Inhibitory Effect of Conjugated Linoleic Acid on Linoleic Acid Elongation in Transformed Yeast with Human Elongase

Lu-Te Chuang[a,b], Amanda E. Leonard[b], Jim-Wen Liu[b], Pradip Mukerji[b], Tammy M. Bray[a], and Yung-Sheng Huang[a,b,*]

[a]The Ohio State University, Department of Human Nutrition and Food Management, Columbus, Ohio 43210, and [b]Strategic-Discovery R&D, Ross Products Division, Abbott Laboratories, Columbus, Ohio 43219

ABSTRACT: Conjugated linoleic acid (CLA; 18:2) refers to a group of positional and geometric isomers derived from linoleic acid (LA; Δ9,12-18:2). Using a growing baker's yeast (*Saccharomyces cerevisiae*) transformed with human elongase gene, we examined the inhibitory effect of CLA at various concentrations (10, 25, 50, and 100 μM) on elongation of LA (25 μM) to eicosadienoic acid (EDA; Δ11,14-20:2). Among four available individual CLA isomers, only *c*9,*t*11- and *t*10,*c*12-isomers inhibited elongation of LA to EDA. The extent of inhibition (ranging from 20 to 60%) was related to the concentration of CLA added to the medium. In the meantime, only these two isomers, when added at 50 μM to the media, were elongated to conjugated EDA (*c*11,*t*13- and *t*12,*c*14-20:2) by the same recombinant elongase at the rate of 28 and 24%, respectively. The inhibitory effect of CLA on LA elongation is possibly due to competition between CLA isomers and LA for the recombinant elongase. Thus, results from this study and a previous study suggest that the biological effect of CLA is exerted through its inhibitory effect on Δ6-desaturation as well as elongation of LA which results in a decrease in long-chain n-6 fatty acids and consequently the eicosanoid synthesis.

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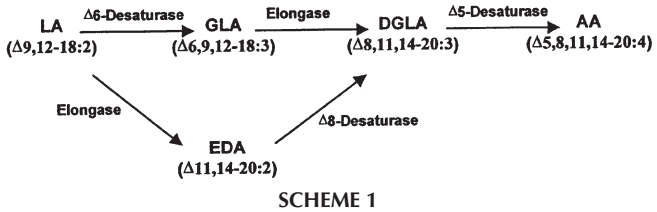
Conjugated linoleic acid (CLA), a mixture of positional and geometric dienoic isomers derived from linoleic acid (LA, Δ9,12-18:2), has many beneficial effects in animals. It can decrease the development of atherosclerosis in rabbits and hamsters (1,2) and modulate the immune function in rats (3). CLA can also decrease breast cancer cell proliferation and inhibit mammary gland, skin, and stomach tumorigenesis in experimental animals (4–9).

The mechanism by which CLA exerts its biological function is still not fully understood. CLA may modulate the immune function through a modification of eicosanoid synthesis (10–12). Results from our previous study demonstrated that CLA significantly inhibited Δ6-desaturation (13), the rate-limiting step for the production of polyunsaturated fatty acid (PUFA) and eicosanoids in mammalian cells. In these cells, when Δ6 desaturation is suppressed, an alternate metabolic pathway for LA to form PUFA could also take place (see Scheme 1). Some LA could be elongated to form eicosadienoic acid (EDA; Δ11,14-20:2) (14), followed by Δ8-desaturation to form dihomo-γ-linolenic acid (DGLA; Δ8,11,14-20:3) and subsequently to arachidonic acid (AA, Δ5,8,11,14-20:4) by the action of Δ5-desaturase (15,16). Since there was a significant decrease of AA in the CLA-fed animals (11,17), it is reasonable to postulate that CLA can inhibit not only the Δ6-desaturation but also the elongation of LA.

The objective of this study was to examine whether CLA could inhibit the elongation of LA by directly competing with the enzyme elongase, the first step of the alternate pathway. This study was performed in a simple system of an established transformed yeast containing the human PUFA-specific elongase gene without the presence of other enzymes in the metabolic pathway of PUFA synthesis.

