Incorporation profiles of conjugated linoleic acid isomers in cell membranes and their positional distribution in phospholipids

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Abstract

Although the conjugated linoleic acids (CLA) have several isomer-specific biological effects including anti-carcinogenic and anti-adipogenic effects, their mechanisms of action remain unclear. To determine their potential effects on membrane structure and function, we studied the incorporation profiles of four CLA isomers (*trans*-10 *cis*-12 (A), *trans*-9 *trans*-11 (B), *cis*-9 *trans*-11 (C), and *cis*-9 *cis*-11 (D)) in CHO and HepG2 cells. All four isomers were incorporated into cellular lipids as efficiently as linoleic acid (LA), with the majority of the incorporated CLA present in membrane rafts. Of the four isomers, only CLA-A increased the cholesterol content of the raft fraction. Over 50% of the incorporated CLAs were recovered in phosphatidylcholine of CHO cells, but in HepG2 the neutral lipids contained the majority of CLA. The desaturation index (18:1/18:0 and 16:1/16:0) was reduced by CLA-A, but increased by CLA-B, the effects being apparent mostly in raft lipids. The Δ^9 desaturase activity was inhibited by CLAs A and C. Unlike LA, which was mostly found in the *sn*-2 position of phospholipids, most CLAs were also incorporated significantly into the *sn*-1 position in both cell types. These studies show that the incorporation profiles of CLA isomers differ significantly from that of LA, and this could lead to alterations in membrane function, especially in the raft-associated proteins.

Key words: Conjugated linoleic acid; membrane raft; desaturation index; positional distribution; stearoyl CoA desaturase; raft cholesterol

Abbreviations:

CLA: conjugated linoleic acid

DHA: docosahexaenoic acid

EPA: eicosapentaenoic acid

FFA: free fatty acid

LA: linoleic acid

PPAR: peroxisome proliferator-activated receptor

PBS: phosphate-buffered saline

PC: phosphatidylcholine

PE: phosphatidylethanolamine

SCD: stearoyl CoA desaturase

1. Introduction

Conjugated linoleic acids (CLA), the naturally occurring isomers of linoleic acid (LA) in the dairy products and meats, have been reported to have several beneficial effects including anti-carcinogenic, anti-inflammatory, and anti-obesity effects [1-3], but the underlying mechanisms are poorly understood. While there are several possible isomers of CLA, *cis*-9 *trans*-11, and *trans*-10 *cis*-12 isomers are the predominant isomers found in the natural products as well as in the commercially available nutritional supplements. With the availability of pure CLA isomers, it became evident that the isomers differ significantly in their biological effects. Thus the *cis*-9 *trans*-11 isomer was shown to be responsible for the majority of the anti-carcinogenic effects, whereas the *trans*-10 *cis*-12 isomer appears to be responsible for the anti-obesity effects in mice [2,3]. Despite extensive studies, the exact mechanisms of action of CLA, especially the isomer-specific effects are unclear. While several investigators reported the isomer-specific effects on the transcription of selected genes [1,4-6], others reported that CLA act as ligands for the transcription factors PPARα and PPARγ [2]. However the pleiotropic effects of CLA are hard to explain simply on the basis of their known gene transcription effects. Furthermore, the PPAR affinities of CLA isomers do not differ from each other [7] or from the more abundant endogenous fatty acids [2]. Therefore the ligand properties of CLA are unlikely to be responsible for the majority of their biological effects or for their isomer-specific effects. Since the CLA, like other polyunsaturated fatty acids, are incorporated into the cell membrane lipids, some of their biological effects could be due to their effects on membrane composition and function, as demonstrated in the case of omega 3 fatty acids [8-10]. Thus, it is known that EPA and DHA

are readily incorporated into the raft and non-raft lipids of cell membranes, and consequently affect the functions of raft-associated proteins [8,11,12]. The possible effects of CLA isomers on membrane structure and function have not been investigated, and no systematic studies on their incorporation profiles, especially in the raft and non-raft lipids, have been carried out. In this study, we investigated the incorporation of the two most abundant natural CLA isomers (*trans*-10 *cis*-12 (CLA-A), and *cis*-9 *trans*-11 (CLA-C)), into the total membranes of CHO cells and HepG2 hepatocytes, as well as into the raft and non-raft lipids of CHO cells. In addition, we determined the positional distribution of CLA in the phospholipids of the cell membranes. To determine the relative effects of the conjugation and the double bond configuration on the incorporation profiles, we have included in the study two unnatural CLA isomers, namely a double *trans* isomer (*trans*-9 *trans*-11 (CLA-B)) and a double *cis* isomer (*cis*-9 *cis*-11, (CLA-D). The results show that all the CLA isomers are incorporated into both raft and non-raft lipids, with the majority in the raft lipids, and that the fatty acid composition of the membranes is altered differentially by the various isomers. The CLAs replaced 18:1 in most cases, and inhibited the Δ^9 desaturase activity in an isomer-specific manner. In addition, we found that unlike the unconjugated 18:2 (LA), which is found predominantly in the *sn*-2 position, a significant percentage of the incorporated CLAs was found in the *sn*-1 position of the phospholipids. The presence of two *cis* or two *trans* double bonds in CLA increased its incorporation into the *sn*-1 position of phospholipids, but decreased the inhibitory effect on Δ^9 desaturation, compared to the natural CLA that contain one *cis* and one *trans* double bond. These results support the hypothesis that CLA may exert part of their biological effects through modification of membrane lipid composition and function, especially in the raft domains.

2. Material and Methods

2.1. Materials:

CHO and HepG2 cells were obtained from ATCC. Essentially fatty acid-free BSA, lyophilized snake venom (*Crotalus atrox*) and linoleic acid were purchased from Sigma chemical Co. Lipid standards for TLC were obtained from Avanti Polar Lipids. The CLA isomers (96+% pure) were obtained from Matreya LLC (Pleasant Gap, PA). Radio-labeled stearic acid (1-^{14}C, 55 mCi/mmol) was obtained from American Radiochemical Corp. Silica gel G TLC plates were purchased from E.Merck. TLC plates containing 5% silver nitrate in siliga gel H were prepared in-house using a Camag spreader. All the solvents were reagent grade, and were purchased from Fisher Scientific.

2.2. CLA treatment of cells:

CHO cells were grown in 75 cm^2 culture flasks to about 50% confluency in Ham's F-12 medium with HEPES buffer and 10% fetal bovine serum, containing Penicillin (50 units/ml) and Streptomycin (50 µg/ml). Growth medium was then replaced with the incorporation medium, composed of Ham's F-12 medium with HEPES, 2% lipoprotein deficient serum (LPDS), 0.4% essentially fatty acid free BSA, and with 50 µM of one of the following; CLA-A (*trans*-10 *cis*-12 or t10c12), CLA-B (*trans*-9 *trans*-11 or t9t11), CLA-C (*cis*-9 *trans*-11 or c9t11), CLA-D (*cis*-9 *cis*-11 or c9c11) or unconjugated 18:2 (LA). CLAs and LA were added as ethanol solutions (5mg/ml), with the final ethanol concentration in the medium at <0.3%. Control flasks containing no added fatty acids in the medium were included in each study. Cells were incubated in the incorporation medium for 48 h at 37 °C

in 5% CO_2. Cells were washed with PBS, trypsinized with 0.05% trypsin-EDTA, harvested by centrifugation, and washed once more with PBS before suspending in 10 mM Tris buffer, pH 7.4. The cells were mechanically lysed by passing them through a 3-inch, 25-gauge needle twenty times, and either directly extracted for the total lipids or fractionated into raft and non-raft fractions as described below. HepG2 cells were treated similarly, except that the growth medium was a 50:50 mixture of F-12 and DMEM, and it also contained 1% non-essential amino acid mixture (Gibco # 11140) in addition to the antibiotics and fetal bovine serum. The trypsinization of HepG2 cells was done with 0.25% trypsin-EDTA.

2.3. Raft –non raft fractionation:

Raft isolation was carried out by a modification of the non-detergent method of Macdonald and Pike [13]. Cells from one 75 cm^2 flask were suspended in 950 µl of 10 mM Tris buffer and 50 µl of Protease Inhibitor II cocktail (Calbiochem) was added. Following cell lysis as described above, 1 ml of 95% sucrose was added, and this mixture was gently layered under 6 ml of 30% sucrose in a Beckman 14 x 89 mm Ultra Clear centrifuge tube, and 2 ml of 5% sucrose was gently layered on the top. Samples were centrifuged for 18 h at 38,000 rpm at 4°C in a Beckman SW41 rotor, and 0.6 ml fractions were collected with an ISCO model 640 fractionator. Aliquots of the fractions were assayed for cholesterol with the Amplex Red kit (Invitrogen), and the raft and non-raft fractions were pooled corresponding to the cholesterol peaks.

