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Dietary conjugated linoleic acid induces peroxisome-specific enzyme accumulation and ornithine decarboxylase activity in mouse liver

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Previous studies have shown that the dietary fatty acids, conjugated linoleic acids (CLA), inhibit carcinogenesis in the colon, mammary gland, forestomach, and skin. Several properties of this chemoprotective polyunsaturated fatty acid suggest it will act as an hepatic peroxisome proliferator. This study evaluated the effect of dietary CLA on the accumulation of enzymes associated with peroxisome proliferation in rodent liver. Female SENCAR mice were fed one of four semipurified diets containing 5% corn oil without CLA ("control diet") or supplemented with incremental levels of CLA (0.5%, 1.0% or 1.5% by weight of diets) for 6 weeks. Hepatic mRNA levels of several enzymes known to be induced during peroxisome proliferation [i.e., acyl-CoA oxidase (ACO), cytochrome P4504A1 (CYP4A1), and liver fatty acid binding protein (FABP)] were measured by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Diets containing 0.5%, 1.0% or 1.5% CLA were associated with approximately 6-, 9-, and 9-fold increases in ACO mRNA, respectively, compared with mRNA levels in mice fed the control diet. The steady state levels of FABP and CYP4A1 mRNA accumulation were maximal in animals fed 1.0% CLA diets and less magnified in mice fed 1.5% CLA diets. Western blot analysis revealed that the relative abundance of ACO protein in livers of mice fed CLA-containing diet groups (0.5%, 1.0%, and 1.5% CLA) were 2.5, 3.0, and 3.0 times the level ACO protein of mice fed the control diet (0.0% CLA). Because most peroxisome proliferators are considered nongenotoxic hepatocarcinogens in rodents, the effect of dietary CLA on ornithine decarboxylase (ODC) activity, a measure of cell proliferation and tumor promotion, was quantified. Activity of hepatic ODC was increased by approximately 10-fold for mice fed 1.0% and 1.5% diets, respectively, compared with those fed the control or 0.5% CLA diets. These data suggest that CLA displays the typical peroxisome proliferation response, i.e., induction of ACO, CYP4A1 and FABP accumulation and cell proliferation in rodent liver. (J. Nutr. Biochem. 8:579–584, 1997) © *Elsevier Science Inc. 1997*

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Introduction

The levels and types of dietary fat contribute substantially to the risk of the development of cancer. In particular, saturated and unsaturated fatty acids modulate carcinogenesis in an organ-, sex-, and species-specific manner (reviewed in Ref. 1). Most omega-6 polyunsaturated fatty acids such as linoleic acid (18:2n6) are associated with increased cancer risk, particularly in the mammary gland, colon, and pancreas. In contrast, the linoleate derivative conjugated linoleic acid (CLA) is chemoprotective in several tissues during multiple stages of carcinogenesis.[1–4] CLA refers to a group of dienoic linoleate isomers with several possible positional (9, 11 or 10, 12) and geometric (*cis* and *trans*) double bond

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configurations. As a component of foods, it is abundant in ruminant meats and dairy products[5] and the concentration generally increases in foods that are heat processed.

Evidence in our laboratory has demonstrated that body weights of mice fed semipurified diets with incremental levels of CLA (0.5 to 1.5% by weight of diet) were significantly lower ($P < 0.05$) than weights of mice fed diets without CLA.[2] In addition, dietary CLA altered the lipid composition of liver; increasing levels of CLA significantly enhanced ($P < 0.05$) lipid accumulation in mouse liver.[6] Furthermore, CLA incorporation into neutral and phospholipids of liver was associated with increased amounts of oleic acid (18:1,c9) and reduced levels of linoleate and arachidonate (20:4, c5, c8, c11, c14). Hepatic lipid accumulation, decreased body weights, and/or altered fatty acid composition of rodent liver have been attributed to effects of the hypolipidemic agent clofibrate,[7] some polyunsaturated fatty acids (including arachidonate and linoleate)[8] and the perfluorinated fatty acid analog, perfluorodecanoate.[9] The similarity in structure and biologic effects of CLA with these hypolipidemic drugs, fatty acids, and fatty acid derivatives suggests that CLA is a member of a group of chemicals known as peroxisome proliferators. Although not chemically related, peroxisome proliferators have two common structural features, including a hydrophobic-rich region and an acidic group. It is thought that peroxisome proliferators exert their biologic effects in rodent liver through activation of the steroid hormone receptor, peroxisome proliferator-activated receptor-α (PPARα).[10,11] Activation of PPARα induces transcription of a number of genes encoding for lipid metabolizing enzymes including the peroxisomal-specific enzyme, acyl-CoA oxidase (ACO), the lipid transporter, liver fatty acid binding protein (FABP), and the microsome-associated cytochrome P4504A1 (CYP4A1). This paper demonstrates CLA induces each of these molecular markers of peroxisome proliferation as well as ODC activity, a hallmark event associated with tumor promotion in mouse liver. These data suggest CLA may be chemoprotective in extrahepatic tissues at the expense of enhancing peroxisome-associated enzyme accumulation in rodent liver. Further studies are needed to determine the modulatory role of CLA in PPARα activation as well as hepatocarcinogenesis in rodents.