MATERIALS AND METHODS

Chemicals. Triheptadecanoin (a synthetic triacylglycerol containing three molecules of heptadecanoic acid, 17:0) LA, EDA, and a mixture of CLA isomers (free fatty acid form, containing 41% of *c*9,*t*11-isomer, 44% of *t*10,*c*12-isomer, and others) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Four individual isomers of CLA (*c*9,*t*11; *t*9,*t*11; *c*9,*c*11; and *t*10,*c*12) and a mixture of conjugated eicosadienoic acid (CEDA) isomers (free fatty acid form, containing 53% of *c*11,*t*13 isomer, 29% of *c*11,*c*13 isomer, and others) were obtained from Matreya Inc. (Pleasant Gap, PA). Yeast minimal medium (YMM) was prepared by mixing 26.7 g Dropout base (DOB) medium and 0.69 g complete supplement mixture



SCHEME 1

*To whom correspondence should be addressed at Strategic-Discovery R&D, Ross Products Division, Abbott Laboratories, 625 Cleveland Ave., Columbus, OH 43215. E-mail: vic.huang@rossnutrition.com

Abbreviations: AA, arachidonic acid (Δ5,8,11,14-20:4); CEDA, conjugated eicosadienoic acid; CLA, conjugated linoleic acid; CSM-LEU, complete supplement mixture minus leucine; DGLA, dihomo-γ-linolenic acid (Δ8,11,14-20:3); DOB, Dropout base; EDA. eicosadienoic acid (Δ11,14-20:2); GC, gas chromatography; GLA, γ-linolenic acid (Δ6,9,12-18:3); LA, linoleic acid (Δ9,12-18:2); MS, mass spectrometry; PUFA, polyunsaturated fatty acid; YMM, yeast minimal medium.

minus leucine (CSM-LEU). Both DOB medium and CSM-LEU were from Bio 101, Inc. (Vista, CA). YPD medium containing yeast extract, peptone, and dextrose was from Difco Laboratories (Detroit, MI). Hexane was ultraviolet grade and other solvents were distilled-in-glass quality.

Plasmids and yeast strain. The pYX242 expression vector was chosen for the construction of the clone pRAE-58. This expression vector has a strong, constitutive triose phosphate isomerase promoter, leucine selection marker, and ampicillin resistance marker. The pYX242 is a 2μ plasmid, which allows it to replicate autonomously in yeast and be stably maintained at 25–100 copies per cell. In this study, two plasmids, pYX242 (vector only) and pRAE-58 (with human elongase cDNA), were constructed and transformed into a host strain of *Saccharomyces cerevisiae*, SC334 (18). The transformation protocol and growth conditions followed the procedures described previously (18).

Incubation conditions and experimental design. Colonies of the transformed yeasts were grown overnight in YPD medium at 30°C. Cultures (1×10^8 cells) were then inoculated into 50 mL YMM. Cell numbers were maintained at the same level in all studies. The cultures were grown at 30°C for 48 h. The culture temperature (30°C) has previously been shown to be optimal for expression of the elongase activity (18). Cells were harvested by centrifugation, and cell pellets were washed once with sterile distilled-deionized H_2O. The yeast transformed with only vector (pYX242) was used as the negative control.

To confirm whether the activity of elongase (conversion of LA to EDA) was expressed in the transformed yeast, LA (100 μM) was provided as the exogenous substrate in the YMM. To study if CLA could affect the conversion rate of LA to EDA, CLA (as a mixture of four isomers or individual isomers) was supplemented to the medium at different levels (10, 25, 50, and 100 μM) while LA was maintained at 25 μM. To examine whether CLA could be elongated to CEDA, 100 μM of four individual isomers were added to the medium separately. To determine the uptake of substrates (i.e., LA and CLA isomers) and conversion rates of LA to EDA and CLA to CEDA by elongase, LA and two CLA isomers (*c*9,*t*11 and *t*10,*c*12) were supplemented at 50 μM.

Lipid extraction and fatty acid analysis. The extraction of yeast lipids was performed according to the procedure described previously (13). Briefly, the rinsed cell pellet was extracted with 20 mL of chloroform/methanol (2:1, vol/vol) containing 16 μg triheptadecanoin (used as the internal standard). After extraction, the yeast lipids were saponified and methylated as described by Yamasaki *et al.* (19). Fatty acid methyl esters were then analyzed by gas chromatography (GC) using a flame-ionization detector and a fused-silica capillary column (Omegawax; 30 m × 0.32 mm, i.d., Supelco, Bellefonte, PA). The identity of CEDA was confirmed by GC–mass spectrometry (MS), using a Hewlett-Packard mass selective detector (model 5972) operating at an ionization voltage of 70 eV with a scan range of 20–500 Da. The mass spectrum of any new peak obtained was compared with that of standard in the database NBS75K.L (National Bureau of Standards). In this study, the conversion of substrates to products was determined based on the ratio of [product]/[product + substrate] × 100%. The amount of LA and CLA isomers taken up by the yeast was calculated from the percentage of LA or CLA isomers in total yeast lipids.