2.4. Fatty acid analysis:

The lipids were extracted by the Bligh and Dyer method [14], after adding 17:0 free fatty acid (FFA) as the internal standard. Methyl esters of the total lipid extract were prepared using methanolic HCl (Alltech), and analyzed in a Shimadzu GC-17A instrument, equipped with a flame ionization detector, employing a Omegawax 250 column (30m x 0.25 mm x 0.25 μm). Hydrogen was used as carrier gas (37 ml/min) and the injection port was set at 250 °C, while the detector was set at 260 °C. The temperature gradient was as follows: initial temperature at 150 °C for 1.0 min, raised to 210 °C at 3.0 °C/min, then to 225 °C at 2.0 °C/min, and finally held at this temperature for 15 min. The fatty acid methyl esters were identified with the help of authentic standards, and the peaks quntitated with EZ Chrom software (Shimadzu). The desaturation (18:3) and elongation (20:2) products of each CLA were identified from the retention times relative to the corresponding unconjugated analogs.

2.5. TLC Separation:

Cells were suspended in 0.4 ml of 10 mM Tris-Cl buffer pH 7.4, the lipids were extracted [14] after adding di-17:0 PC, di-PE and 17:0 free fatty acid (FFA) as internal standards. The lipid extract was separated on a silica gel TLC plate using the mobile phase of chloroform: methanol: acetic acid: 0.15 M NaCl (60:30:10:3 by vol). Standards of sphingomyelin, PC, PE, phosphatidylinositol (PI), and oleic acid (FFA) were spotted on the plate, and the lipids were visualized by exposure to iodine vapors. The spots corresponding to PC, PE, and neutral lipids (free fatty acids, triacylglycerol and cholesteryl ester) were scraped and eluted [15], methylated, and analyzed by GC as described above. Very little of CLA were incorporated into sphingomyelin. Some incorporation was found into PI, but is not included in the analyses.

2.6 Positional distribution of CLA:

The total lipids of the cells were extracted by Bligh and Dyer procedure [14], after adding di-17:0 PC and di-17:0 PE as internal standards. The samples were separated on a silica gel TLC plate using the solvent system of chloroform: methanol: water (65:25:4 by vol). The PC and PE spots were identified with the help of standards after visualizing the lipids by brief exposure iodine vapors, and the spots were scraped and eluted [15]. The solvent was evaporated and the lipids were redissolved in 5 ml diethyl ether, and treated overnight at room temperature with 50 µl of a 1 mg/ml solution of crude snake venom (*Crotalus atrox*) in 10 mM Tris-Cl pH 7.4, containing 10 mM $CaCl_2$. Following evaporation of the remaining ether, the lipids were extracted [14], and separated on a TLC plate with the solvent system of chloroform: methanol: water (65: 25: 4 by vol). The lysophospholipid and free fatty acid spots were identified with the help of standards, eluted [15], methylated, and analyzed by GC as described above. The fatty acid composition of lyso PC or lyso PE represents the composition of the *sn*-1 position of the corresponding diacyl phospholipid, while the fatty acid composition of free fatty acid spot represents the *sn*-2 acyl composition. The amount of CLA at each position was calculated with the help of the corresponding value for the internal standard (17:0).

2.7. Assay of Δ^9 desaturase activity

CHO cells were grown in 6-well plates, and treated with various CLA isomers and LA (50 µM) for 44 h in 2% LPDS as described above, and further incubated with 2µM C^{14}-stearic acid (100 nCi, added in 5 µl ethanol) for 4 h. The cells were washed with PBS, trypsinized, and again washed with 10 mM Tris buffer and finally re-suspended in 0.4ml of 10mM Tris

pH 7.4, and homogenized by passing 10x through a 25 gauge 3-inch needle. Unlabeled stearic and oleic acids (100 µg each) were added as carriers to samples and the total lipids were extracted [14]. The lipids were methylated with methanolic HCl (Analtech #18053, 1 h at 50°C), evaporated under nitrogen, and re-dissolved in 50 µl of chloroform. The methyl esters were separated on 5%$AgNO_3$ silica gel H TLC plates with the solvent system of hexane: diethyl ether (9:1, v/v). The lipids were visualized by spraying with 2'7'-dichlorflourescein and the spots corresponding to the standards of methyl stearate and methyl oleate were scraped and their radioactivity determined in a scintillation counter. The enzyme activity is expressed as percent of labeled stearate converted in 4 h /10^5 cells.

3. Results

3.1. Cellular fatty acid profiles:

The fatty acid profiles of CHO cells after a 48 h treatment with LA or one of the four CLA isomers is shown in Table 1. There was no detectable CLA in the cells in the absence of the fatty acids (control) or when LA was the fatty acid in the medium. The enrichment of the cell membranes with the CLA isomers was comparable to, or higher than that with LA, indicating that the cells do not discriminate against the conjugated fatty acids in the uptake or incorporation into membranes. However, the incorporation rates of individual CLA isomers, differed from each other significantly. The incorporation of CLA-A (t10c12) was greater than that of LA or other CLAs. The incorporation of the two unnatural isomers, which contained either two *trans* (t9t11) or two *cis* double bonds (c9c11), was lower than that of the natural CLA isomers which contain one *cis* and one *trans* double bond. At least two of the metabolites of CLA, the desaturation product (18:3), and the elongation product (20:2) were detected in most of the samples, indicating the ability of the cellular enzymes to metabolize CLA by pathways similar to that of unconjugated 18:2.

The endogenous fatty acids that decreased in CHO cells in response to t10c12 CLA, c9t11 CLA, and LA were mostly 18:1 (n-9), 18:1 (n-7) and 16:1 (n-7). However, the saturated fatty acids (16:0 and 18:0) also decreased in presence of the double *trans* CLA and double *cis* CLA (although not statistically significant), indicating that the 'unnatural' CLA replaced the saturated fatty acids in addition to the monounsaturated fatty acids. The concentration of 20:4 decreased in all cases, including when the cells were treated with LA, the precursor of 20:4. Similar decrease in 20:4 was also reported for MCF-7 breast cancer

cells treated with LA or CLA [16], and this is possibly due to low activities of Δ^6 and Δ^5 desaturase activities in the cells.

The fatty acid composition of HepG2 cells incubated with LA or CLA isomers for 48 h is shown in **Table 2.** The incorporation rates of all fatty acids were significantly lower than seen in CHO cells, probably reflecting the differences in metabolic activities and membrane turnover between the two cell lines. The relative incorporation rates of the various CLA isomers were similar to that found in CHO cells, with t10c12(CLA-A) being incorporated more efficiently than the other CLA isomers or LA. Although there was a decrease in the concentration of all monounsaturated fatty acids in presence of CLA-A and CLA-C, the decreases were not statistically significant. Furthermore, in contrast to CHO cells, there was no significant decrease in saturated fatty acids even in presence of double *cis* or double *trans* CLA. In fact the concentration of 18:0 increased in presence of CLA-A, relative to control and LA treated cells. It may be pointed out that the HepG2 cells have relatively low 18:0 compared to CHO cells at the baseline. Similar to the CHO cells, the concentration of 20:4 decreased in presence of LA, CLA-A and CLA-C. All CLAs also decreased the concentration of plasmalogens, as reflected by the decrease in dimethyl acetal which is formed from the alkenyl group during methylation step. The dimethyl acetal was not detected in the CHO cells.

3.2. CLA distribution among cellular lipids:

In CHO cells, more than 50% of the total fatty acid incorporated into CHO cells was recovered in PC in the case of all CLA isomers as well as LA (**Fig. 1**). The next major lipid

incorporating CLA was PE, which contained from 18-35% of the total recovery. Less than 20% of the CLA or LA was recovered in the neutral lipids which included free fatty acids, cholesteryl esters, and triacylglycerol. In contrast, in the HepG2 cells, the CLA were incorporated more into neutral lipids than into phospholipids, although only the incorporation of c9t11 was statistically significant from LA because of high standard deviations. Unlike the CLA, the majority of LA was recovered in PE fraction in HepG2. Previous studies in mouse liver [17], rat liver [18], and HepG2 [19] also reported that the majority of CLA was recovered in the neutral lipids, predominantly in tracylglycerol. Thus the incorporation profiles of CLA appear to be tissue-dependent.

