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RESEARCH ARTICLE

Montaser M. M. E. Metwally
Afifi M. Eid
Fawzy M. Mohemed
Ahmed Kh. El-Dawy

CONJUGATED LINOLEIC ACID ISOMERS REGULATE FUEL-METABOLIC ENZYMES VIA PPAR-B IN MALE ALBINO RAT

ABSTRACT:

Obesity of many peoples all-over the world urges them to change their life style and food habits, one of these habits is the recent world-wide use of conjugated linoleic acid (CLA) as a dietary supplement in many countries to control body weight. Many studies were done on the conjugated linoleic acid addressing its role in decreasing the body weight, however many of them have conflicting results and didn't consider the effect of CLA isomers on gene expression level. This study aimed to monitor the possible mechanisms of different CLA isomers in controlling body weight and lipid oxidation at gene expression of the main lipolytic enzymes in rats. From the present results it can be concluded that the different isomers of CLA especially $T_{10}C_{12}$ isomer able to control body weight via induction of lipolysis through activation of peroxisome proliferators-activated receptor-β(PPAR-β) and its inducible enzymes as carnetine palmetoyl transferase(CPT1), hormone sensitive lipase(HSL) and lipoprotein lipase(LPL).

KEY WORDS:

Body weight, CLA (Conjugated Linoleic Acid), CPT1 (carnitine palmetoyl transferase), HSL (hormone sensitive lipase), LPL (lipoprotein lipase), PPAR-β (peroxisome proliferators-activated receptor-β)

CORRESPONDENCE:

Metwally Mohemed El-Sayed Metwally Montaser

Faculty of Science, Al-Azhar University, Cairo, Egypt

Biotechnology Department, Faculty of Science, Taif University, 888 Hawyia, Taif, KSA

E-mail: Montaser1968@yahoo.com.

Mohemed Eid Afifi

Medhat Mohemed Fawzy

Khalifa El-Dawy Ahmed

Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt

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INTRODUCTION:

CLAs are trans fats that are beef and dairy constituents. Despite nearly a decade of study of the role of CLA in modulating the development of atherosclerosis and hepatic lipoprotein metabolism, considerable uncertainty still exists as to the potential cardiovascular benefits or risks associated with these unusual fatty acids. No consistent improvement in the plasma lipid or lipoprotein profiles has been detected in animal or human studies, although emerging evidence suggests that CLA could affect the metabolism of fatty acids in the liver. More work is needed to understand the mechanisms of CLA action in the hepatocytes, especially with regard to isomer-specific effects on hepatic lipid metabolism and the role of gene expression patterns that can be altered by CLA (McLeod *et al.*, 2004)

Until recently, the influence of CLA on lipid metabolism and gene expression in the liver and extrahepatic tissues was largely unknown (Kennedy *et al*., 2007). Dietary CLA alters the levels of other (non-conjugated) fatty acids in phospholipids and neutral lipids in the liver. Trends that have been observed include alterations of oleic and palmitoleic acids. In hepatic neutral lipids palmitoleic and oleic acids decrease in mice (Park *et al.*, 1999) and rats (Sisk *et al.*, 2001). However, the ability of CLA to lower monounsaturated fatty acids seems to be somewhat specific for the strain and/or species of animal.

In particular, CLA modulates lipid metabolism, in part, by a mechanism dependent on the activation of the group of nuclear transcription factors, peroxisome proliferator-activated receptors (PPARs). In the liver, PPARα is a critical transcription factor for lipid metabolism, because several genes coding for enzymes involved with oxidation (either in peroxisomes or mitochondria) contain a functional peroxisome proliferator–responsive element in their enhancer regions (e.g., acyl-CoA oxidase, liver fatty acid–binding protein, cytochrome p 450A, hepatic lipoprotein lipase, and others) (Schoonjans *et al.*, 1996 and Kennedy *et al*.,

2007). In fact, several isomers of CLA are high-affinity ligands and activators of PPAR.

