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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.

1. Introduction

Conjugated linoleic acids (CLA), the naturally occurring isomers of linoleic acid (LA), 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. Although there are several possible isomers of CLA, the *cis*-9 *trans*-11, and *trans*-10 *cis*-12 isomers are predominant in the dairy products and meats, 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 [2–4]. CLA have been reported to act as ligands for the transcription factors PPARα and PPARγ [2], and to modulate the transcription of selected genes in an isomer-specific manner [1,5–7]. However the pleiotropic effects of CLA cannot be explained simply on the basis of their known gene transcription effects. Furthermore, the PPAR affinities of CLA isomers do not differ from each other [8] 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 [9–11], 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 [10,12–15]. 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, or CLA-A, and *cis*-9 *trans*-11, or 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, we have included in the study two unnatural CLA isomers, namely a double *trans* isomer (*trans*-9 *trans*-11, or CLA-B) and a double *cis* isomer (*cis*-9 *cis*-11, or CLA-D). The results show that each CLA isomer is incorporated into both raft and non-raft lipids, with the majority in the raft lipids. The fatty acid composition of

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

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the membranes is altered differentially by the various isomers. The CLAs replaced 18:1 in most cases, inhibited the Δ^9 desaturase activity in an isomer-specific manner, and unlike the unconjugated 18:2 (LA), were incorporated significantly into the *sn*-1 position of phospholipids. 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). Radiolabeled 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 silica 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 U/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, 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 [16]. 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 14×89 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 [17], 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 by gas chromatography in a Shimadzu GC-17A instrument, equipped with a flame ionization detector, employing an Omegawax 250 column (30 m×0.25 mm×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 quantitated 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 [17] 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 [18], 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 the Bligh and Dyer procedure [17], 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 [18]. 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 [17], 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 [18], 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 or LA (50 μM) for 44 h in 2% LPDS as described above, and further incubated with 2 μM 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