3.3. Distribution of CLA between raft and non-raft domains:

The distribution of CLA isomers and LA between the raft and non-raft fractions of CHO cells, isolated by a non-detergent method, is shown in **Fig. 2**. More than 50% of the incorporated fatty acid was present in the raft fraction in all cases. Although there was a slight decrease in the incorporation of double *trans* CLA isomer into the raft fraction, compared to the LA-treated cells, this was not statistically significant. The effect of CLA incorporation on the distribution of cholesterol between raft and non-raft fractions is shown in **Fig. 3**. The only noticeable effect on cholesterol distribution that approached statistical significance occurred in the CLA-A (t10c12) treated cells which showed an increase in the amount of raft cholesterol, compared to the LA-treated cells.

The fatty acid composition of raft and non-raft fractions of CHO cells are shown in **Tables 3 and 4**. In general, the effects of CLA on the fatty acid composition were more

noticeable in the raft fraction than in non-raft fraction. Thus, the raft lipids showed a decrease in the monounsaturated fatty acids after treatment of the cells with t10c12, c9t11, and c9c11 (compared to control), and a decrease in saturated fatty acids after treatment with t9t11 and c9c11 (compared to both control cells and LA-treated cells). In contrast, the decrease in saturated fatty acids in the non-raft lipids was not significant with any of the CLA, and the only significant decrease in 18:1 (n-9) occurred with t10c12. Furthermore, there was a significant decrease in arachidonate in the raft lipids in presence of CLA-A, but no significant changes in this fatty acid in the non-raft lipids after treatment with any of the CLA. These results suggest that the majority of the effects of CLA seen in whole cells are due to the changes in the raft lipid composition.

3.4. Desaturation index:

Several studies have previously shown that CLA inhibit the fatty acid desaturase activities, especially the Δ^9 desaturase, and that the effects are predominantly due to the t10c12 isomer [19-21]. However, some unconjugated fatty acids also inhibit the expression and activities of desaturase [21,22], and therefore the structural requirements for the desaturase inhibition by the CLA isomers need to be investigated. Since we have used four different positional isomers of CLA in addition to LA, it is possible to draw some tentative conclusions on the relationship between the structure of CLA and its effect on desaturase activity based on the changes in the fatty acid composition. We calculated the ratios of 16:1(n-7)/16:0 (desaturation of 16:0), 18:1(n-9)/18:0 (desaturation of 18:0, as well as 18:1(n-7)/16:0 (desaturation of 16:0, followed by elongation) in the CHO cells and in the raft and non-raft fractions of the same cells. The results in **Fig. 4** show that LA itself decreases the

desaturation indices significantly compared to control, as previously reported [23]. CLA-A (t10c12) inhibited the desaturation of both 16:0 and 18:0, but c9t11 significantly inhibited only the desaturation of 16:0. In contrast, neither the double *cis* CLA nor the double *trans* CLA inhibited the desaturation of either 16:0 or 18:0. Compared to LA, t10c12 showed more inhibition of 16:0 desaturation, although not of 18:0 desaturation, whereas all other CLA isomers showed an actual increase in desaturation rather than a decrease. Most of the effects on the desaturation present in the whole cell lipids are present in the raft lipids but the differences in the non-raft lipids were less significant (**Fig. 4**). In HepG2 cells, only the t10c12 isomer showed a significant inhibition of the desaturation index (**Fig. 5**). Although previous studies by Yee et al [24] suggested that the desaturation of 16:0 is not inhibited by t10c12, we found significant inhibition of desaturation of both 16:0 and 18:0. In contrast to the CHO cells, however, the effect of LA on the desaturation index was not significant in HepG2 cells.

3.5. Stearoyl CoA desaturase (SCD) activity:

The SCD activity was assayed in intact CHO cells by incubation of the cells with ^{14}C- labeled stearic acid (18:0) and measuring its conversion to oleic acid (18:1) in a 4 h period. As shown in **Fig. 6**, both t10c12 and c9t11 inhibited the enzyme reaction significantly, compared to the control, with the former being more effective. The double *trans* and the double *cis* isomers showed little inhibitory effect. Although LA decreased the desaturation index in the cells (**Fig 4**), it did not show direct inhibitory effect on the enzyme activity, indicating that it may affect the enzyme levels in the long term treatment, rather than

directly inhibiting the enzyme activity. In addition, the decrease in desaturation index could also be due to the substitution of 18:1 by exogenous 18:2.

3.6. Positional distribution:

The positional distribution of incorporated CLA in the cellular PC and PE was determined, employing snake venom phospholipase A_2 **(Fig. 7)**. Free fatty acids released by this enzyme represent the composition at *sn*-2 position, while the acyl composition of lyso PC or lyso PE represents the fatty acids present at *sn*-1 position. As expected, most of the LA was present in the *sn*-2 position in both PC and PE of both CHO and HepG2 cells. However, the distribution of CLA isomers clearly differed from that of LA, with a significant amount of CLA appearing in the *sn*-1 position (decrease in sn-2) of PC and PE in both the cell types. In the PC of CHO cells, about 60% of the two natural CLA (t10c12 and c9t11) were recovered in *sn*-2 position. Only about 30% of the double *trans* CLA (t9t11) was incorporated into *sn*-2 of both PC and PE, thus behaving more like a saturated fatty acid. The double *cis* CLA (c9c11) on the other hand showed differential distribution between PC and PE. In PC, the majority was present at *sn*-2 position, whereas in PE the majority of it was recovered in *sn*-1 position. In HepG2 cells, the presence of CLA in the *sn*-2 position was even lower than that in CHO cells. Less than 50% of the total of all CLA isomers (except the double *cis* isomer) was recovered in the *sn*-2 position of PC. The double *cis* isomer, however was present to a greater extent in the *sn*-2 of both PC and PE. These results show that the acyltransferases and other enzymes of the cells discriminate between the isomers of CLA. Furthermore the decrease in the saturated fatty acids in presence of double *cis* and double

trans CLA isomers appear to be due to the replacement of saturated fatty acids from the *sn*-1 position of phospholipids, rather than activation of the desaturase activity.

4. **Discussion**

The present studies show that all CLA isomers are incorporated efficiently into cell membranes, and metabolized comparably to the unconjugated LA. They further show that more than 50% of CLA was present in the raft domains of the membranes, in contrast to the omega-3 fatty acids which tend to excluded from the raft domains [25]. It should be noted that the introduction of conjugation in the middle of the acyl chain makes the chain more rigid because of the inflexibility the methylene carbon between the two double bonds. A further difference between CLA and omega 3 fatty acids is the poor affinity of the latter to cholesterol, which is consistent with their exclusion from the raft domains. On the other hand, our previous studies showed the PCs containing CLA at *sn*-2 position readily bind cholesterol, although with differing affinities [26]. Of the two natural CLA, t10c12 CLA binds cholesterol more avidly than c9t11 CLA or LA, when present at the *sn*-2 of PC. The present studies in cultured cells support the in vitro data, since in cells incubated with t10c12 CLA, more cholesterol was found in the raft domains, compared to the cells treated with either LA or c9t11 CLA. In general, the effects of CLA, especially t10c12 CLA, on the raft composition appear to be opposite to those of DHA. It remains to be seen whether the physiological effects of CLA and DHA are related to their effects on raft composition.

One of the known effects of CLA in experimental animals and in cultured cells is the inhibition of the expression, as well as the activity, of Δ^9 desaturase, the enzyme that

converts the saturated fatty acids to monounsaturated fatty acids, and which plays a critical role in lipogenesis and adipogenesis [27]. This enzyme is bound to endoplasmic reticulum (ER) membranes, and has a short half life [28]. In addition to being regulated at the transcriptional level, the enzyme activity is regulated by the rapid turnover of the protein because of its short half life. It has been shown by several studies that t10c12 CLA is the more effective isomer in the inhibition of the desaturase [19,20], although the underlying mechanism for this specificity is not known. In addition to the transcriptional regulation of the enzyme through the SREBP1 (sterol regulatory element binding protein 1)-mediated pathway, a direct inhibition of the enzyme activity by t10c12 CLA has been reported in cell-free system [21]. Based on the present data, the following conclusions can be drawn (at least for the CHO cells) with regard to the structural requirements for the inhibition of SCD activity. 1). Introduction of conjugation alone in LA without a change in the double bond geometry (c9c11 vs LA), appears to negate the inhibitory effect of LA on the desaturation, although it is also possible that the shifting of the double bond from carbon 12 to carbon 11 may also be responsible for the loss of inhibition. 2). Substitution of one of the *cis* double bonds with a *trans* double bond (c9t11 vs c9c11), partially restores the inhibitory effect, but the substitution of both the *cis* double bonds (t9t11 vs c9c11) does not have much effect. 3). The presence of a *cis* double bond at carbon 12 (n-6) may be more important for the inhibition of 18:0 desaturation, while the 16:0 desaturation is affected both by the position and geometry of the double bond. Previous studies by Park et al. [29] also concluded that the presence of *cis* 12 double bond is a key requirement for the direct inhibition of SCD activity. It appears that the presence of both conjugation and a *cis* 12 double bond provide the maximum effect.