This study was designed to monitor the ability of two different CLA isomers in controlling the neutral fat content in hepatic tissue to evaluate their effect on the main lipolytic enzymes on the level of gene expression and activity, as well as the effect on PPARβ.

MATERIAL AND METHODS:

Animals:

Fifty Male Sprague-Dawley rats, 150 ± 10 g BW, were kept on standard diet (Degrace *et al.*, 2004) during this study. Rats were allocated into 5 groups, 10 rats each; negative control group (NC group) treated with normal saline, Positive control group (PC group) received corn oil and the other 3 groups treated with preparations enriched in CLA ethyl ester: group I (G I) treated with c_9t_{11} CLA isomer, group II (G II) treated with $t_{10}c_{12}$, and group III received mixed equal parts of both CLA isomers. However, treatment dose was 1.5 g /kg BW of rat in each group for 21 days and rats had free access for water and diet. CLA mixture from Crola Company (USA), t_{10}, c_{12} and c_9, t_{11} CLA from Loredo Lab. (Sweden).

Sampling:

After 24 hours from the last dose, Rats were anesthetized; blood and liver samples were taken.

Blood samples:

Blood samples were collected from heart into clean sterile anticoagulant-free tube; serum was separated and used for determination of (Triacylglycerol- TAG, total lipids, total cholesterol, and leptin.)

Liver samples:

Small parts were immediately taken, weighted and kept in liquid nitrogen until RNA extraction. Other part of liver was used in preparation of liver homogenate for the estimation of hepatic carnitine palmitoyl-transferase 1 (CPT1) and hepatic sinusoidal lymphocytes (HSL) activates.

Biochemical assays:

CPT1 activity assay in liver was done according to Markwell and Bieber (1976), lipase activity assay was done according to the method described by Lott *et al.* (1986), and concentration of serum-leptin was done according to Frieman and Halaas (1998). Concentration of total lipids was done according to Kornberg and Horecker (1955). Triacylglycerides' concentration was done after Fossati and Prencipel (1982). Total cholesterol was done according to the methods of Allain *et al.* (1974)

Molecular assays:

Determination of PPARβ, CPT1, ACO and HSL gene expression using a semi-quantitative RT-PCR was carried out according to Meadus (2003). Total RNA was extracted with EZNA-spin column RNA extraction kit (Omega Biotech),The first strand cDNA was synthesized by using Revert-Aid™ First Strand cDNA Synthesis Kit (Fermentas), the PCR amplification was carried out using 2X PCR Master Mix from (Fermentas). PCR were carried out in a volume of 50 µl consisting of 1 µg of cDNA and variable amount of both forward and reverse Primer according to Jones *et al.* (1999).

A primer pair for β-Actin (Ambion, Austin, TX) was used as an internal control for PCR analysis. Samples underwent a 10 min initial denaturing step to release DNA polymerase activity (hot start PCR), followed by the 30 cycles of 94°C/1min, annealing /1 min, and 72°C/1 min. (Table 1). The final extension step was 7 min at 72°C. PCR products were resolved on 1.5% agarose, visualized by UV trans-illumination and digitized with a Gel-Doc Fluorchem (MJ research). Image-analysis of the amplified fragments was carried out by using AlphaEaseFC (V 4.0.0). The intensities (IDV= Integrated density values) of PCR products (PPAR β, CPT1 and ACO) were corrected to that of β-actin (internal control).

Table 1. The primer sequences and PCR conditions

NO.	Name	Accession No	Primer Sequence (5'→3')	T-annealing C°	Product Size (bp)
1	PPARβ	M21060.1	Fd: `GGTCCGATTCTTCCACTGC Rv: TCCCCTCCTGCAACTTCTC	62	387
2	CPT1	FQ210484.1	Fd: GAGACACCAACCCCAACATC Rv: GTCTCTGTCCTCCCTTCTCG	55	290
3	ACO oxidase	NM_017340	Fd: AGCTTCACGCCCTCACTG Rv: ACCACCCACCAACTTCCC	60	245
4	HSL	NM_012859	Fd: CTGCTTCTCCCTCTCGTCTG Rv: TCAGACACACTCCTGCGC		322
3	B-Actin	NM_031144.2	Fd: CACCCGCGAGTACAACCTTC Rv: CCCATACCCACCATCACACC	60.4	726

Statistics:

The Mann-Whitney U-rank test was used to test for the differences in MT/β-actin ratios between the tested groups compared to the control group. In all the statistical tests, difference was considered significant when $P \leq 0.05$.

