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DIETARY CONJUGATED LINOLEIC ACID IN HEALTH: Physiological Effects and Mechanisms of Action[1]

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■ **Abstract** Conjugated linoleic acid (CLA) is a group of polyunsaturated fatty acids found in beef, lamb, and dairy products that exist as positional and stereo-isomers of octadecadienoate (18:2). Over the past two decades numerous health benefits have been attributed to CLA in experimental animal models including actions to reduce carcinogenesis, atherosclerosis, onset of diabetes, and body fat mass. The accumulation of CLA isomers and several elongated/desaturated and β-oxidation metabolites have been found in tissues of animals fed diets with CLA. Molecular mechanisms of action appear to include modulation of eicosanoid formation as well as regulation of the expression of genes coding for enzymes known to modulate macronutrient metabolism. This review focuses on health benefits, metabolism, and potential mechanisms of action of CLA and postulates the implications regarding dietary CLA for human health.

CONTENTS

[1]Abbreviations: CLA, conjugated linoleic acid; PPAR, peroxisome proliferator-activated receptor; ZDF, Zucker diabetic fatty (fa/fa).

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids that exist as positional and stereo-isomers of conjugated dienoic octadecadienoate (18:2). The predominant geometric isomer in foods is the c9t11-CLA isomer (36, 69) [also called "rumenic acid" (59)], followed by t7,c9-CLA, 11,13-CLA (c/t), 8,10-CLA (c/t), and the t10c12-CLA isomer (36). The three-dimensional stereo-isomeric configuration of CLA may be in combinations of cis and/or trans configurations. CLA is found in foods such as beef and lamb, as well as dairy foods derived from these ruminant sources (20, 38, 69). Synthetically prepared oils of CLA are composed of an isomeric composition somewhat different than isomers found naturally in foods. A method of preparation for synthetic CLA oil has traditionally relied on an alkaline-catalyzed reaction using linoleate as substrate. The isomeric composition of synthetic CLA oil with ~90% purity that is prepared using linoleate (18:2cis9cis12) as a substrate is: c9t11/t9c11-CLA (~42%) and t10c12-CLA (~43%), with c9c11-CLA, c10t12-CLA, t9t11/t10t12-CLA, 7,9-CLA, 8,10-CLA, and 11,13-CLA comprising minor amounts. In addition, this 90% pure CLA contains residual substrate (0.5% linoleate) plus some oleate (~5.5%) and unidentified fatty acid (4.0%). Aside from preparation, the purification of synthetic compositions of CLA oil and individual isomers warrants attention. Due to high cost and/or lack of availability, very few studies conducted in vivo have used highly purified isomers or naturally extracted CLA oil. Thus, for the most part, studies conducted in experimental animals and humans to demonstrate the physiological effects of CLA are attributable to the synthetic mixture of isomers (predominantly c9t11-CLA and t10c12-CLA) (Figure 1). Little has been done in vivo to determine the activity and mechanisms of isomers other than these two. Except where noted, the remainder of this review focuses primarily on studies using the synthetic mixture of CLA oil in vivo or purified isomers isomers in cultured cells in vitro.

HEALTH PROPERTIES OF CONJUGATED LINOLEIC ACID

Numerous physiological properties have been attributed to CLA including action as an antiadipogenic, antidiabetogenic, anticarcinogenic, and antiatherosclerotic agent (Table 1) [reviewed in (12, 13)]. In addition, CLA has effects on bone formation and the immune system as well as fatty acid and lipid metabolism and gene expression in numerous tissues (8, 12, 31, 41, 64, 85).