Another important finding in the present study is the incorporation of significant amounts of CLA into the *sn*-1 position of PC and PE. It is well known that the *cis* unsaturated fatty acids are located predominantly in the *sn*-2 position of phosphoglycerides whereas the *trans* unsaturated fatty acids tend to be incorporated into the *sn*-1 position [30]. Our results show that in the natural CLA, which contain one *cis* and one *trans* double bond, the effect of the *trans* double bond appears to predominate, because they are incorporated significantly into *sn*-1 position. In addition, the presence of conjugation alone appears to result in the altered positional distribution because c9c11 CLA was incorporated significantly in to *sn*-1 of PE in CHO cells. Again, the rigidity imposed by the conjugation may be responsible for this behavior. The additive effects of *trans* double bond and conjugation are apparent in the predominant occurrence of t9t11 CLA in the *sn*-1 position of both PC and PE. The physiological consequences of the presence of CLA in the *sn*-1 position are not known. In our previous study with synthetic phospholipids, where we used PCs that contained CLA only in the *sn*-2 position and 16:0 at *sn*-1 [26], significant differences in physicochemical properties were found between the various CLA isomers. Thus, the presence of t10c12 at *sn*-2 position of PC resulted in increased binding to cholesterol, and decreased membrane permeability, compared to PC containing c9t11 at the same position. However, the presence of some CLA in the *sn*-1 position may have additional effects, especially depending upon the nature of the fatty acid occupying the *sn*-2 position. Since the *sn*-2 position of such phospholipids will presumably contain an unsaturated fatty acid, the overall fluidity of the membrane may be increased by the presence of CLA at *sn*-1 position. Furthermore, the turnover of fatty acids at *sn*-1 should be slower than that of *sn*-2 fatty acids because of the

predominant occurrence of phospholipases A_2 compared to phospholipases A_1. Therefore, the effects of CLA may last longer than the other dietary polyunsaturated fatty acids such as omega-3 fatty acids which are predominantly in the *sn*-2 position of membrane phospholipids. In conclusion, our studies show that CLA are readily incorporated into membrane lipids, especially the raft lipids, and have unusual positional distribution in phospholipids. Combined with their known inhibitory effect on fatty acid desaturation, this unusual pattern of incorporation could contribute to their biological properties.

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Legends for Figures.

Figure 1: Distribution of CLA isomers in the major lipid fractions of CHO (top) and HepG2 (bottom) cells.

The fatty acid composition of PC, PE, and neutral lipids was analyzed after a 48 h incubation with various CLA isomers or LA, as described in section 2.5. The values shown are mean ± S.D. of 3 separate experiments. * $p<0.05$ compared to LA.

Figure 2: Distribution of CLA isomers between raft and non-raft fractions of CHO cells.

CHO cells were treated with 50 μM CLA or LA for 48 h, and the raft and non-raft fractions were separated by a non-detergent method as described in section 2.3. The total lipid extracts were analyzed for the fatty acid composition after adding 17:0 FFA as internal standard. The values shown are mean ± S.D. of three separate experiments. No statistically significant differences were found between samples.

Figure 3: Effect of CLA on cholesterol distribution in CHO cells.

The raft and non-raft fractions were isolated after 48 h treatment, as described in section 2.3. Cholesterol was determined in the fractions by the Amplex Red method. The values shown are mean ± S.D. of three experiments. Only the difference between t10c12 CLA and LA approached statistical significance by Student's t test.

Figure 4: Effect of CLA isomers on the desaturation indices in CHO cells.

The ratios of 16:0/16:1, 18:1(n-9)/18:0, and 16:1 + 18:1 (n-7)/ 16:0 were calculated in the lipid extracts of whole cells, and raft and non-raft fractions. The results shown are mean ± S.D. of three separate experiments.

Figure 5: Desaturation indices in HepG2 cells.

The ratios were calculated from the fatty acid composition of the total cell extracts, as described in section 3.4. The values shown are mean ± S.D. of four experiments.

Figure 6: Stearoyl CoA desaturase activity in CHO cells:

Stearoyl CoA desaturase (Δ^9 desaturase) activity was determined from the conversion of ^{14}C labeled stearate (18:0) to oleate (18:1, n-9) by the intact cells (see section 2.7). The cells were first incubated with the various CLA isomers or LA for 44 h, and then incubated for further 4h in presence of ^{14}C stearate. The total lipid extract was methylated and the methyl esters separated on silver-nitrate impregnated TLC plates as described in the text. The values shown are mean ± S.D. of three separate experiments, each performed in triplicate.

Figure 7: Positional distribution of CLA in PC and PE

Following a 48 h incorporation of CLA or LA, the total lipids were extracted after adding di17:0 PC and di17:0 PE as internal standards. The PC and PE were isolated by silica gel TLC, and treated with snake venom phospholipase A_2 (see section 2.6). The liberated free fatty acids (*sn*-2) and lysophospholipids (*sn*-1) were analyzed for the fatty acid composition by GC. The amount of CLA in each position was calculated

from the values of 17:0 internal standard. Only the percentage in *sn*-2 position is shown, the rest being present in *sn*-1 position. The values shown are mean ± S.D. of three experiments for HepG2, and four experiments for CHO cells. * $p<0.05$ compared to LA control.

Table -1. Whole cell lipids in CHO (% of total)

	Control	LA	CLA-A	CLA-B	CLA-C	CLA-D
14:0	0.86 ± 0.74	1.02 ± 0.86	1.90 ± 1.55	1.46 ± 0.98	1.25 ± 0.89	1.19 ± 0.81
16:0	19.71 ± 3.05	22.42 ± 7.29	18.02 ± 1.63	16.52 ± 4.32	18.87 ± 1.75	16.79 ± 4.14
16:1	3.74 ± 1.65	1.31 ± 0.17†	0.91 ± 0.10*†	5.31 ± 1.72*	1.41 ± 0.35†	3.40 ± 1.24
18:0	17.45 ± 3.63	15.45 ± 4.30	17.00 ± 3.86	11.36 ± 4.50†	14.70 ± 2.06	11.43 ± 3.21†
18:1 (n-9)	32.61 ± 8.89	15.59 ± 5.05†	11.28 ± 3.81†	20.34 ± 4.75†	15.98 ± 6.26†	20.25 ± 5.17†
18:1 (n-7)	9.03 ± 3.95	6.76 ± 0.90	4.59 ± 1.20*†	6.84 ± 1.92	6.86 ± 1.98	7.84 ± 1.99
18:2	3.53 ± 0.94	19.75 ± 6.39†	2.43 ± 0.86*	3.69 ± 0.63*	2.74 ± 0.53*	3.26 ± 0.79*
20:1	1.47 ± 0.65	0.66 ± 0.74	0.85 ± 0.95	1.16 ± 1.76	1.54 ± 1.67	0.95 ± 1.09
20:2	0.08 ± 0.17	4.82 ± 2.32†	0.00 ± 0.00*	0.00 ± 0.00*	0.05 ± 0.12*	0.00 ± 0.00*
20:3	0.11 ± 0.25	0.41 ± 0.75	0.07 ± 0.16	0.10 ± 0.23	0.06 ± 0.14	0.00 ± 0.00
20:4	5.37 ± 0.76	3.56 ± 1.37†	4.17 ± 0.68†	5.39 ± 0.27*	4.03 ± 0.64†	4.43 ± 0.56
20:5	0.03 ± 0.07	0.00 ± 0.00	0.07 ± 0.16	0.43 ± 0.97	0.03 ± 0.07	1.35 ± 1.59
22:4	2.59 ± 1.33	2.30 ± 0.90	1.72 ± 0.56	1.97 ± 0.76	2.13 ± 0.73	2.03 ± 0.69
22:5	1.85 ± 0.55	1.52 ± 0.45	1.39 ± 0.56	1.65 ± 0.70	1.51 ± 0.35	1.61 ± 0.49
22:6	1.87 ± 0.45	1.54 ± 0.40	1.72 ± 0.26	1.95 ± 0.29	1.59 ± 0.27	1.73 ± 0.31
CLA			29.31 ± 6.74*	19.38 ± 3.68	24.11 ± 5.93	21.66 ± 3.69
CLA 18:3			1.82 ± 1.50	1.92 ± 2.06	0.71 ± 0.75	0.40 ± 0.31
CLA 20:2			2.75 ± 1.11	0.51 ± 0.56	2.32 ± 0.63	1.69 ± 1.42