Methods and materials

Materials

Enzymes, cofactors and nucleotides necessary for internal standard construction and RT-PCR were purchased from Promega Corporation (Madison, WI USA) and primers were obtained from Genosys Biotechnologies (The Woodlands, TX USA). Solvents and other chemicals were of the highest grade commercially available.

Diets and animals

Four AIN-76 formulated diets[12] containing various levels of CLA shown to modulate mammary and skin tumorigenesis[2] were used in these studies (*Table 1*). All diets contained 5% corn oil providing approximately 2.8% linoleic acid by weight of diets. The "control diet" contained no supplemental CLA and experimental diets contained increasing levels of CLA (0.5%, 1.0% or 1.5% by weight) at the expense of dextrose. Because oleate, linoleate, and arachidonate may also act as peroxisome proliferators,[8] the relative amounts of each of these fatty acids supplied in the diets are shown in *Table 1*. Diet components were purchased from Dyets, Inc. (Bethlehem, PA USA) with the exception of CLA, which was purchased from NuChek Prep, Inc. The isomeric distribution of CLA was 43% c9,t11- and t9,c11-CLA, 45% t10,c12-CLA, 6% c9,c11-, c10,c12-, t9,t11-, t10,t12-CLA, 2% linoleate, and 4% unidentified compound as reported by the manufacturer and confirmed by gas chromatography analysis.[6] Diets were fed every other day in clean powder feeders with stainless steel grids and mice were allowed free access to food and water. Body weights were measured weekly and food disappearance as an index of food consumption was determined periodically.[6] After maintaining mice on experimental diets for 6 weeks, animals were euthanized and livers excised and frozen in liquid nitrogen. Tissue was stored at −80°C until analyses were performed. Final body weights, hepatic lipid concentration, and fatty acid composition of hepatic lipids are shown in *Table 2* and have been described previously.[6]

Table 1 Composition of experimental diets and dietary oils

	Control	0.5% CLA	1.0% CLA	1.5% CLA
Diets[a]				
Casein	20.0	20.0	20.0	20.0
Dextrose	65.0	64.5	64.0	63.5
Cellulose	5.0	5.0	5.0	5.0
DL-Methionine	0.3	0.3	0.3	0.3
Choline	0.2	0.2	0.2	0.2
Mineral Mix[b]	3.5	3.5	3.5	3.5
Vitamin Mix[c]	1.0	1.0	1.0	1.0
Corn Oil	5.0	5.0	5.0	5.0
CLA[d]	–	0.5	1.0	1.5
Dietary oils[e]				
Oleate (18:1)	25.01	21.37	20.88	18.16
Linoleate (18:2)	56.94	48.92	46.82	40.88
CLA (18:2; 9,11 and 10,12)	0.17	12.53	15.82	24.62
Arachidonate (20:4)	N.D.	0.02	0.02	0.01
CLA:Linoleate	0.094	0.256	0.338	0.602

Diets were prepared on the premises and stored in sealed containers under inert conditions for no longer than 2 weeks at −10°C. For details, see Ref. 6.
[a]g/100g.
[b]AIN-76
[c]AIN-76A
[d]Free fatty acid.
[e]Fatty acid composition of dietary oils expressed as percent total fat.[6]