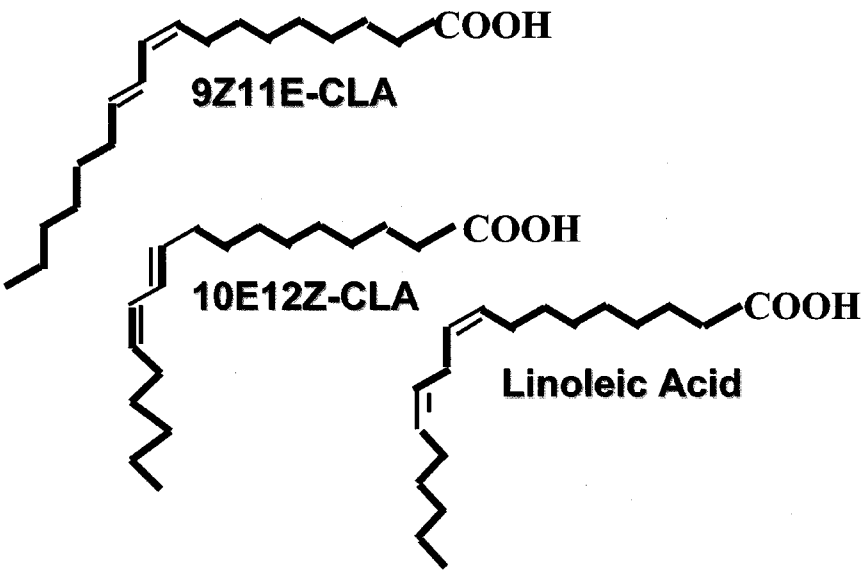

Figure 1 Structures of cis9trans11-CLA, trans10cis12-CLA and linoleic acid (18:2cis9cis12). These isomers are also referred to as c9t11-CLA or 9Z11E-CLA and t10c12-CLA or 10E12Z-CLA, respectively.

Conjugated Linoleic Acid Reduces Adipose Tissue

A plethora of data demonstrates that CLA modulates body composition, especially by reducing the accumulation of adipose tissue, in experimental animals. In mice, rats, pigs, and humans, dietary CLA reduces adipose tissue depots (29, 80, 100, 101). Early work to demonstrate the adipose-mass lowering effect of CLA was performed in growing mice where postweanling mice (6 weeks old) were fed a diet containing 1.0% CLA for 28–32 days (80). Total adipose tissue mass was reduced by over 50% compared with mice fed a control diet (without CLA). Further work demonstrated that the dietary CLA reduction of adiposity could be sustained in mice even after CLA was removed from the diet (81). Subsequent studies in nonobese mice demonstrated that some depots of fat mass [especially retroperitoneal and epididymal white adipose tissue masses (26, 108) and brown adipose tissue (108)] might be more sensitive to CLA-mediated reductions. In contrast to findings in nonobese rats, obese Zucker rats (100), but not Zucker diabetic fatty (ZDF) rats (41), exhibited an adipose-enhancing effect of dietary CLA (100). 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, increased fat accumulation in the liver, and eventual development of insulin resistance (108).

TABLE 1 Physiological properties of conjugated linoleic acid

Major function	Physiological model	References
Body composition	↓ Adiposity in chicks, mice, and rats	24, 80, 100
	↑ Adiposity in obese Zucker rats	100
	↓ Adiposity in ZDF rats	41
	↓ Adiposity is isomer specific (t10c12-CLA)	84
	↓ Adiposity in human subjects	101, 105
	↔Adiposity in human subjects	116
Diabetes	↓ Onset of diabetes in ZDF male rats	41
	Aids in the management of metabolic parameters in human subjects with type 2 diabetes	M.A. Belury, unpublished data
	↓ Insulin sensitivity in mice	108
Carcinogenesis	↓ Chemically induced mammary carcinogenesis in rats	46
	↓ Chemically induced mammary carcinogenesis in rats by either c9t11-CLA or synthetic CLA	47
	↓ Chemically induced mammary carcinogenesis in rats regardless of level of fat or esterification of CLA (in triglyceride) vs. free fatty acid	45, 52
	↓ Growth of transplantable breast cancer tumor cells in nude mice	42,109
	↓ Growth of transplantable prostate cancer tumor cells in nude mice	19
	↓ Stages of chemically induced skin tumorigenesis in mice	11, 39
	↓ Chemically induced colon carcinogenesis in rats	65
	↔ Carcinogenesis in Min mice	87
	↓ Chemically induced forestomach	40
Atherosclerosis	↓ Atherosclerotic plaque formation in hamsters	113
Bone formation	↓ Eicosanoid production	64
Immune system	↓ Eicosanoid and histamine production	104, 112
	↑ Onset of lupus in mouse model	115

↓, decreases; ↑, increases; ↔, no effect; CLA, conjugated linoleic acid; ZDF, Zucker diabetic fatty.

In addition to some indications that the effect of CLA on adiposity may be dependent on preexisting adiposity, the effects of CLA on body composition may be gender specific. Male rat pups are more responsive to dietary CLA, resulting in reduced adipose and increased muscle mass compared with female pups. In addition, there appeared to be an isomer-specific effect of CLA on adiposity: t10c12-CLA was much more effective at lowering adipose tissue mass than the c9t11-CLA isomer in mice (84). In addition, t10c12-CLA appeared to be the effective isomer for modulating gene expression in cultured 3T3-L1 preadipocytes (21). 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 (9, 26, 80). In fact, CLA reduces leptin in rats (13) and humans (M.A. Belury, unpublished data).