* $p<0.05$ vs LA; † $p<0.05$ vs Control

Table-2. Whole cell lipids in HepG2 (% of total)

	Control	LA	CLA-A	CLA-B	CLA-C	CLA-D
14:0	3.47 ± 2.12	3.32 ± 2.40	2.49 ± 1.17	2.61 ± 2.56	2.74 ± 2.98	1.79 ± 0.96
16:0	22.86 ± 1.14	21.81 ± 2.34	25.71 ± 3.36	23.23 ± 4.79	21.08 ± 10.79	20.13 ± 1.75
16:0 DMA	1.93 ± 0.12	1.61 ± 0.87	1.23 ± $0.45^{\dagger}$	1.08 ± $0.38^{\dagger}$	0.80 ± $0.25^{\dagger}$	1.50 ± $0.28^{\dagger}$
16:1	7.16 ± 1.61	6.07 ± 2.16	5.59 ± 1.53	7.92 ± 2.65	5.94 ± 2.21	6.08 ± 1.48
18:0	4.05 ± 1.91	5.72 ± 0.82	7.77 ± $1.98^{\dagger}$	4.16 ± 0.62*	5.46 ± 1.53	4.97 ± 0.99
18:1 (n-9)	25.14 ± 3.74	22.74 ± 2.04	19.26 ± 3.07	25.49 ± 3.07	19.13 ± 4.52	22.25 ± 1.18
18:1 (n-7)	17.37 ± 3.78	13.35 ± 2.87	11.40 ± 3.86	13.17 ± 4.78	11.98 ± 3.32	14.41 ± 1.10
18:2	1.18 ± 0.34	6.13 ± $1.17^{\dagger}$	1.08 ± 0.18*	0.92 ± 0.18*	1.04 ± 0.26*	0.84 ± 0.51*
20:1	0.65 ± 0.60	0.47 ± 0.35	0.31 ± 0.29	1.34 ± 0.89	1.60 ± 0.66*	0.72 ± 0.54
20:2	2.40 ± 0.61	1.74 ± 0.85	1.25 ± $0.57^{\dagger}$	1.12 ± 1.12	1.56 ± $0.44^{\dagger}$	2.29 ± 0.55
20:3	0.55 ± 0.08	0.74 ± 0.27	0.82 ± 1.04	0.63 ± 0.87	0.50 ± 0.14	0.67 ± 0.12
20:4	4.48 ± 0.63	3.95 ± 1.07	3.48 ± 0.80	2.72 ± $1.24^{\dagger}$	3.54 ± 1.37	3.86 ± 1.17
20:5	2.24 ± 2.09	3.44 ± 3.73	0.44 ± 0.27	0.55 ± 0.60	0.98 ± 0.89	1.05 ± 0.41
22:4	0.06 ± 0.08	0.33 ± 0.46	0.11 ± 0.16	0.29 ± 0.22	0.47 ± $0.05^{\dagger}$	0.10 ± 0.14
22:5	0.10 ± 0.14	0.16 ± 0.23	0.20 ± 0.17	0.33 ± 0.33	0.35 ± 0.12	0.14 ± 0.20
22:6	1.92 ± 0.99	1.71 ± 0.79	2.00 ± 0.97	1.52 ± 0.86	1.56 ± 0.94	1.55 ± 1.03
CLA			11.34 ± 4.31*	7.60 ± 3.42	8.57 ± 4.45	8.58 ± 2.76
CLA 18:3			0.53 ± 0.50	1.06 ± 0.97	0.25 ± 0.21	0.34 ± 0.33
CLA 20:2			0.23 ± 0.33	0.22 ± 0.34	1.32 ± 0.41	0.00 ± 0.00

DMA: dimethyl acetal (from plasmalogens) * $p<0.05$ vs LA; † $p<0.05$ vs Control

Table -3 : Fatty acid composition in raft lipids of CHO cells. (% of total)

	Control	LA	CLA-A	CLA-B	CLA-C	CLA-D
14:0	1.32 ± 0.33	1.53 ± 0.33	1.88 ± 0.51	1.85 ± 0.52	1.32 ± 0.48	1.37 ± 0.53
16:0	19.08 ± 2.29	20.59 ± 2.29	17.85 ± 2.58	16.39 ± 2.27	17.65 ± 2.42	17.72 ± 3.99
16:1	2.44 ± 1.51	1.13 ± 1.51	0.93 ± 0.23	3.57 ± 1.84	1.01 ± 0.23	2.32 ± 0.78
18:0	17.42 ± 3.04	15.16 ± 3.04	17.23 ± 1.99	8.66 ± 0.92*†	14.54 ± 0.89	10.64 ± 0.49*†
18:1 (n-9)	29.82 ± 3.84	15.64 ± 3.84†	13.49 ± 4.90†	21.69 ± 5.92	20.00 ± 4.01†	19.82 ± 5.21†
18:1 (n-7)	10.09 ± 1.56	7.02 ± 1.56	4.67 ± 1.25†	6.76 ± 2.03	6.94 ± 1.98	6.77 ± 1.90
18:2	2.55 ± 0.67	20.31 ± 0.67†	2.11 ± 0.20*	2.99 ± 0.46*	2.04 ± 0.30*	2.71 ± 0.78*
20:1	1.97 ± 1.07	1.29 ± 1.07	1.21 ± 0.50	3.41 ± 1.07*	1.77 ± 0.68	2.26 ± 1.79
20:2	0.77 ± 0.40	5.57 ± 0.40†	1.39 ± 0.53*	1.03 ± 0.39*	0.55 ± 0.18*	0.86 ± 0.14*
20:3	0.68 ± 0.24	0.88 ± 0.24	1.01 ± 0.74	1.38 ± 0.85	0.76 ± 0.50	1.57 ± 1.17
20:4	5.69 ± 0.42	3.85 ± 1.34	4.36 ± 0.49†	5.29 ± 0.18	4.39 ± 0.91	3.54 ± 1.61
20:5	1.19 ± 0.91	1.27 ± 0.91	1.90 ± 1.00	1.51 ± 0.50	1.24 ± 0.69	1.85 ± 0.71
22:4	3.11 ± 1.13	2.52 ± 1.13	1.65 ± 0.37	1.57 ± 0.59	2.14 ± 0.53	2.02 ± 0.77
22:5	1.85 ± 0.23	1.58 ± 0.23	1.68 ± 0.67	1.58 ± 0.39	1.75 ± 0.49	1.62 ± 0.68
22:6	2.02 ± 0.45	1.65 ± 0.45	1.83 ± 0.75	1.87 ± 0.45	1.93 ± 0.63	1.85 ± 0.75
CLA			24.49 ± 7.61	16.91 ± 1.06	21.85 ± 7.20	18.71 ± 3.66
CLA 18:3			0.73 ± 0.16	0.00 ± 0.00	0.92 ± 0.54	0.90 ± 0.10
CLA) 20:2			1.58 ± 0.94	1.31 ± 0.36	2.28 ± 1.48	2.02 ± 0.90