Isolation of total RNA and protein

Total RNA and protein were isolated from mouse liver using Tri Reagent™ according to the manufacturers protocol[13] (Molecular Research Center, Cincinnati, OH USA). RNA and protein extracts were frozen (−80°C) until analyses were performed.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using recombinant RNA (rcRNA) as an internal standard was performed as described by Vanden Heuvel et al.[11,14] In brief, reverse transcription of RNA occurred in a 20 μL volume of Tris-HCl buffer with 2.5 units MMLV reverse transcriptase (Gibco Life Technologies, Gaithersburg, MD USA), 2.5 mM oligo$(dT)_{16}$ (Promega Co.), 5 mM $MgCl_2$, 0.1 μg total RNA, and varying

Table 2 Effects of dietary CLA on body weight and hepatic lipid composition

	Diet group			
	(0.0% CLA)	(0.5% CLA)	(1.0% CLA)	(1.5% CLA)
Body weight[a]	37.80 ± 2.47*[d]	34.86 ± 1.20*†	32.90 ± 0.91†	32.46 ± 2.25†
Lipid concentration[b]	77.5 ± 5.84*	99.0 ± 6.30†	113.3 ± 15.90†	140.3 ± 9.19§
Fatty acid composition[c]				
16:0	22.23 + 0.18*	23.18 + 0.33*	24.27 + 1.39*	26.54 + 0.83†
16:1;7	3.49 + 0.13	3.31 + 0.18	3.30 + 0.77	4.02 + 0.13
18:0	7.17 ± 0.80*	6.97 ± 1.70*	5.15 ± 1.63*	3.45 ± 0.13†
18:1;9	40.26 ± 0.61*	38.62 ± 2.98*	42.68 ± 1.18*	45.26 ± 1.27†
18:1;11	4.74 ± 0.29*	4.97 ± 0.52*	4.65 ± 0.31*	3.75 ± 0.26†
18:2;9,12 (LA)	10.86 ± 0.76*	11.5 ± 0.99*	8.41 ± 1.75†	6.28 ± 0.31†
18:2; 9,11 and 10,12 (CLA)	0.00*[e]	0.67 ± 0.05*	0.94 ± 0.06*	1.06 ± 0.03†
20:1;9	0.37 + 0.07	0.43 + 0.06	0.35 + 0.06	0.30 + 0.01
20:4; 5,8,11,14	7.70 ± 0.05*	7.11 ± 1.00*	5.12 ± 1.11*	2.85 ± 0.05†
CLA:LA	—	0.06	0.11	0.17

[a]Mice (n = 12 per diet) were fed assigned diets for 6 weeks.[6] Final body weights were determined before sacrifice of animals. Values represent mean ± S.D. of body weight (g).
[b]Values represent mean ± S.D. of lipid extracted (mg)/liver tissue (g) (n = 3 to 6 samples).
[c]Mice were fed experimental diets for 6 weeks and total lipids were extracted from liver, fatty acids methylated, and quantified by gas chromatography as we have described previously.[6] Values represent mean ±S.D. of percentage total fatty acid (n = 5 to 6 liver samples per diet group).
[d]Values with different symbols (*, †, §) in each row are significantly different ($P < 0.05$).
[e]Not detected in measurable quantities and estimated to account for less than 0.09% total fatty acid.

amounts of rcRNA internal standard. Each gene has its own specific rcRNA template to be used as an internal standard and all internal standards, primers, and reaction conditions have been optimized as described previously.[14] The sequences for the RT-PCR primers are shown in *Table 3*. Samples were incubated for 15 min at 42°C and reverse transcriptase inactivated by heating at 99°C for 5 min. Prepared cDNA samples were incubated with PCR master mix (total volume, 50 μL) containing 4 mM $MgCl_2$, 2.5 units Taq polymerase (Promega Co.) and 6 pmol forward and reverse primers. The reactions contained primers for β-actin, ACO, CYP4A1, or FABP that were chosen using the primer selection programs, Oligo™ (National Biosciences, Hamel, MN USA) or Primer Select (DNASTAR, Madison, WI USA). Reactions were heated to 94°C for 3 min and immediately cycled 28 to 34 times through a 30-sec denaturing step at 94°C, a 30-sec annealing step at optimal temperatures (50 to 56°C depending on primers used) and a 60-sec elongation step at 72°C. After the final cycle, a 5-min elongation step at 72°C was executed. Aliquots of the PCR reaction were electrophoresed on a 2.5% NuSieve/agarose (3:1;w/w, FMC BioProducts, Rockland, ME USA) gel. PCR fragments were visualized with ethidium bromide and intensity of staining quantified from a photograph using pdi® scanning densitometry (Huntington Station, NY USA).