PUTATIVE MECHANISMS OF CONJUGATED LINOLEIC ACID REDUCTION OF ADIPOSITY Mechanisms of how CLA reduces adiposity in lean animals, and perhaps in humans, may revolve around pathways that regulate energy expenditure (111). In fact, feeding a semipurified diet containing CLA (1.0%) to male AKR/J mice for 6 weeks resulted in significantly increased metabolic rates and reduced nighttime respiratory quotients (111). When Std ddY mice were gavaged with CLA (5 ml/kg body weight), the increased oxygen consumption was associated with significantly increased oxidation of fat, but not carbohydrate (79). The cellular basis of the enhanced oxidation of lipids is not thought to require peroxisomal β-oxidation (25, 70). Because the hormones noradrenaline and adrenaline were also significantly higher in mice gavaged with CLA (79), the data suggest that CLA enhances sympathetic nervous activity that leads to increased energy metabolism and eventual reduction of adipose tissue mass.

The ability of CLA to reduce adipose tissue mass has also been linked with induction of adipocyte apoptosis and/or differentiation. Induction of apoptosis by CLA occurred in preadipocyte cultures (33). In addition, female mice fed a diet with 1.0% CLA for 8 months exhibited increased apoptosis in brown and white adipose tissues (108). The induction of apoptosis of adipose tissues was associated with induction of TNF-alpha and uncoupling protein-2. Uncoupling protein-2 is a member of the mitochondrial uncoupling protein family and functions to "uncouple" the transfer of electrons over the inner mitochondrial membrane, resulting in thermal dissipation of energy as heat in place of adenosine triphosphate. The induction of uncoupling protein-2 in muscle by CLA was also demonstrated in ZDF (fa/fa) rats (91) and therefore may be a mechanism of increased energy expenditure in mice fed CLA (110). A diet with CLA (1.0%) did not induce uncoupling protein mRNA in muscle of mice, although the same mice exhibited increased energy expenditure. Therefore, it was concluded that uncoupling protein-2 may not be a significant mediator of effects of CLA on energy utilization and adiposity.

Induction of markers of differentiation of adipose tissue by dietary CLA was first shown in vivo in male ZDF (fa/fa) rats fed 1.5% CLA for 2 weeks (41). In this study the adipocyte lipid binding protein (ap2), a marker of adipocyte differentiation (95), was increased approximately fivefold over levels in rats fed a control diet (without CLA). In vitro studies using 3T3-L1 preadipocytes demonstrate mixed effects of CLA: At least one study showed that CLA enhanced differentiation of 3T3-L1 preadipocytes (as assessed by lipid accumulation in cells) (93), whereas another study showed that CLA inhibited differentiation (17). Because of the limitations of using a programmed preadipocyte, interpreting these data and extrapolating them to an in vivo setting is difficult. In fact, it was recently shown that timing of

CLA treatment in cultured preadipocytes is critically important to determining the differential effects of CLA on inducing differentiation (applied early) or inhibiting differentiation (applied after 3 days of programmed culturing) (33).

In addition to modulating apoptosis and differentiation, CLA may reduce adipose tissue mass by minimizing accumulation of triglycerides in adipocytes. t10c12-CLA inhibits activity of lipoprotein lipase in vivo (83). Because lipoprotein lipase aids in the incorporation of fatty acids into triglycerides in adipocytes, these data suggest that the adipose-lowering effects of t10c12-CLA result from reduced uptake of fatty acids into adipocytes. In fact, triglycerides and glycerol levels were reduced in 3T3-L1 cells (33). Notably, the addition of linoleate could partially restore the content of triglycerides in cultured preadipocytes (17).

CONJUGATED LINOLEIC ACID HAS DIFFERENTIAL EFFECTS ON BODY FAT IN HUMANS In adult humans the ability of CLA to lower adipose tissue mass has been demonstrated in some (15, 101, 105) but not all (72, 116) studies. For example, when overweight or obese human subjects were supplemented with CLA (3.4–6.0 g/day) for 12 weeks, a significant reduction of fat mass was observed (15). However, in people consuming 3.0 g/day for 12 weeks, no benefit was observed for body weight or adiposity (116). More recent studies have demonstrated that CLA supplementation reduces body weight, leptin, and body adiposity in people [(101, 105); M.A. Belury, unpublished data]. It is likely that dose, duration (short- or long-term), and the isomeric composition of CLA each impact the ability of CLA to affect obesity in humans. In addition, how strain/species-, age-, and sex-specific effects of various isomers of CLA affect adipose tissue accumulation, either in obese humans or those seeking to prevent adipose gain, is yet to be determined.