RESULTS AND DISCUSSION:

According to the present data, it can be observed a significant ($p \leq 0.05$) parallel decreases in body weight with liver weight due to treatment with CLA-isomers (table 2). Different isomers of CLA (t_{10}-c_{12} and c_9-t_{11}) and their mixture (1:1) increased the activities as well as the gene expression of HSL (table 4 and Fig. 2) that hydrolyze the neutral fat in the liver and adipose tissue ,the t_{10}-c_{12} isomer has the highest ability for controlling the body weight and activation of HSL gene expression. Two different forms of CLA were tested by Cherniack (2008**)** in a double-blinded, randomized, placebo-controlled trial of 180 men and women ages 18-65, BMI 25-30. After one year there was a small but statistically significant 1.8-kg weight reduction; 0.6 kg/m2 reduction in body mass index (BMI) when CLA was given as a triglyceride (4.5 g/day, 76% CLA-triacylglycerol). Subjects were given a written questionnaire asking about dietary and exercise habits at baseline, six months, and one year. In a one-year, open-label extension of the trial no further decrease in weight was observed. Adverse events were noted in less than four percent of subjects, were not serious, and were similar for CLA and placebo groups. When CLA was given as free fatty acids rather than a triglyceride, weight loss was not observed**.** Smedman and Vessby (2001) reported that CLA modulates body composition, especially by reducing the accumulation of adipose tissue in experimental animals. Park *et al.* (1997) reported that the total adipose tissue mass was reduced by over 50% in mice feed diet containing CLA compared with that fed a control diet (without CLA). Long-term feeding of CLA (1.0% CLA for 8 months) appears to have a lipodystrophic effect in female C57BL/6J mice, leading to complete ablation of brown adipose tissue, reduced leptin, a hormone known to regulate feed intake (Tsuboyama-Kasaoka *et al.*, 2000). The ability of CLA to reduce adipose tissue mass occurs regardless of food intake or fat level (6.5–20.0%) in mice, so feed efficiency may be improved, in fact; CLA reduces leptin in rats and humans (Belury and Vanden 1999).

The concentrations of serum total lipids, triacylglycerol and total cholesterol were significantly ($p \leq 0.05$) increased in all treated groups (Table 3).

The obtained data concerning the gene expression of PPAR β, CPT1, Acyl COA and HSL (Figs 1 & 2) showed that CLA treatment either given each isomer alone or in mixture leads to a high significant induction of these genes expression. The hepatic CPT1 and HSL activities were significantly ($p \leq 0.05$) decreased (Table 4) in all treated groups. In contrast, the serum lipten concentration was decreased in all treated groups (Table 3).

Also the decrease of body fat mass leads to decrease the concentration of leptin. The activation of HSL as well as LPL leads to increasing the hydrolysis of tissue and serum lipids this leads to decrease the serum total lipids and TAG, that are converted in to fatty acids and glycerol, CLA isomers leads also to the activation of the entrance of fatty acids into the liver and muscle cells through activation of fatty acid pending protein that activated by PPAR-β that stimulated by CLA , then fatty acids are enter to peroxisome and mitochondria through the activation of the CPT and the peroxisomal fatty acid oxidation is stimulated through the activation of the rate limiting enzyme acyl-CoA oxidase (Belury and Vanden 1999).