* $p < 0.05$ vs LA; † $p < 0.05$ vs Control

Table -4 : Fatty acid composition of non-raft lipids (CHO cells), % of total

	Control	LA	CLA-A	CLA-B	CLA-C	CLA-D
14:0	1.83 ± 0.70	1.42 ± 0.99	2.31 ± 1.44	1.72 ± 0.81	1.78 ± 0.64	1.26 ± 0.39
16:0	19.34 ± 4.60	17.54 ± 5.88	17.88 ± 4.72	16.48 ± 4.64	18.33 ± 4.10	22.35 ± 14.62
16:1	3.03 ± 1.29	1.78 ± 1.33	0.99 ± 0.10†	4.94 ± 1.65	1.58 ± 0.53	2.30 ± 0.76
18:0	16.60 ± 2.84	14.93 ± 5.57	18.72 ± 3.49	12.53 ± 3.77	14.31 ± 2.21	13.76 ± 3.64
18:1 (n-9)	28.32 ± 6.71	17.96 ± 8.87	14.64 ± 5.73†	21.97 ± 7.42	17.87 ± 7.99	19.44 ± 7.13
18:1 (n-7)	10.64 ± 2.73	7.47 ± 2.90	5.86 ± 1.95†	7.44 ± 2.70	7.47 ± 2.79	7.22 ± 2.63
18:2	4.33 ± 0.97	16.93 ± 9.00†	3.81 ± 0.82*	4.19 ± 0.64*	3.69 ± 0.71*	4.81 ± 1.65*
20:1	3.12 ± 2.08	2.15 ± 1.62	2.54 ± 1.36	3.49 ± 0.54	3.32 ± 3.03	2.15 ± 1.97
20:2	0.76 ± 0.37	2.62 ± 1.55	0.75 ± 0.42	0.54 ± 0.34*	1.48 ± 0.92	1.26 ± 0.67
20:3	0.83 ± 0.30	1.00 ± 0.77	0.85 ± 0.57	0.64 ± 0.32	1.18 ± 0.62	0.65 ± 0.28
20:4	4.82 ± 1.13	3.92 ± 2.55	4.82 ± 0.82	5.45 ± 0.44	4.46 ± 0.70	4.27 ± 1.28
20:5	1.81 ± 0.77	1.25 ± 0.68	1.79 ± 0.72	1.97 ± 0.54	1.59 ± 0.33	1.77 ± 0.40
22:4	1.69 ± 0.66	1.73 ± 0.94	1.49 ± 0.74	1.46 ± 0.59	1.53 ± 0.58†	1.38 ± 0.56
22:5	1.58 ± 0.24	1.83 ± 1.65	1.85 ± 0.11	1.57 ± 0.30	1.54 ± 0.36	1.38 ± 0.59
22:6	1.85 ± 0.66	1.76 ± 1.23	2.29 ± 0.52	2.01 ± 0.40	1.80 ± 0.50	1.71 ± 0.74
CLA			24.35 ± 1.42	17.99 ± 3.28	20.56 ± 1.44	13.67 ± 7.00
CLA 18:3			1.45 ± 1.14	0.00 ± 0.00	1.27 ± 0.78	0.81 ± 0.07
CLA) 20:2			2.05 ± 0.51	1.29 ± 0.24	2.26 ± 1.17	1.24 ± 0.41

