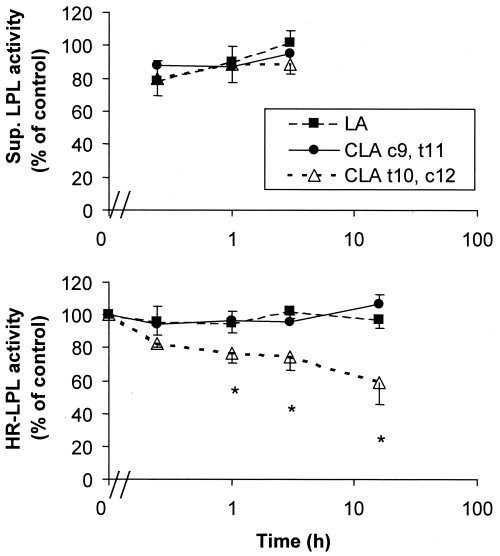

Fig. 3. Time course of fatty acids on LPL activity in medium supernatant (upper panel) and HR-LPL activity (lower panel) in the presence of insulin. 3T3-L1 adipocytes were cultured in medium containing indicated fatty acids (100 μmol/L) and insulin (6 μg/ml) for 15 min, 1 h, 3 h or 16 h. After incubation, the media were collected and centrifigated at 8000 rpm for 3 min to get rid of cell debris. The LPL activity in the medium supernatant was determined. HR-LPL activity was determined as described in Figure 1. Data are presented as percentage of (HR-)LPL activities of the respective BSA controls, which were 11–26 (for supernatant LPL) and 37–70 (for HR-LPL) milli-units, depending on the independent experiments. Reported values are means ± SE, collected from three independent experiments and each of them was conducted in triplicate. * Indicates significant difference from BSA control at the respective time point ($P < 0.05$).

LPL activity, while LA showed its inhibitory effect at 1000 μmol/L.

3.2. Effects of CLA on LPL activity in the presence of insulin or TNFα

It is known that insulin stimulates LPL activity in the body [20] and that TNFα has a suppressing effect [21]. We further evaluated our test-system by incubating 3T3-L1 adipocytes in the presence of insulin and TNFα. Results, as showed in Figure 2, revealed that insulin remarkably increased and that TNFα decreased intracellular and heparin releasable LPL activity. These data indicate that 3T3-L1 adipocytes can sensitively regulate LPL activity in response to the stimulation of insulin or TNFα. In the absence of insulin, both 100 μmol/L c9,t11 CLA and t10,c12 CLA suppressed HR-LPL activity. Insulin could override the inhibitory effect of c9,t11 CLA, but not of t10,c12 CLA. In the presence of insulin t10,c12 CLA more pronouncedly inhibited HR-LPL activity (−41%) as compared to that in the absence of insulin (−25%). The intracellular LPL activity was not reduced by the presence of the experimental fatty acids compared with corresponding BSA control. By contrast, t10,c12 CLA partially abolished the TNFα-induced decrease of cellular LPL activity. In the presence of TNFα, t10,c12 CLA did not suppress HR-LPL activity. No adverse effect of LA, c9,t11 CLA and t10,c12 CLA on cell viability was observed in the presence of insulin or TNFα as indicated by MDH activity.

3.3. The time course of fatty acids on HR-LPL activity in the presence of insulin

When cells were cultured in the media with insulin, the HR-LPL activities increased by 31, 28, 60, and 67% above the corresponding values of control (without insulin) at 15 min, 1h, 3h or 16 h, respectively. Figure 3 (lower panel) shows that in the presence of insulin, t10,c12 CLA (100 μmol/L) suppressed HR-LPL activity to 77% of the control level within 1 h. In 16 h incubation the suppressing effects

of t10,c12 CLA was more pronounced. In contrast to t10,c12 CLA, both LA and c9,t11 CLA had no effect on HR-LPL activity when insulin was included in the medium. These results are consistent with those shown in the Figure 2. It has been reported that fatty acids can release LPL from cell membrane by competing with LPL for the binding sides [22]. In order to elucidate this direct LPL-releasing effect of fatty acids, adipocytes were cultured in the presence of the indicated fatty acids for 15 min to 3 h and LPL activity was measured in the cell culture media without heparin-adding. The results are shown in Figure 3 (upper panel). LPL activity in the medium-supernatant was not increased by fatty acids. Additionally, an isolated HR-LPL had been incubated for 1 h with 100 μmol/L LA and CLA isomers, respectively, results showed that these fatty acids had no direct effect on enzyme activity (data not shown).

4. Discussion

The present study documents that in 3T3-L1 adipocytes the modulation effects of (HR-) LPL activity by insulin, cytokine (TNFα) or fatty acids are promptly (within 1 h although more pronouncedly in 16 h) (Figure 3), in contrast with chicken adipocytes which required more than one day to change the HR-LPL activity in response to fatty acids [23]. These results indicate that 3T3-L1 adipocytes provide a good model for the study of lipid metabolism in adipose tissues in vitro. Recently, Park et al investigated the chronic effect of fatty acids on HR-LPL activity by using 3T3-L1 adipocytes and reported that t10, c12 CLA suppressed HR-LPL activity in 48-h cell-culture [24]. This chronically inhibitory effect is difficult to be extrapolated to the situation in vivo. The present study investigated the acute (from 15 min to 16 h) effects of fatty acids on HR-LPL activity. These results may have more physiological relevance since the fatty acid concentrations in the plasma are changed within hours.