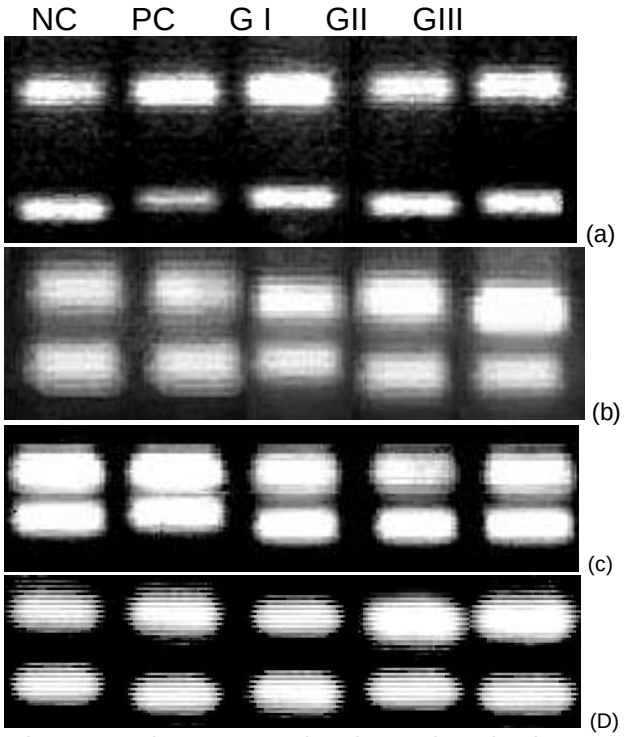
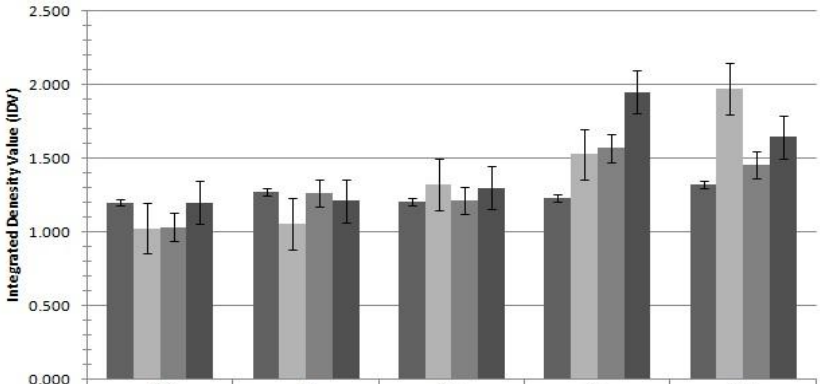


Fig. 1. Panel represents the electrophoretic pictures for PCR products of (a) PPARβ = 404 bps; (b) CPT1= 295 bps; (c) Acyl COA oxidase = 245 bps; (d) HSL - 322 bps. All products were correlated to β-Actin product (207 bps) as an internal control. PC (positive control group), NC (negative control group), G I; GII; GIII(Treated groups I, II and III)



	NC	PC	G I	GII	G III
ACO	1.201	1.273	1.206	1.233	1.322
CPT	1.027	1.053	1.324	1.528	1.975
HSL	1.034	1.266	1.214	1.570	1.455
PPAR β	1.200	1.211	1.300	1.952	1.647

Fig. 2. The expression levels (semi-quantitation of) for PPARβ and the enzymes ACO and CPT represented in IDV histograms. PC (positive control group), NC (negative control group), G I; GII; GIII (Treated groups I, II and III)

Table 2. Body parameters (Body weight and liver weight in grams) for rats treated with c9, t11 CLA, t10, c12 CLA and mixture of CLA isomers

Groups	Body weight	Liver weight
NC	284.2 ± 4.4[a]	14.2 ± 0.3[a]
PC	281.5 ± 3[a]	14.3 ± 0.2[a]
G I	227.4 ± 2[b]	11.4 ± 0.1[b]
G II	216.6 ± 3[b]	10.8 ± 0.3[b]
G III	226.3 ± 5[b]	11.3 ± 0.2[b]
LSD	11.09	0.59