Conjugated Linoleic Acid Modulates Metabolic Parameters of Type 2 Diabetes

There are numerous risk factors for the development of type 2 diabetes including the presence of impaired glucose tolerance, ethnicity, age, gender, and genetics. Central to all of these risk factors is obesity (27). Based on the fact that CLA reduces adiposity in experimental animals, we designed a study to elucidate the role of CLA in the development of type 2 diabetes in male ZDF (fa/fa) rats. Male ZDF rats were fed semipurified diets containing no CLA (control), 1.5% CLA, or the antidiabetic thiazolidinedione drug, troglitazone (0.02%) for two weeks. Rats fed the CLA or thiazolidinedione diet exhibited significantly reduced ($p < 0.05$) fasting glucose, insulinemia (41), triglyceridemia, free fatty acid levels, and leptinemia (13) compared with control rats. In addition, dietary CLA induced aP2 mRNA, a marker of adipose differentiation, in vivo (41). A recent study using a similar protocol, but with various sources of CLA, demonstrated that a mixture of CLA isomers induces adipose-lowering effects in ZDF rats and enhances glucose uptake into muscle of ZDF rats (91). In contrast, butter enriched with c9t11-CLA

exerted little, if any, ability to reduce glucose tolerance, lower adipose tissue, or modulate glucose uptake into muscle. The authors (91) speculated that the t10c12-CLA isomer may be the biologically active isomer in delaying the onset of diabetes reported earlier (41). However, the role of specific CLA isomers in delaying the onset and/or reducing the severity of type 2 diabetes is yet to be measured directly.

Whereas CLA reduces fasting insulin in diabetic animals, it modestly increases fasting serum insulin in nondiabetic swine (103), mice (108), and humans (71). Because fasting insulin may be used as a surrogate marker for insulin resistance, these data suggest that CLA reduces insulin sensitivity under a normoglycemic state. In agreement, after long-term feeding (8 months) of a CLA-diet, an induction of insulin resistance was observed in C57Bl/6J male mice (108). CLA-induced insulin resistance was associated with lipodystrophy. The impact and significance of CLA for reducing insulin sensitivity and/or altering lipodystrophy for people who are normoglycemic is unknown.

Because CLA was able to delay the onset of diabetes in the ZDF rat model, CLA as an aid in the management of type 2 diabetes in humans was examined (M.A. Belury, unpublished data). A double blind, randomized study to determine the effect of daily supplementation with CLA or placebo (safflower oil) on metabolic parameters of diabetes was conducted. Subjects with type 2 diabetes were provided with supplements with CLA or placebo, instructed to maintain a healthy diet using the Food Guide Pyramid, and asked not to change their diet or activity habits for the 8-week intervention period. CLA supplementation (6.0 g CLA/day) significantly decreased fasting blood glucose, plasma leptin, body mass index, and weight. Low density lipoprotein levels significantly increased, but less in the CLA-supplemented group than in the placebo group. In addition, body fat (%) was modestly decreased ($p < 0.08$) in subjects supplemented with CLA. Fasting insulin, HbA_{1c}, triglycerides, cholesterol, and high density lipoprotein were not significantly affected by CLA. According to 3-day diet records, energy intake was not significantly different between groups at baseline or throughout the study. We concluded that supplementation with CLA for 8 weeks could be associated with favorable alterations of several metabolic parameters of subjects with type 2 diabetes. Further work is needed to determine the therapeutic potential of CLA in the management of type 2 diabetes.

Dietary Conjugated Linoleic Acid Inhibits Carcinogenesis

Dietary CLA inhibits numerous cancer models in experimental animals. In particular, it inhibits skin tumor initiation and forestomach neoplasia (39, 40). In addition, the synthetic mixture of CLA isomers inhibits chemically induced skin tumor promotion as well as mammary and colon tumorigenesis when added to semisynthetic diets (11, 46, 65). Importantly, the inhibitory effect of CLA on mammary carcinogenesis is independent of type or level of fat in the diet and occurs in a dose-dependent manner (45, 46). When transplanted into nude mice, growth of mammary (109) or prostate (19) cancer cell lines was significantly reduced if animals were fed a diet with CLA (1.0%). The inhibition of chemically induced

mammary carcinogenesis occurred whether CLA was fed as a free fatty acid or triglyceride (52). Furthermore, the 9,11-CLA and 10,12-CLA isomers appear to be equally active in inhibiting mammary carcinogenesis in rats (54).