Statistical analyses. Data were analyzed by analysis of variance and Fisher's protected least significant difference to determine differences between means of the uptake rates and between means of the conversion rates. Means differences were considered significant at the $P \leq 0.05$ level.

RESULTS

When the recombinant human elongase, expressed in yeast strain 334(pRAE-58), was incubated with 100 μM linoleic acid (LA) for 48 h, a substantial portion (13%) of LA was elongated to form EDA (Fig. 1B). There were also increases in the levels (relative concentration as well as absolute amount) of Δ11-18:1, the elongation product of palmitoleic acid (Δ9-16:1), and Δ13-20:1, the elongation product of Δ11-18:1. No increases in these elongation products (Δ11-18:1, Δ13-20:1, and EDA) were observed in the control 334(pYX242) yeast (Fig. 1A).

To examine the effect of CLA on elongation of LA to EDA in the transformed yeast, various concentrations of CLA mixture (10, 25, 50, or 100 μM) were added to the growth medium containing 25 μM of LA. Results in Figure 2A show that addition of the CLA mixture inhibited the conversion of LA to EDA in the transformed yeast. Elongation of LA to EDA was inhibited by 50% when equal concentrations of

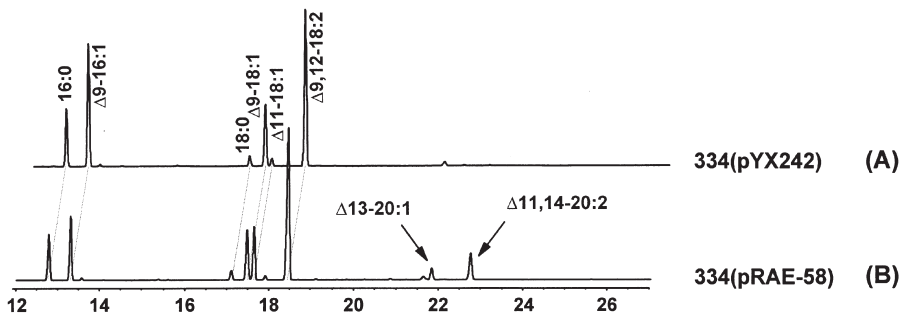


FIG. 1. Gas chromatographic analysis of fatty acid methyl esters (FAME) from the total lipids of the transformed yeast with (A) only the vector [334(pYX242)] or (B) the elongase gene [334(pRAE-58)]. All yeast cells were incubated in the medium containing 100 μM linoleic acid (LA). Arrows indicate the appearance of Δ13-20:1, the elongation product of Δ11-18:1, and eicosadienoic acid (Δ11,14-20:2, EDA), the elongation product of LA.