Target mRNA (i.e., ACO, CYP4A1, or FABP) was quantified by first determining the approximate optimum concentration of internal standard required to result in a 1:1 intensity of internal standard:target PCR product. Following, a constant amount of optimal internal standard in combination with RNA samples in triplicate were amplified by RT-PCR. A standard curve was prepared by using incremental concentrations of internal standard and constant concentration of RNA for amplifications and the log (ratio of band intensity) versus log (internal standard added) plotted. The ratio of internal standard to target mRNA PCR product intensity was used to quantify β-actin, ACO, CYP4A1, or FABP mRNA levels.[14]

Western blot analysis

Proteins were solubilized in 1% SDS and samples (50 μg protein/lane) loaded onto an 8.0% SDS-polyacrylamide gel with 3.5% stacking gel. Electrophoretically resolved proteins were blotted onto nitrocellulose and incubated with goat anti-rat albumin serum or rabbit anti-rat ACO serum. Blots were washed in TBS-Tween-20 and incubated with horseradish peroxidase-linked secondary antibody (BioRad Corp., Richmond, CA USA). Immunoreactive bands were detected using enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL USA) and films quantified using pdi® scanning densitometry.

Table 3 Sequences for RT-PCR primers and size[a] of PCR products

	Primer sequence	Target PCR-product size	Internal standard PCR-product size
β-Actin forward	5′ CCT CTA TGC CAA CAC AGT 3′	125	153
β-Actin reverse	5′ AGC CAC CAA TCC ACA CAG 3′		
ACO forward	5′ ATT CGG TGT TGT AAG TGC 3′	417	340
ACO reverse	5′ TTG GTG GGT GGG TGT TGA 3′		
CYP4A1 forward	5′ CAG GCC ATT GGG AAC TTG AA 3′	448	348
CYP4A1 reverse	5′ GGT CCA GGT GAT CCC AGG TAA 3′		
FABP forward	5′ GCG ATG GGT CTG CCT GAG 3′	243	307
FABP reverse	5′ CAC GGA CTT TAT GCC TTT GAA 3′		

[a]basepairs

Ornithine decarboxylase activity

Liver tissue homogenates were suspended in ice-cold sodium potassium phosphate buffer and ODC activity quantified in the supernatants (12,000 × g) under conditions described previously.[15] Total protein was determined by Coomassie blue reaction (BioRad Corp.).

Statistical analysis

Data were analyzed by ANOVA (General Linear Model, Tukey) using Statistical Analysis System (Cary, NC).

Results

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Because quantitative RT-PCR is an accurate and sensitive alternative to Northern blot analyses, this method was used to quantify accumulation of β-actin, ACO, CYP4A1, and FABP mRNA.[12] The inclusion of an internal standard in competitive RT-PCR reactions allows for the quantification of target mRNA by negating tube-to-tube variability in amplification efficiency. No significant differences in accumulation of β-actin mRNA were observed among diet groups (data not shown). Diets containing 0.5, 1.0, and 1.5% CLA were associated with approximately 6-, 9-, and 9-fold induction respectively of hepatic ACO mRNA accumulation compared with levels of ACO mRNA in livers of mice fed control diet (containing no CLA; *Figure 1*). In contrast, the steady state levels of FABP and CYP4A1 mRNA were maximal in livers of mice fed 1.0% CLA resulting in a 16-fold increase of FABP mRNA and a 10,000-fold increase in CYP4A1. The more dramatic increase in CYP4A1 mRNA may be attributable to the lower steady state level of this transcript in livers of mice fed 0.0% CLA diets (approximately 100,000 molecules of CYP4A1 per 0.1 μg RNA compared with 1,000,000 molecules of FABP per 0.1 μg RNA).