Table 3. Serum lipid profile and serum leptin for rats treated with c9, t11 CLA, t10, c12 CLA and mixture of CLA isomers. Total lipids, triacylglycerol and total cholesterol (mg / dL), leptin (ng / ml)

Groups	Serum leptin	Total lipids	TAG	Cholesterol
NC	6.4318± 0.59[a]	422.6± 1[a]	91.5± 2[a]	151.5± 4.[a]
PC	6.2656± 0.45[a]	414.7± 1.[a]	90.7± 2[a]	149± 4[a]
G I	2.8040± 0.26[b]	362.6± 2	68.3± 2[b]	105.7± 1[b]
G II	0.9408± 0.05[c]	292.6± 9[c]	46.4± 1[c]	93.3± 4[c]
G III	0.9475± 0.043[c]	324.4± 2	47.1± 1[c]	92.5± 3.[c]
LSD	0.05	35.63	5.005	10.07

Each value represents the mean of 10 rats ± S.E

Table 4. Hepatic CPT1 and ACO activities (nmol/ mg protein) and hepatic lipase activity (U/ L) homogenate in rats treated with c9, t11 CLA, t10, c12 CLA and mixture of CLA isomers

Group	CPT1	HSL	Acyl COA oxidase
NC	2.07 ± 0.038[b]	3.76 ± 0.02[b]	0.51 ± 0.006[d]
PC	2.114 ± 0.02[b]	3.59 ± 0.06[b]	0.08 ± 0.0005[e]
G I	2.142 ± 0.03[b]	8.62 ± 0.80[a]	0.72 ± 0.008[c]
G II	2.612 ± 0.03[a]	8.99 ± 0.85[a]	1.72 ± 0.09[b]
G III	2.661 ± 0.02[a]	10.3 ± 0.61[a]	1.8 ± 0.08[a]
LSD	0.08	1.68	0.018

Tables 2, 3, & 4: Each value represents the mean of 10 rats ± S.E statistically analyzed using one way F-test. Means which share the same letter are not significantly different. Means which have different letters are significantly different (P < 0.05). PC (positive control group), NC (negative control group), G I; GII; GIII (Treated groups I, II and III) and LSD (Least Significant Difference).

Considerable effort is now in progress to understand the mechanisms of the changes associated with CLA supplementation. Much of the work so far centers on the role of CLA as PPAR ligands. PPARα is an important member of this nuclear receptor family that is expressed in the liver and modulates hepatic lipid metabolism. With use of a scintillation proximity assay, CLAs were shown to be high-affinity fatty acid ligands for PPARα (McLeod *et al.*, 2004).

CLA isomers differ in their effects on gene expression. Some studies suggested that FA and acyl derivatives up regulate the expression of both L-CPT 1 and ACO genes through PPAR , but other findings showed that long-chain FA regulate L-CPT I through a PPARα-independent pathway . Both CLA-isomers studied, to a greater extent, were demonstrated to be potent ligands for PPARα .Both dietary CLA 1 & 2 effectively increased the peroxisomal ACO expression, but CLA, which was recovered in far greater amounts in liver lipids than CLA2 , did not increase L-CPT I gene expression. Therefore, L-CPT I regulation was independent of PPARα activation, as already discussed. In mice fed CLA2, which was mainly recovered as desaturated and elongated metabolites the hepatic effects may originate from complex mechanisms, causing at least the induction of L-CPT I and ACO gene expression. Interestingly, transcription of M-CPT I, CPT II, and ACO genes has been shown to be activated by FA via PPARα and also significantly via either CLA isomers, which suggest that some effects of both CLA may depend on PPARα (Degrace *et al.*, 2004).