* p< 0.05 vs LA; † p<0.05 vs Control

Figure 1

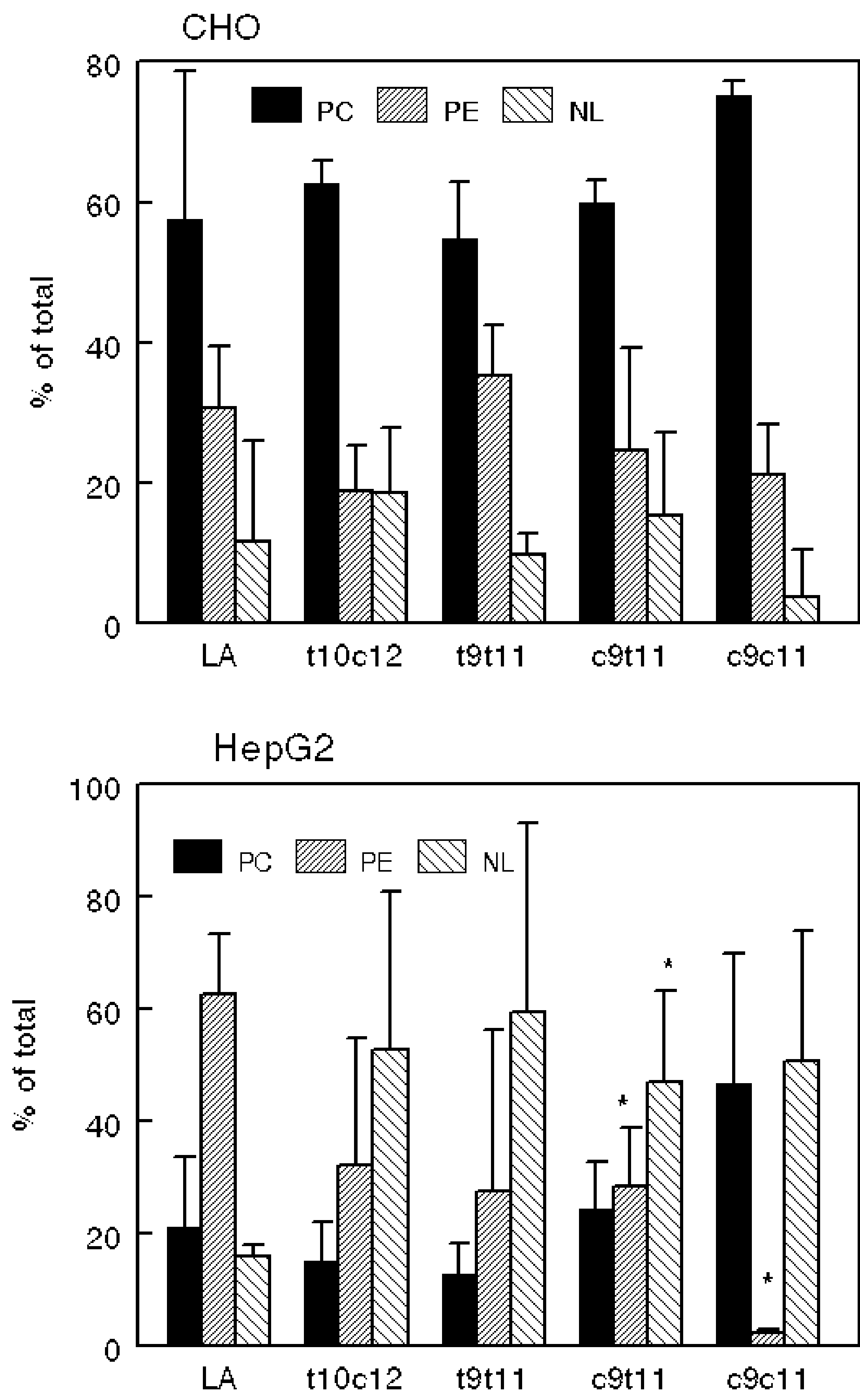

Figure 2

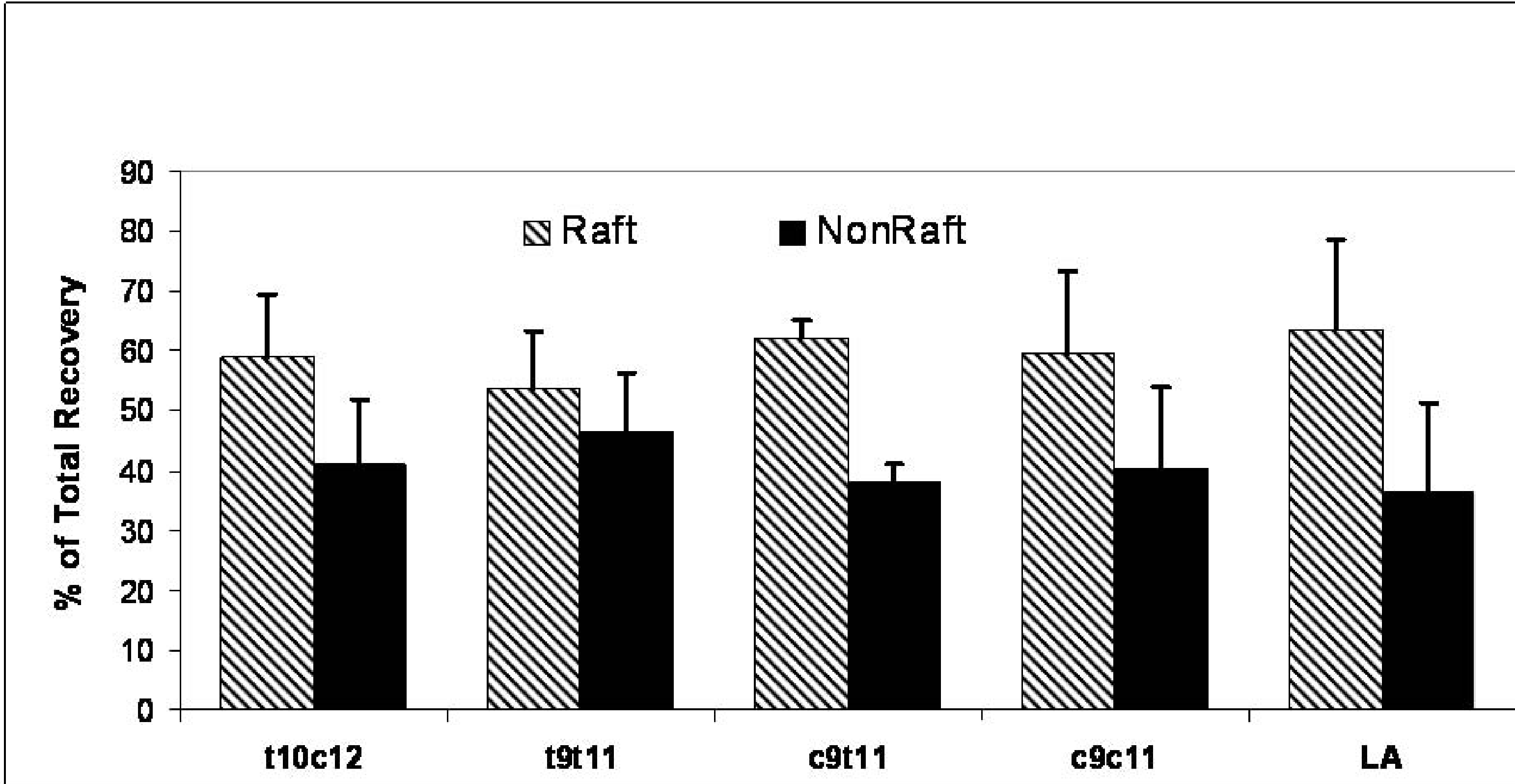

Figure 3

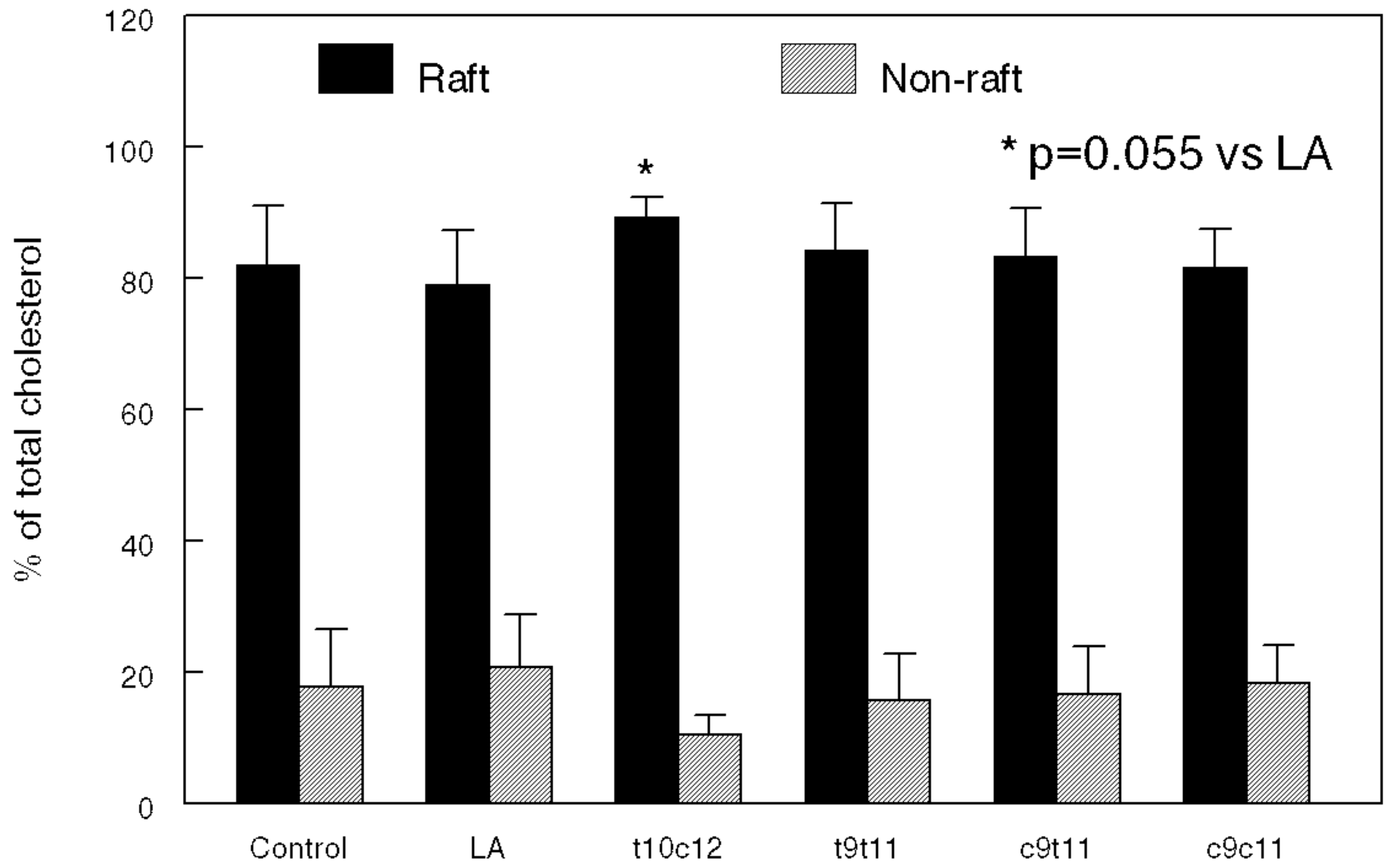