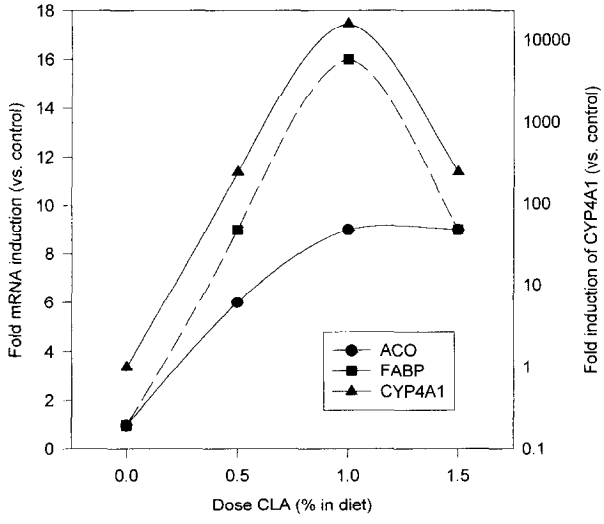

Figure 1 Effect of dietary CLA on hepatic ACO, FABP, and CYP4A1 mRNA accumulation. After groups of mice were fed experimental diets containing no (control), 0.5, 1.0, or 1.5% CLA for 6 weeks, livers were excised and frozen at −80°C until RNA was isolated ($n = 3$) (performed in triplicate). Data were quantified relative to the amount of β-actin mRNA present in each sample and are expressed as an index of molecules of target mRNA (ACO, FABP, or CYP4A1) in dietary CLA diet groups versus control (1.00).

Western blot analysis

To determine whether dietary CLA-associated changes in mRNA levels of peroxisome-specific genes is associated with enhanced protein expression, the level of protein for ACO, the rate limiting enzyme in peroxisomal β-oxidation was measured by Western blot analysis. A significant ($P < 0.05$) but less dramatic trend was observed for ACO protein levels compared with ACO mRNA. The levels of ACO protein increased approximately 3-fold in livers of mice fed 1.0% or 1.5% CLA diets compared with ACO protein expression in mice fed 0.0% CLA (*Figure 2*). The levels of albumin were not influenced by CLA (data not shown).

Ornithine decarboxylase activity

Because most peroxisome proliferators are associated with enhanced hepatocarcinogenesis, these studies begin to address the role of dietary CLA in hepatic cell proliferation by