Although the inhibitory role of CLA is convincing, not all studies consistently demonstrate that CLA inhibits carcinogenesis. In fact, CLA was unable to alter the growth of transplanted prostate (96) and breast (114) cancer cells in some studies and did not reduce tumorigenesis in an intestinal model of colon carcinogenesis using the Apc Min mouse model (87). No studies report that CLA enhances tumorigenesis. In contrast to effects of CLA on carcinogenesis, the n-6 and n3 fatty acids, linoleate (18:2c9c12) and eicosapentaenoate (20:5n3), have differential effects (from no effects to potent enhancing or inhibitory effects) depending on the tumor model and tissue studied [reviewed in (34)]. Therefore, the ability of CLA to inhibit multiple models of carcinogenesis appears to be specific for this group of fatty acids. Furthermore, some of the mechanisms and functions of CLA are likely to be unique among polyunsaturated fatty acids [reviewed in (7, 43); (51)].

PROPOSED MECHANISMS OF INHIBITION OF CARCINOGENESIS BY CONJUGATED LINOLEIC ACID Efforts have been made to elucidate the mechanistic role of CLA in modulating carcinogenesis by determining the effects on the stages of carcinogenesis known as initiation, promotion, and progression [reviewed in (12)]. In fact, the anticarcinogenic property of CLA was first identified during the initiation stage of the mouse skin multistage carcinogenesis model (39), where the stages of initiation, promotion, and progression are operationally separable (28). In this initial study a lipid fraction extracted from fried ground beef was topically applied to mouse skin prior to initiation of the carcinogen, 7,12-dimethylbenz(a)anthracene. Tumor yield (average number of tumors per mouse) after 16 weeks of promotion with phorbol ester was inhibited by approximately 45%. In a manner independent from its antiinitiator activity, CLA was then shown to inhibit tumor promotion (11). Mice were fed semipurified diets containing various levels of synthetically prepared CLA (5% corn oil plus 0%, 0.5%, 1.0%, or 1.5% CLA) after initiation and for the duration of promotion with the phorbol ester, 12-0-tetradecanoylphorbol-13-acetate (35 weeks). Mice fed 1.5% CLA exhibited an inhibition of tumor yield of ~30% compared with mice fed control diets (containing no CLA). In chemically induced rat mammary carcinogenesis the stages of initiation and promotion are not readily separable. Nevertheless, when fed before or after carcinogen treatment (postinitiation), dietary CLA inhibited carcinogenesis as well (45, 52).

Whereas a great deal of evidence demonstrates that dietary CLA inhibits the initiation and promotion stages of carcinogenesis, the role of CLA in the progression stage of carcinogenesis has not been comprehensively addressed. In transplantable tumor models, dietary CLA reduced the growth rates of cells when implanted in vivo (19, 109). In addition, at least one study demonstrated that CLA (0.5–1.0%) inhibited the growth of transplanted mammary cancer cells to form secondary tumors in mice (42). Furthermore, the CLA-responsive chemically induced mammary carcinogenesis model (46) is a model for human breast cancer ductal carcinomas in

situ. Therefore, data showing that CLA inhibits tumorigenesis of this model are consistent with the possibility that CLA reduces metastasis resulting from breast cancer. However, no studies have addressed the role of CLA in the prevention of metastatic cancer. It is critical to understand how CLA modulates malignant tumor formation and metastasis because the growth of secondary tumors is the major cause of morbidity and mortality in people with cancer.

CONJUGATED LINOLEIC ACID MODULATES NUMEROUS EVENTS DURING TUMOR PROMOTION In order to elucidate the anticarcinogenic mechanisms of CLA, early work focused on events associated with initiation. As an antiinitiator, CLA may modulate events such as free radical–induced oxidation, carcinogen metabolism, and/or carcinogen-DNA adduct formation in some tumor models [reviewed in (7)]. In recent years, attention has focused on elucidating the mechanisms of action of CLA that inhibit carcinogenesis during promotion, particularly in the mammary and skin carcinogenesis models (52). The promotion stage involves the clonal expansion of initiated cells to form a benign tumor. This stage of carcinogenesis represents a premalignant state in which tumors arise from cells that have increased cell proliferation, reduced programmed cell death (or apoptosis), and/or dysregulated differentiation. In cultured cells, CLA reduced proliferation of mammary tumor cells in vitro (30, 99) and in vivo (53). In cultured mammary epithelial cell organoids, CLA (64 μM) or c9t11-CLA (128 μM) inhibited cell growth (48). In vivo, rats that were initiated with methylnitrosourea and then fed a diet with CLA (1.0%) exhibited reduced proliferation of terminal end bud and lobuloalveolar bud structures of mammary epithelium (using histochemical analyses of bromodeoxyuridine staining) (106). Importantly, the terminal end bud is the site of tumor formation for both rat and human breast cancer. The inhibition of proliferation by CLA was accompanied by a reduction in density of the terminal end bud (106). More recently, the reduction of cell proliferation in terminal end bud structures by dietary CLA was accompanied by reduced levels of two cyclins known to regulate the cell cycle, cyclin D1 and cyclin A (47). These data suggest that CLA modulates molecular signaling events that impact the cell cycle, ultimately regulating cell proliferation.