The mechanisms by which t10,c12 CLA suppressed HR-LPL activity are clearly different from that by TNFα. This is based on the fact that the suppressed HR-LPL activity by TNFα was accompanied by a decreased intracellular LPL activity. In contrast, t10,c12 CLA did not decrease intracellular LPL activity. Additionally, t10,c12 CLA partially prevented TNFα-induced decrease of intracellular LPL activity. It has been reported that TNFα diminishes LPL mRNA level in 3T3-L1 adipocytes by down-regulating LPL gene transcription [25]. These data suggest that t10,c12 CLA induced-decrease of HR-LPL activity might not be mediated by down regulation of intracellular LPL production. Free fatty acids have been observed to cause dissociation of the HR-LPL from its binding to the cellular heparan sulfate proteoglycans in vivo [22]. However, in the present study the LPL activities in the supernatant of the medium were not increased by the supplement of fatty acids (Figure 3). These results do not support the hypothesis that inhibitory effect of CLAs is via dissociation of LPL from the membrane of adipocytes. The HR-LPL is derived from the intracellular LPL, which is secreted by the adipocytes. Therefore numerous possibilities exist with regard to control of HR-LPL activity on its way to the site of action, for example by influencing intracellular transport and secretion. In the present study the total LPL activity (cellular LPL activity + HR-LPL) (Figure 2) was not altered by t10,c12 CLA supplemented to medium with or without TNFα. It is tempting to postulate that t10,c12 CLA might suppress intracellular LPL transport to the cell membrane. Further studies are required to confirm this hypothesis.

LPL activities in the vascular and myocardial tissue from rat heart have been shown to be down-regulated by fatty acids [26]. The present study demonstrated that the regulation of LPL activity by fatty acids is in a dose dependent manner, e.g. up-regulation of intracellular and HR-LPL activity at lower ($<$30 μmol/L) and down-regulation of HR-LPL activity at higher concentration ($>$100 μmol/L) (Figure 1). LA has less effect in suppression of HR-LPL activity than CLAs. The former began to inhibit HR-LPL activity at concentration of 1 mmol/L. This property of fatty acids on regulating HR-LPL activity may have important physiological significance. Under fasting conditions, plasma fatty acid concentrations are low, which increases triglyceride hydrolysis by stimulating HR-LPL activity. In the postprandial phase, plasma fatty acid concentrations are higher than 100 μmol/L [27], which may suppress HR-LPL activity to prevent overload of fat in adipocytes. Interestingly, insulin remarkably increased (HR-) LPL activity and completely override the inhibitory effects of c9,t11 CLA on HR-LPL activity. These results indicate that insulin may play a principal role in the regulation of LPL activity in the body. However, the suppressing effect of t10,c12 CLA was not influenced by insulin. This finding suggests that t10,c12 CLA is the unique fatty acid which might exert down-regulatory effects of HR-LPL activity in postprandial phase in the body when plasma insulin concentration is increased.

One of the physiological functions of HR-LPL is to hydrolyze triglycerides contained in VLDL and chylomicrons. This action leads to the formation of smaller cholesterol-rich lipoproteins, e.g. low density lipoproteins (LDL) and chylomicron remnants. The HR-LPL activity-inhibitory effects of t10,c12 CLA observed in the present study might explain, at least partly, the previous finding that diet supplemented with t10,c12 CLA moderately increase plasma VLDL-triglyceride level in hamsters [5]. Since VLDL is the precursor of LDL, inhibition of HR-LPL activity might obstruct the conversion of VLDL into LDL. This concept appeared to be corroborated by the fact that hamsters fed with CLA supplemented diets had a lower plasma LDL concentrations [5]. However, an opposite finding has been reported that a mixture of CLA-isomers decreased plasma VLDL-triglyceride in hamsters [4] and mice [28]. The discrepancy of these two reports might be attributed to different dosage and/or different isomers of CLA supplemented

to the diets. The present study clearly shows that CLA isomers and their dosage have different effects on HR-LPL activity in 3T3-L1 cells.

HR-LPL is known to play an important role in the uptake of triglyceride-derived fatty acids by adipose tissue for fat-storage. The LPL expression in adipose tissue has therefore been related to the initiation and/or development of obesity. A higher activity of LPL in adipose tissue has been observed in obese rats [29] and human subjects [8]. The increased accumulation of fat in obese rat adipocytes is caused by increased HR-LPL activity due to a hyperresponsiveness to insulin on LPL synthesis. The unique inhibitory property of t10,c12 CLA, on HR-LPL activity in the presence of insulin suggests that it might have anti-obesity effects. In support of this, a recent study conducted by Park et al showed that t10,c12 CLA decreased body fat in mice [24].

Recently, the number of the studies concerning the relationship between LPL and atherosclerosis is increased due to the findings of numerous novel properties of LPL, which are associated with the development of atherosclerosis. LPL deficiency is supposed to be a risk factor in the development of cardiovascular diseases, which is associated with an elevated VLDL triglyceride level [30]. However, decreased LPL activity may decrease plasma LDL levels [31]. LDL is more artherogenic than VLDL. Additionally, it has been recently demonstrated that LPL could function as a monocyte adhesion protein to enhance macrophage accumulation within arteries, and macrophage LPL promotes foam cell formation and atherosclerosis development in vivo [32]. Therefore, some investigators pointed out that LPL might be directly involved in the atherogenic process [33]. Furthermore, Semenkovich et al recently demonstrated that heterozygous LPL deficiency in the vascular wall prevented diet-induced atherosclerosis in mice [34]. Taken together, these data suggest that regulating LPL activity of t10,c12 CLA might be associated with its anti-atherosclerosis effect. More studies are required to elucidate the physicological and pathological relevances of the HR-LPL inhibitory effect of t10,c12 CLA in the development of atherosclerosis.

In conclusion, our data showed that CLAs differ from linoleic acid in regulating HR-LPL activity, and t10,c12 CLA appeared to be more effective than c9,t11 CLA. The inhibitory effect is not accompanied by the suppression of intracellular LPL activity.

Acknowledgments

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