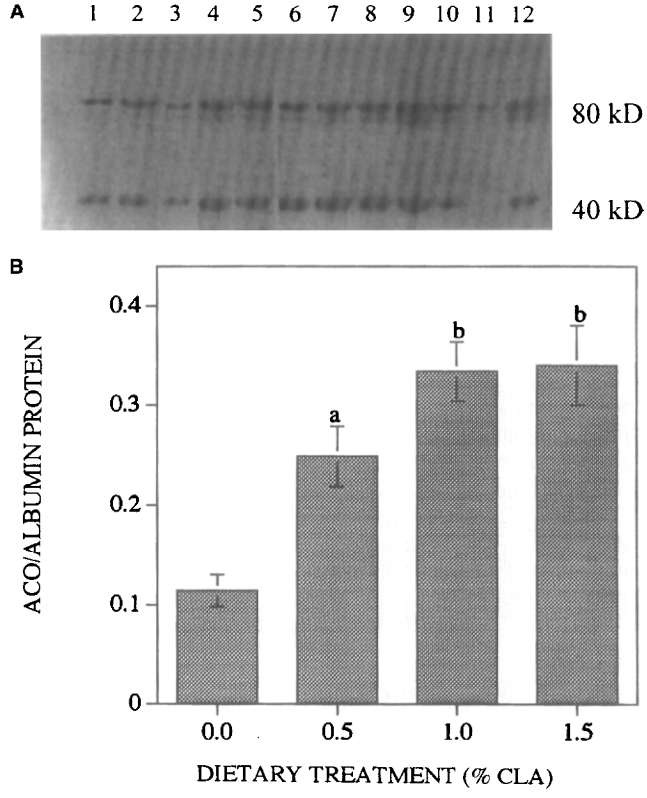

Figure 2 Effect of dietary CLA on ACO protein accumulation. Western blots were prepared and hybridized to primary antibody for ACO as described in Methods and materials. Panel A: Western blots with ACO bands labeled with arrows used for densitometric measurements. Lanes 1–3, 0.0% CLA; Lanes 4–6, 0.5% CLA; Lanes 7–9, 1.0% CLA; Lanes 10–12, 1.5% CLA diet groups. Panel B: Densitometric scans were quantified with pdi® scanning denistometry. The data are arbitrary densitometric units (mean ± SEM) after normalization with albumin. Superscripts indicate values that are statistically different from the value of mice fed control (0.0% CLA) diets.

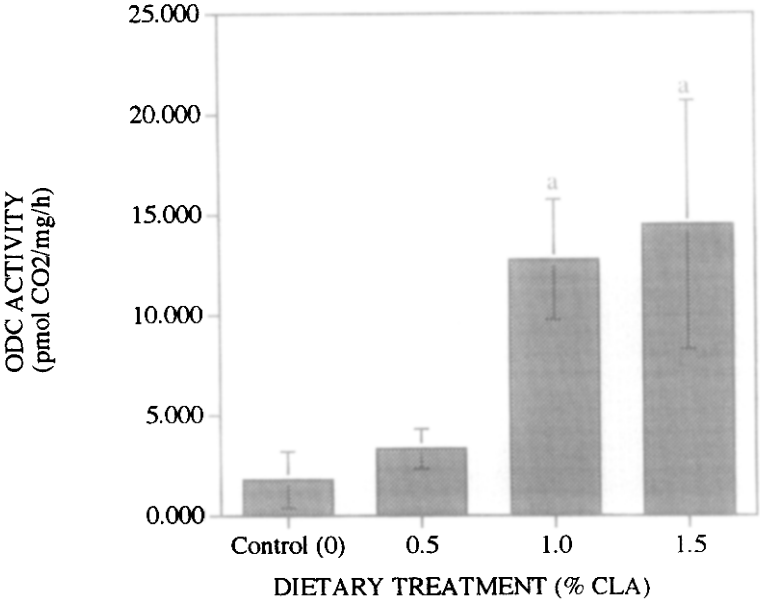

Figure 3 Dietary CLA modulation of ornithine decarboxylase activity. Mice were fed diets containing incremental levels of CLA (control, 0.5, 1.0, or 1.5% by weight) for 6 weeks. ODC activity was measured in protein extracts of liver homogenates as described in Methods and materials. Values represent mean ± S.E.M. $n = 5$ mice per diet group and each liver preparation was assayed in duplicate. Superscripts denote values that are significantly different ($P < 0.05$) from the value of control diet group.

measuring ODC activity in mouse liver. Diets containing 1.0% and 1.5% CLA fed to mice for 6 weeks supported approximately 10-fold greater ($P < 0.05$) levels of ornithine decarboxylase activity in livers compared with control (0.0% CLA) or 0.5% CLA diets (*Figure 3*).

Discussion

Two distinct theoretical mechanisms have been proposed to explain how peroxisome proliferators such as CLA cause the prototypical response associated with these chemicals; i.e., altered gene expression.[9,16,17] One theory is that because treatment with most peroxisome proliferators results in accumulation of hepatic lipids, a substrate overload effect (caused by lipid accumulation) is created. In this mechanism, induction of PPAR-regulated gene expression is secondary to lipid accumulation in the liver. A second theory is that peroxisome proliferators act as ligands for PPARα, resulting in activation of the receptor and induction of transcription of genes downstream from the PPAR-responsive elements (PPREs).