Studies to determine the role of CLA in modulating cell proliferation in models of carcinogenesis other than mammary carcinogenesis have been elusive. In contrast to findings in mammary carcinogenesis, there was no relationship between dietary CLA and markers of cell proliferation of mouse epidermis (hyperplasia, ornithine decarboxylase activity, or c-myc mRNA expression) (56). These data suggest that inhibition of skin tumor promotion by CLA may not occur through inhibition of cell proliferation of mouse epidermis. In rat liver, increasing levels of dietary CLA (0.5–1.5%) increased cell proliferation in diethylnitrosamine-induced focal lesions (68), demonstrating that the ability of CLA to reduce cell proliferation may be tissue-specific and/or tumor model–specific. In other models of carcinogenesis, the mechanistic role of CLA in modulating cell proliferation has not been identified.

As a counterbalancing event in promotion, apoptosis offers protection to carcinogenesis via programmed death of cancer cells. Dietary CLA induced apoptosis in numerous tissues including mammary (48), liver (68), and adipose (108) tissues and in cultured mammary epithelial cells (50). In mammary tissue initiated with methylnitrosourea, dietary CLA induced apoptosis of cells in the terminal end bud and in premalignant lesions known as intraductal proliferation lesions (48). In these studies, CLA induction of apoptosis was associated with a reduction of bcl-2, a signaling protein known to suppress apoptosis. These data suggest that CLA may inhibit promotion by inducing signaling events leading to enhanced apoptosis.

CLA induces markers of differentiation in the noncancer model, adipose tissue (41, 93). Therefore, it is possible that CLA inhibits carcinogenesis via induction of differentiation. In fact, the finding that CLA fed during the time of mammary gland development and maturation has long-lasting protective effects on mammary carcinogenesis (52, 106) suggests that the role of CLA in protecting against mammary carcinogenesis may be, in part, by modulating tissue differentiation.

Dietary Conjugated Linoleic Acid Modulates Atherosclerotic Plaque Formation

There is a growing body of evidence that CLA reduces atherosclerotic plaque formation in experimental animals. When CLA (0.5 g/rabbit/day) was added to a hypercholesterolemic diet and fed to rabbits for 12 weeks, serum triglycerides and low density lipoprotein cholesterol levels were significantly reduced compared with rabbits fed a diet without CLA (60). Importantly for heart disease risk, aortas of rabbits fed the CLA-containing diet exhibited less atherosclerotic plaque formation. In a subsequent study, hamsters were fed a diet with or without CLA designed to induce hypercholesterolemia (76). The diet with CLA (1.0%) reduced plasma total cholesterol, non–high density lipoprotein-cholesterol, and early aortic atherosclerosis relative to a diet with linoleate (113). In a similar model in hamsters fed a hypercholesterolemic diet, c9t11-CLA, the sole CLA isomer in the diet, had no effect on plasma lipids (37). Because dietary CLA was associated with significantly reduced formation of dienes, it was concluded that the ability of CLA to reduce aortic plaque formation could be due to changes in low density lipoprotein oxidative susceptibility. In contrast to protective effects of CLA on atherosclerotic plaque formation in rabbits and hamsters, CLA induced the formation of aortic fatty streaks in C57Bl/6 mice fed an atherogenic diet (75).

The effects of CLA on thrombotic properties of blood cells have been studied in cultured platelets in vitro and in human subjects. In cultured platelets, CLA, c9t11-CLA, and t10c12-CLA inhibited collagen- or arachidonate-induced platelet aggregation (107). These findings were associated with reduced production of the proaggregatory cyclooxygenase products of ^{14}C-arachidonate, ^{14}C-thomboxane-A2, and ^{14}C-thromboxane-B2. In human subjects supplemented with CLA (3.9 g/day) or placebo (sunflower oil) for 93 days, there were no differences in platelet aggregation or prothrombin time (14).