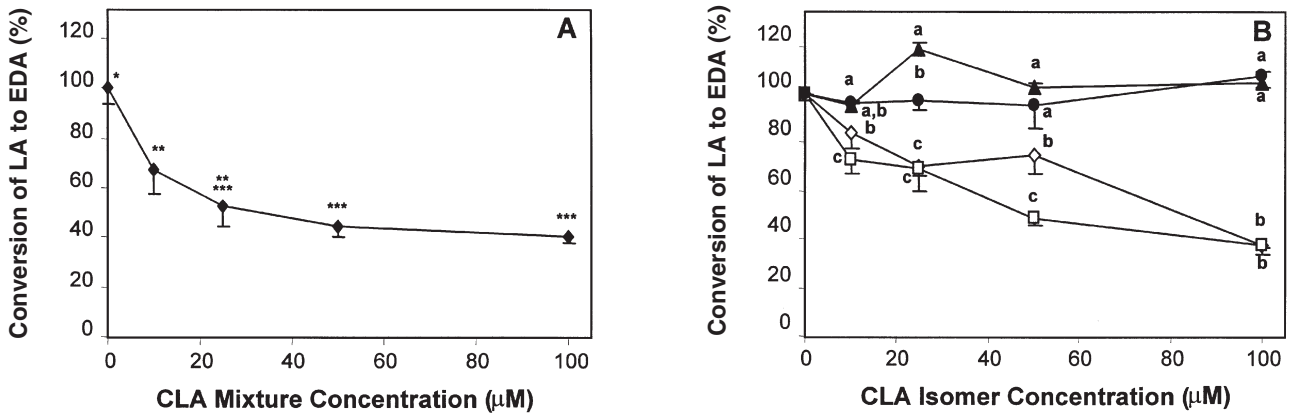


FIG. 2. Effect of different concentrations of conjugated linoleic acid (CLA) isomers in mixture (A) or individually (B) on elongation of LA to EDA in yeast transformed with human elongase gene. LA was maintained at 25 μM in the medium. Conversion of LA to EDA was calculated as [product/(product + substrate)] × 100%. Yeasts incubated with medium containing only 25 μM LA were designed as the control (100%). Each value point represents the mean of three incubations. At same substrate concentration, values with different letters (a,b,c) or superscripts (*,**,***) indicate a significant difference ($P < 0.05$) in the decrease in elongation of LA to EDA. *c*9,*t*11-CLA isomer (◇); *t*10,*c*12-CLA isomer (□); *c*9,*c*11-CLA isomer (▲); and *t*9,*t*11-CLA isomer (●).

CLA (25 μM) and LA (25 μM) were added to the medium. No additional inhibitory effect was observed when the concentration of CLA was greater than 25 μM.

The effect of individual CLA isomer on the elongation of LA to EDA was also examined. Figure 2B depicts that only *c*9,*t*11- and *t*10,*c*12-CLA isomers inhibited the conversion of LA to EDA, whereas the other two isomers (*c*9,*c*11- and *t*9,*t*11-CLA) exerted no such effect.

To examine whether CLA itself could be elongated by the same recombinant elongase, the transformed 334(pRAE-58) yeast were incubated with four individual CLA isomers separately for 48 h. Results in Figure 3 show that only two isomers, *c*9,*t*11- and *t*10,*c*12-CLA, could be elongated to form *c*11,*t*13-20:2 and *t*12,*c*14-20:2, respectively (Figs. 3A, 3B). No detectable elongation metabolites were observed in the 334(pRAE-58) yeasts incubated with either *c*9,*c*11- or *t*9,*t*11-CLA isomers (Figs. 3C, 3D).