Based on the current knowledge of peroxisome proliferator-mediated gene expression and our findings, it can be speculated that peroxisome proliferators and

CLA share a mechanism to explain their hypolipidemic and hypocholesterolemic effects. At the extracellular level, the hypolipidemic effect induced by peroxisome proliferators may be due to an increased hydrolysis by lipoprotein lipase of triglycerides derived from chylomicrons and very low density lipoprotein particles. Evidence suggests that the plasma triglyceride clearance processes are regulated by peroxisome proliferators and, in some steps, directly by hepatic PPARα as lipoprotein lipase expression is induced by PPAR activators. Inside the cell, free fatty acids (FFAs) are transported by FABP. This binding protein is also up-regulated through a PPAR-mediated mechanism, with one functional PPAR response element (PPRE) regulating its expression. FFAs regulate other lipid-metabolizing enzymes of the peroxisome, microsome, or cytosol via activation of PPARα. In peroxisomes, the rate-limiting enzyme for ß-oxidation of ACO and the enzyme responsible for the second and third step in ß-oxidation enoyl-CoA hydratase-3-hydroxyacyl-CoA dehydrogenase have functional PPREs. Other lipid metabolizing enzymes regulated via PPAR are also found in mitochondria (acyl-CoA synthase, medium chain acyl-CoA dehydrogenase, ß-hydroxy-ß-methylglutaryl-CoA synthase), in microsomes (CYP4A, ω-hydroxylase, and in cytosol (phosphoenol pyruvate carboxy kinase, CLA regulates lipid metabolism through binding and activation of PPARα therefore, CLA may be beneficial for those metabolic disorders related to impaired lipid metabolism, such as non-insulin-dependent diabetes mellitus, atherosclerosis, obesity and cancer.

CONCLUSION:

From the obtained results it can be concluded that both CLA isomers have the ability to regulate the body fuel metabolism on the molecular levels through activation of PPAR-β which reflected on the activation of the main enzymes of β-oxidation of fatty acids, and glucose oxidation enzymes which leading to decrease in the level of serum TAG, total lipids, and blood glucose and increase hepatic glycogen.

Both CLA isomers having the ability for decrease Body weights and liver weight.

The present results proved also that CLA c10t12 not cause hepatomegaly or insulin resistance as maintained by some authors. However, further studies are required to strengthen this conclusion.

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نظائر حمض اللينوليك تتحكم في إنزيمات أيض الدهون في الفئران البيضاء عبر PPAR-B

متولي محمد السيد متولي منتصر[1&3]، محمد عيد متولي[2]، مدحت محمد فوزي[2]، خليفة الضوي أحمد[2]

[1]-قسم علم الحيوان، كلية العلوم، جامعة الأزهر، القاهرة، مصر.

[2]-قسم الكيمياء الحيوية، كلية الطب البيطري، جامعة الزقازيق، مصر

[3]-قسم التقنية الحيوية، كلية العلوم، جامعة الطائف، الطائف، المملكة العربية السعودية.

تفرض السمنة علي شعوب كثيرة في العالم تغيير نمط الحياة و العادات الغذائية مثل الاستخدام المتزايد لحمض اللينوليك لإنقاص الوزن . أجريت دراسات عديدة علي حمض اللينوليك لتوضيح دوره في إنقاص وزن الجسم . ولقد جاءت بعض النتائج بتناقضات كما أن معظمها لم يتطرق لأثر نظائر الحمض علي مستوي التعبير الجيني. يهدف هذا البحث دراسة اثر نظائر حمض اللينوليك (النظير C_9 T_{11} و النظير $T_{10}C_{12}$) علي وزن الجسم و أكسدة الدهون في الفئران علي المستوي الكيموحيوي و البيولوجي الجزيئي لجينات إنزيمات ايض الدهون. حيث أظهرت الدراسة زيادة معنوية في التعبير عن الجينات CPT1 و HSL مصاحبة لزيادة الجرعة من الحمض حسب النظير . و قد خلصت الدراسة إلي أن النظائر المختلفة للحمض خاصة النظير تستطيع التحكم في وزن الجسم من خلال تنشيط المستقبلات PPAR-β و الإنزيمات التي يثيرها مثل إنزيمات CPT1 و HSL و LPL .