Figure 4

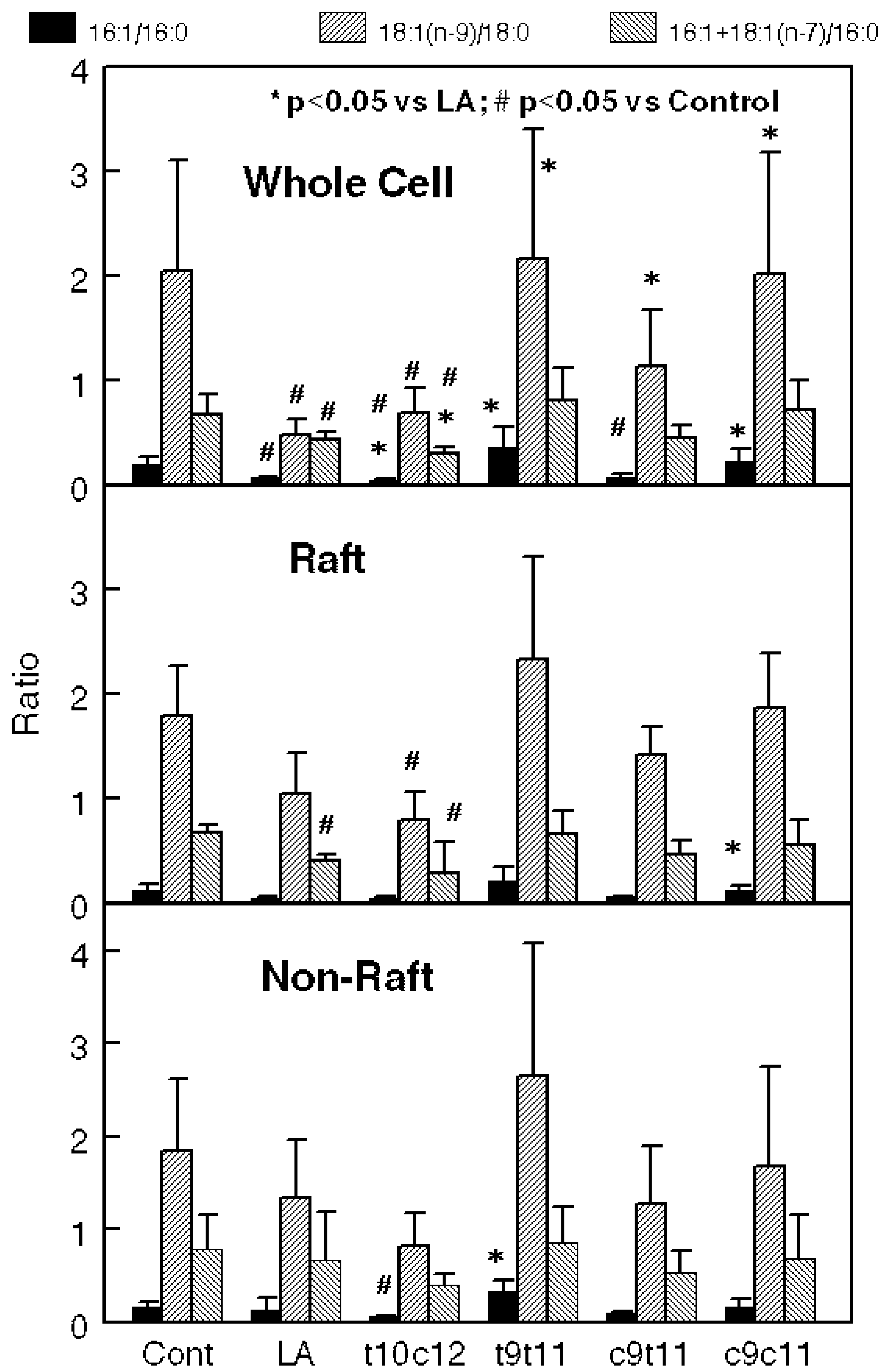

Figure 5

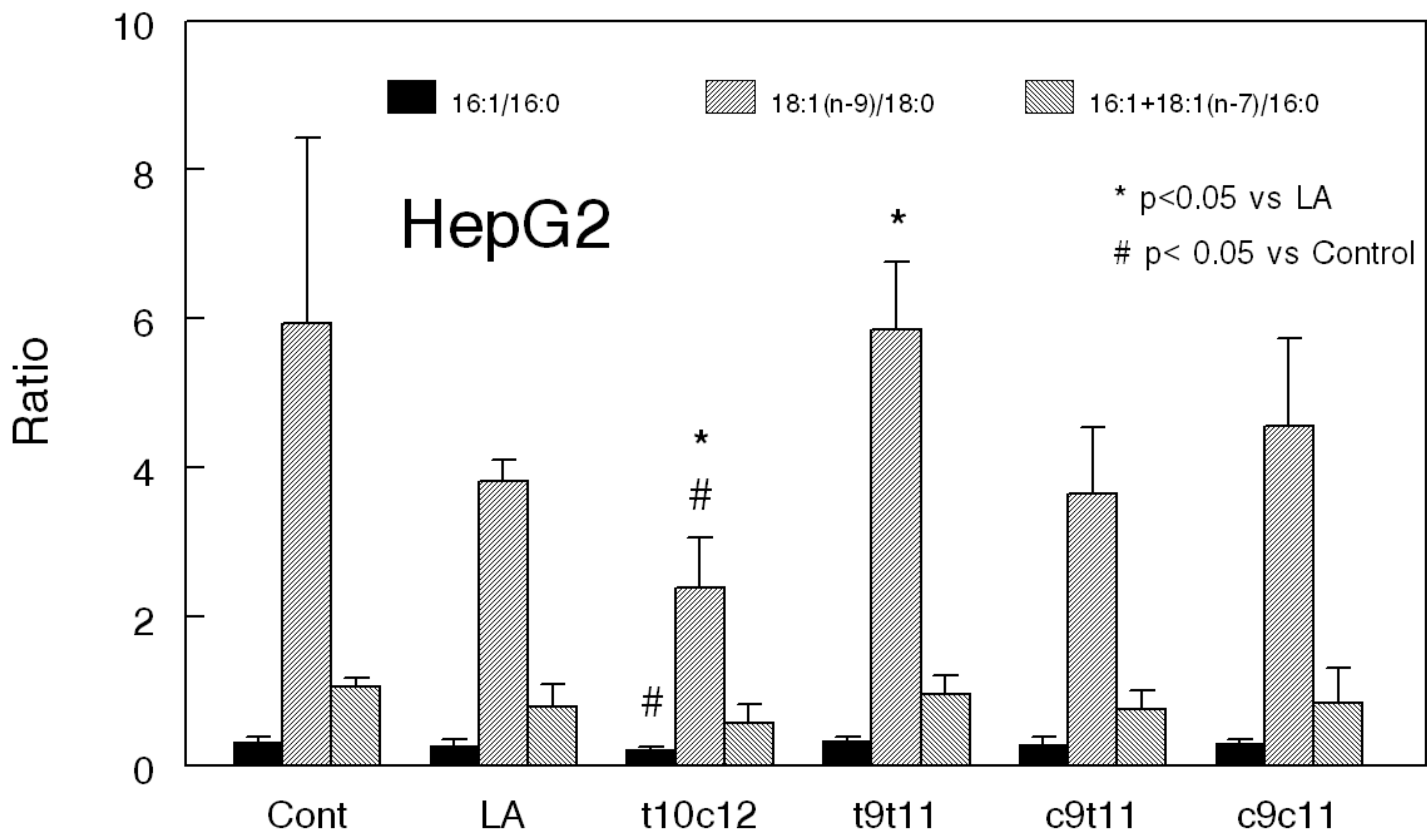

Figure 6

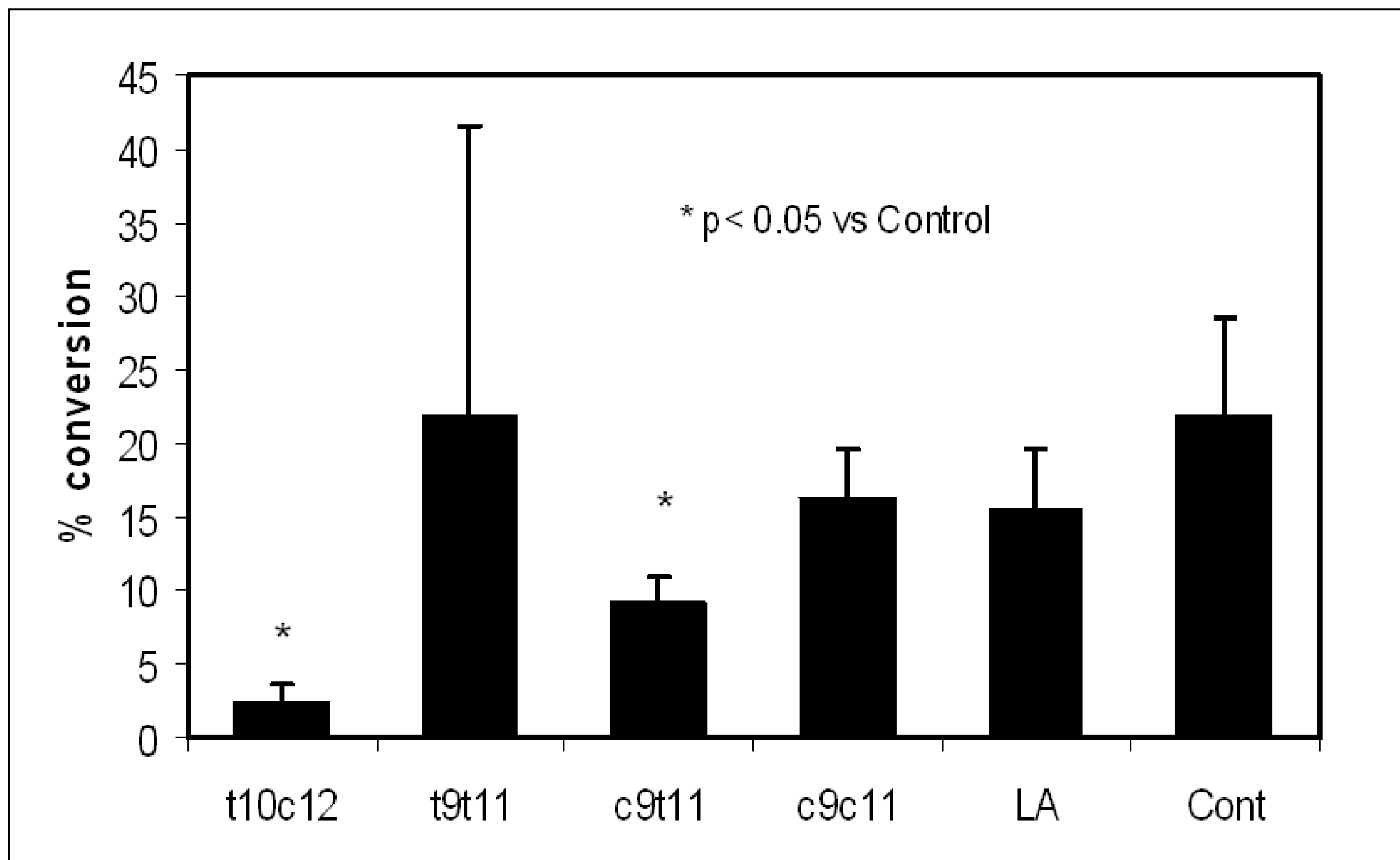

Figure 7