PPAR is a ligand-activated transcription factor that heterodimerizes with retinoic acid-X-receptor-α and binds to the PPRE. A number of genes contain PPREs including ACO,[18] CYP4A1,[19] and FABP.[20] Activators of PPAR that have been identified include fibrate hypolipidemic drugs, phthalate ester plasticizers, trichloroacetic acid herbicides, perfluorinated fatty acid analogues, as well as endogenous fatty acids such as linoleate, α-linolenate (18:3n3), arachidonate (20:4n6), eicosapentaneoate (20:5n3), and docosahexanoate (22:6n3; 9). Recent findings demonstrate that the arachidonate metabolite, prostaglandin J_2, is a direct ligand and activator for the PPARγ subtype.[21] In addition, the lipoxygenase product of arachidonate, leukotriene B_4, and the cyclooxygnease inhibitor, indomethacin, were recently found to be potent ligands for PPARα and PPARγ.[22,23] Several peroxisome proliferators including Wy-14,643 [(4-chloro-6-(2,3-xylindino)-2-pyrimidinylthio)-acetic acid], arachidonate, and eicosatetraynoate activate PPARα to induce transcription in a reporter gene assay system. Ongoing studies in our lab are being conducted to determine whether CLA is a direct ligand and/or activator of PPARα.

Peroxisome proliferators result in dramatic alterations in hepatic lipid levels. Specifically, several genes involved in lipid metabolism have been identified downstream from PPREs suggesting that PPAR has evolved to maintain cellular lipid homeostasis. ACO, the rate-limiting enzyme in peroxisomal β-oxidation, is highly induced during peroxisome proliferation and is the biomarker most often used to assess peroxisome proliferation.[18] CYP4A1 is a lipid-metabolizing enzyme involved in ω-hydroxylation of fatty acids allowing for increased rates of β-oxidation. FABP is a cytosolic lipid transport protein thought to be involved with shuttling fatty acids to and from the plasma membrane during phospholipid turnover.[20] Although the role of CLA in lipid metabolism is largely unknown, induction of several of these fatty acid catabolic enzymes may shed some light on how CLA modulates fat metabolism in the liver.

Peroxisome proliferators are generally classified as non-genotoxic carcinogens meaning they do not bind directly to DNA to initiate carcinogenesis. Instead, peroxisome proliferators may enhance tumorigenesis in liver, testes, and pancreas by acting as promoters resulting in enhanced cell proliferation, altered cell differentiation, and inhibition of apoptosis of initiated cells. Based on the fact that not all hepatocarcinogens are peroxisome proliferators[24] and some peroxisome proliferators (i.e., certain fatty acids like linoleate, arachidonate, and perfluorodecaenoate) have no effect or are weak promoters of liver tumorigenesis,[24,25] it is difficult to predict the role of CLA in hepatocarcinogenesis. To begin to determine whether CLA induction of peroxisome proliferation is associated with enhanced risk for hepatocarcinogenesis, this study determined that incremental levels of dietary CLA were associated with enhanced ornithine decarboxylase activity, a marker of tumor promotion, and cell proliferation.[15] Our data suggest that CLA will modulate liver tumor promotion like a

prototypical peroxisome proliferator; however, studies are still needed in this area.

Because CLA has several physiological (i.e., reduced body weights and enhanced hepatic lipid content[6]) and structural similarities to the fatty acid-like class of chemicals known to be peroxisome proliferators, the fact that CLA acts as a peroxisome proliferator is intriguing for two important reasons: First, CLA induction of peroxisome proliferation suggests that chemoprotection by CLA in extrahepatic tissues may occur at the expense of enhanced hepatocarcinogenesis; and second, if CLA induction of peroxisome proliferation in rodent liver occurs through activation of PPARα, the biologic activity of this chemoprotective compound may occur through a tissue specific, receptor-mediated mechanism to alter fat metabolism, cell proliferation and/or differentiation and apoptosis. Activation of PPARα and modulation of each of these events would be critical links between dietary fats such as CLA, altered gene expression, and carcinogenesis.

These studies are the first to demonstrate that increasing levels of dietary CLA induce peroxisome-associated enzyme accumulation. These data suggest not only a mechanism of action of, the beneficial effects of CLA (i.e., chemoprevention in extrahepatic tissues) but also predict some negative effects of this dietary fatty acid (i.e. increased risk for liver tumor promotion). Currently, studies are in progress to determine the role of dietary CLA in the promotion of hepatocarcinogenesis in rodents and to bind to and activate hepatic PPARα. Therefore, these studies have important ramifications for those interested in using CLA as a cancer preventive dietary agent in humans.

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