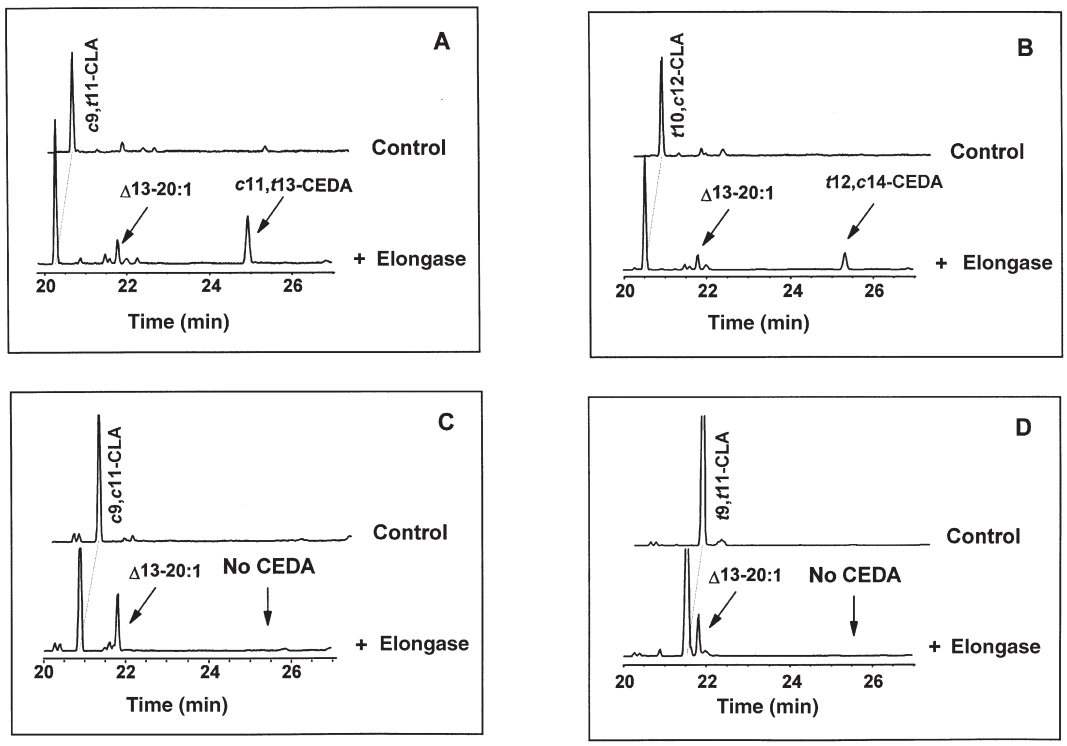


FIG. 3. Gas chromatographic analysis of FAME from the total lipids of yeasts transformed with only the vector (Control) or the elongase gene. Yeast cells were incubated in their respective meda containing *c*9,*t*11-CLA (A); *t*10,*c*12-CLA (B); *c*9,*c*11-CLA (C); and *t*9,*t*11-CLA (D). Arrows indicate the appearance Δ13-20:1 and conjugated eicosadienoic acid (CEDA) isomer. For other abbreviations see Figures 1 and 2.

The identity of *c*11,*t*13-20:2, the elongation product of the *c*9,*t*11-18:2, was confirmed by its retention time (25 min) in GC; and the mass peak (*m/z* = 322) and fragmentation pattern in the GC–MS spectrum were identical to the authentic *c*11,*t*13-CEDA standard (data not shown). The elongation product of *t*10,*c*12-CLA also had the same mass peak (*m/z* = 322) and a similar fragmentation pattern, but it was different in intensity from *c*11,*t*13-CEDA (data not shown). Although no authentic standard was available for comparison, evidence from later in this report suggests that the peak was *t*12,*c*14-CEDA.

The uptake of the individual CLA isomers (100 μM) and the elongation of individual CLA isomer were also examined. Figure 4 illustrates that *c*9,*t*11- and *t*10,*c*12-CLA were the two active isomers. Approximately 25% of these isomers were taken up by the transformed yeast, and significant amounts of these isomers were elongated to their respective CEDA. In contrast, approximately 40% of *c*9,*c*11- and 70% of *t*9,*t*11-isomers were taken up, but no detectable amounts of elongation products were observed.

To examine the substrate specificity of the recombinant elongase, equal amounts (50 μM) of LA and the two active forms of isomers (*c*9,*t*11- and *t*10,*c*12-CLA) were added separately into the medium, and the percentage of elongation of each substrate was determined. Results in Figure 5 show that the amounts of LA and CLA taken up by the yeast were similar. Approximately 12% of LA was elongated to EDA, but more than 25% of the two active isomers were elongated to CEDA.

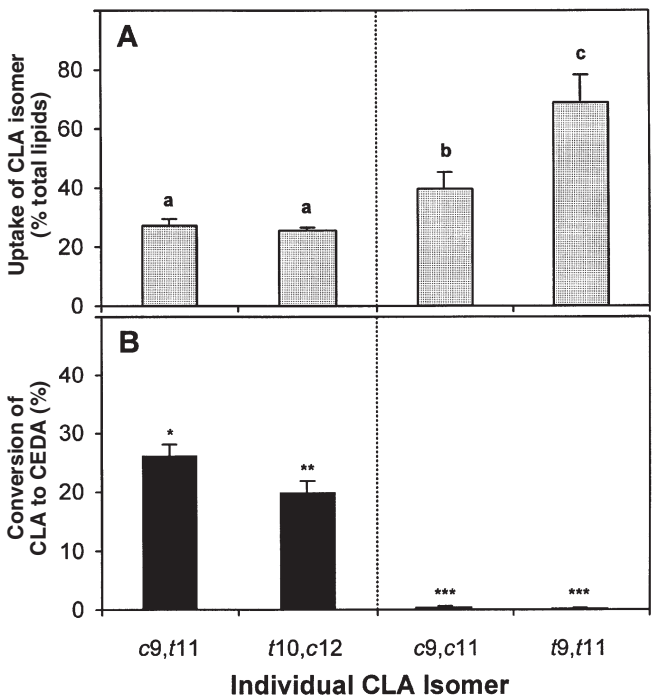


FIG. 4. Uptake of CLA (A) and conversion of CLA to CEDA (B) in yeasts transformed with human elongase gene. The yeast cells were cultured in 100 μM of four individual CLA isomers, respectively. The conversion of substrates to products was defined as the same as the legend of Figure 2. The uptake of CLA isomers was calculated based on the sum of CLA and CEDA isomers (% total lipids) in yeast lipids. All results are mean ± SE of three incubations. Values with different symbols or letters significantly differ from each other at $P < 0.05$. For abbreviations see Figures 2 and 3.