Because CLA appears to exert differential effects on lipid profiles as well as atherogenic markers in various animal models, further work is needed to demonstrate the mechanisms of CLA for the prevention of atherosclerosis and its role in reducing cardiovascular disease risk in humans.

Other Health Properties of Conjugated Linoleic Acid

As a group of fatty acids, CLA displays numerous benefits in experimental animals. In addition to reducing the onset and/or severity of carcinogenesis, obesity, diabetes, and atherosclerotic plaque formation, CLA may affect the rate of bone formation in rats (63). However, combined with an omega-3-rich menhaden oil, dietary CLA exerted no further beneficial effects on bone formation. In support of a positive role of CLA in bone formation, rat pups exposed to CLA (0.5%), either in utero or during the first 7 days of life, had significantly longer tail lengths (a measure of skeletal growth) compared with pups fed a diet without CLA (88).

CLA modulates several events in immunity that may revolve around modulating eicosanoid formation. Arachidonate-derived eicosanoids, derived through cyclooxygenase and lipoxygenase pathways, are produced by numerous types of immune cells and are thought to regulate cytokine synthesis and inflammation. Initial studies demonstrated that CLA protects against *Escherichia coli*-induced weight loss in chicks and mice (23) and reduces histamine-induced prostaglandin-E2 production in Guinea pig trachea (112). However, in a model of the autoimmune disorder, lupus erythematosis, dietary CLA accelerated the onset of proteinuria (115). In this same study, CLA was protective against the development of end-stage symptoms of lupus. These data suggest CLA may have differential effects on disorders involving immunity since CLA exacerbates early stage, but delays late stage, symptoms of lupus.

In rats assigned to a diet with CLA (1.0%), levels of leukotriene-B4 and leukotriene-C4 in spleen and lung were reduced (104). The inhibition of leukotriene levels was associated with significantly reduced non-stimulated histamine. However, in humans supplemented with CLA (3.9 g/day) for 93 days, no apparent alterations in eicosanoids (e.g., prostaglandin E-2, leukotriene B-4) or cytokines (e.g., interleukin-1 beta or tumor necrosis factor alpha) were observed (57). The discrepancy of data between different models of immune function and between animal and human models argues that further study of the role of CLA in immunity is needed. In particular, it is important to determine the modulation of various immune disorders, especially autoimmunity and immunodeficiency by individual isomers, dosages, and duration of dietary CLA.

METABOLISM OF CONJUGATED LINOLEIC ACID

It is well established that when provided in the diet or as a supplemental oil, CLA isomers accumulate in tissues of animals and humans [reviewed in (7); (55)]. In addition, isomers of CLA are readily metabolized in vivo via multiple

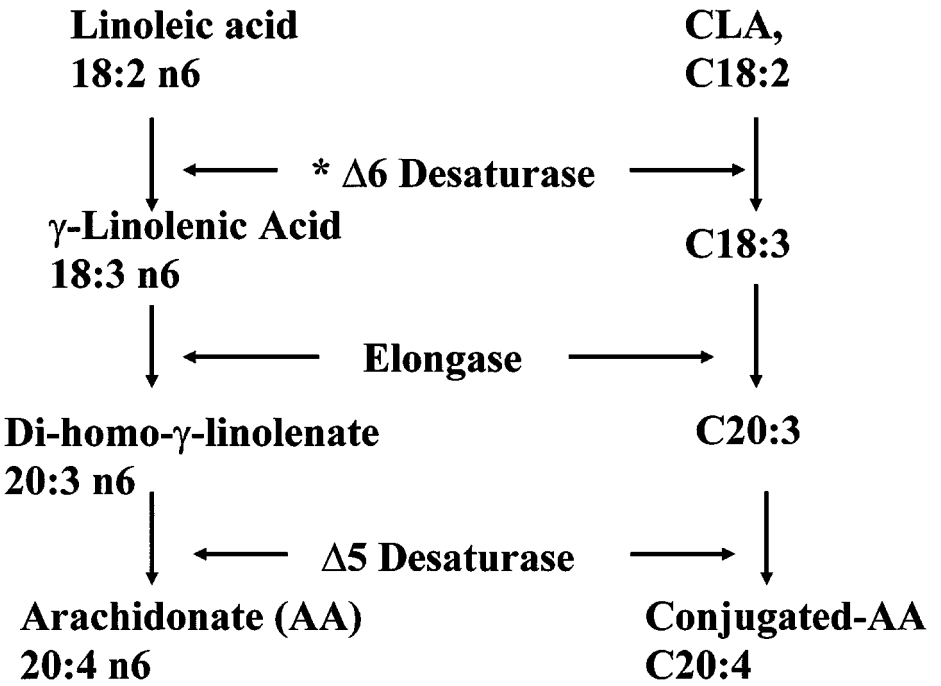

Figure 2 Pathway for desaturation and elongation of CLA. *Δ6 desaturase, rate limiting step. AA, arachidonate.