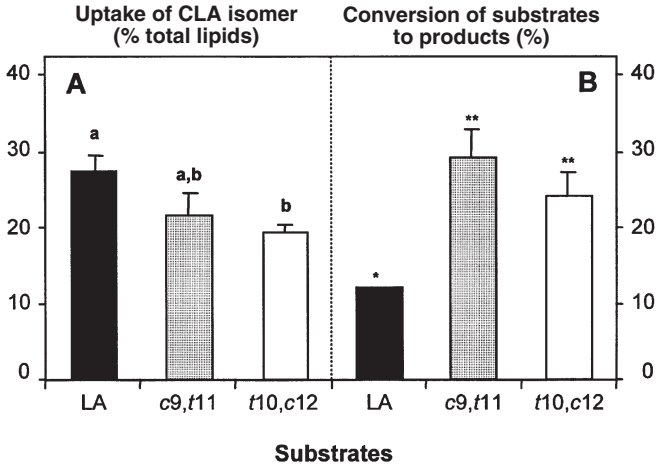


FIG. 5. Comparison of uptake of LA and CLA isomers (A) and conversion of LA to EDA and CLA to CEDA (B) in yeasts transformed with human elongase gene. The concentration of LA or CLA in the medium was 50 μM. All results are mean ± SE of three incubations. Values with different symbols or letters indicate a significant difference ($P < 0.05$). For abbreviations see Figures 1–3.

DISCUSSION

Using a yeast transformed with human PUFA-specific elongase gene, we demonstrated that CLA significantly inhibited the alternate pathway of LA metabolism, i.e., elongation of LA to EDA (Fig. 2A). We also demonstrated that only *c*9,*t*11- and *t*10,*c*12-CLA isomers, among the four available isomers, could inhibit LA elongation and then themselves be elongated (Figs. 3A, 3B). There were no elongation metabolites found from the other two isomers (*c*9,*c*11 and *t*9,*t*11) (Figs. 3C, 3D), despite these two CLA isomers being taken up more readily and incorporated into the 334(pRAE-58) yeast than the two active CLA isomers. Thus, the inability to elongate these two CLA isomers was due to the substrate specificity of the recombinant elongase. It is possible that the recombinant elongase recognize only *cis*-, *trans*- or *trans*-,*cis*- configuration, but not *cis*-,*cis*- or *trans*-, *trans*- configuration of CLA. These findings suggest that the inhibitory effect of CLA on LA elongation was a result of competition between the two *c*9,*t*11- and *t*10,*c*12-CLA isomers and LA as substrate for the recombinant elongase in the transformed yeasts. Interestingly, these two isomers have been previously identified as the most biologically active CLA isomers (9,13, 20,21).

CLA plays an important role in modulating immune functions (10). The decrease of eicosanoid synthesis is attributed to the competitive role of CLA in n-6 PUFA metabolism (10–12). Another hypothesis suggests that incorporation of CLA into the cell membrane might change membrane fluidity which, in turn, influences the mobility of receptors and membrane proteins and affects signal transduction, antigen recognition, receptor-ligand interactions, and cell cycle induction (22). Unfortunately, no direct evidence to support this interesting hypothesis is yet

available. On the other hand, results from the present study and from our previous study (13), demonstrate that the two active forms of CLA (*c*9,*t*11- and *t*10,*c*12-) can inhibit the two metabolic pathways of LA (Δ6-desaturation and elongation) and hence decrease PUFA (e.g., AA) synthesis. This in turn could decrease the amount of AA entering the cyclooxygenase/lipoxygenase pathways for eicosanoid synthesis in mammalian cells.

In conclusion, we demonstrated in this study that CLA can inhibit the elongation of LA to EDA, and thus repress the alternate metabolic pathway of LA. The inhibition is likely due to the competition between LA and two active CLA isomers (*c*9,*t*11- and *t*10,*c*12-) for the enzyme elongase. Only *c*9,*t*11- and *t*10,*c*12-CLA isomers could be metabolized by the transformed yeast with recombinant elongase, and only these two isomers exerted the suppressive effect on conversion of LA to EDA. Together with a previous study, we suggest that the *c*9,*t*11- and *t*10,*c*12-isomers are the two active forms of CLA, and their ability to modulate LA metabolism may be responsible at least in part for their biological effects.

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