metabolic pathways. Elongated and desaturated metabolites of CLA (Figure 2) (e.g., conjugated-18:3, conjugated-20:3, and conjugated-20:4) have been identified in the liver (3, 4, 49) and mammary tissue (2) of rats and adipose tissue and sera of humans (1; M.A. Belury, unpublished data) (Table 2). In fact, ^{14}C-CLA is metabolized to the same extent (to form ^{14}C-conjugated-18:3) as ^{14}C-linoleic acid when compared in an enzymatic assay using an hepatic isolate of Δ6 desaturase enzyme (8). In addition to forming desaturase and elongase products, CLA is readily oxidized to β-oxidation products (16:1 and 16:2), presumably from peroxisomal β-oxidation of downstream elongated/desaturated metabolites of CLA (1, 49).

The role of metabolites of CLA to modulate tissue responses such as adipose tissue mass, glucose sensitivity and/or carcinogenesis is pending further investigation. The biological activities of CLA metabolites are hampered by the lack of availability of purified metabolites (e.g., conjugated gamma-linolenate, 18:3; conjugated eicosatrienoate, 20:3; and conjugated eicosatetraenoate, 20:4) for use in cell culture and in vivo feeding experiments. As an alternative approach, a 19-carbon conjugated fatty acid, conjugated nonadecadienoate (19:2), which is assumed to be metabolized to different downstream products of CLA, was fed to

TABLE 2 CLA isomers and metabolites

Isomers in foods[a]	Δ6 Desaturase	Conjugated anabolites formed elongase	Δ5 Desaturase		
c9t11-CLA	c6c9t11-octadecatrienoate (18:3)	c8c11t13-eicosatrienoate (20:3)	c5c8c11t13- eicosatetraenoate (20:4)		
	—	c8c11t13-eicosatrienoate (20:3)		Rat Liver Rat Mammary 2 Human Plasma Human Serum	1, 3, 97; M. Belury and S. Banni, Unpublished data
t7,c9-CLA	N.D.[b]	N.D.	N.D.	—	
11,13-CLA (c/t)	N.D.	N.D.	N.D.	—	
8,10-CLA (c/t)	N.D.	N.D.	N.D.	—	
t10c12-CLA	c6t10c12-octadecatrienoate (18:3)	c8t12c14-eicosatrienoate (20:3)	c5c8c11t13-eicosatetraenoate (20:4)	Rat Liver Rat Mammary 2 Human Plasma Human Serum	1, 3, 97; M. Belury and S. Banni, Unpublished data
	—	t12c14-eicosadienoate (20:2)			

[a]Listed in order of prevalence in most foods (36).

[b]N.D., not determined.

mice (0.3% of diet) (83). Mice fed nonadecadienoate exhibited significantly lower adipose tissue accumulation (by ~81%) compared with mice fed a control diet. In contrast, mice fed a diet with CLA exhibited a 25% reduction in adipose tissue. In 3T3-Li preadipocytes conjugated nonadecadienoate and CLA had similar efficacy on reducing heparin-releasable lipoprotein lipase and lipid accumulation (17). Because of the difference in hydrocarbon chain length between CLA and conjugated nonadecadienoate, it is likely that metabolites, including those from Δ6 desaturase, are different. Therefore, the authors concluded that the Δ6 desaturase metabolites of CLA may not be important for the alterations in gene expression induced by CLA. It is likely that the biological activities of some metabolites may overlap with biological properties of the parent conjugated octadecadienoates (18:2), whereas others may not.

CONJUGATED LINOLEIC ACID MODULATES LIPID METABOLISM

Conjugated Linoleic Acid Modulates Fatty Acid Composition of Phospholipids and Alters Eicosanoid Formation

Like most other dietary polyunsaturated fatty acids, isomers and metabolites of CLA are readily incorporated into phospholipid and neutral lipid fractions of numerous tissues (8, 40, 45, 46, 73). In some studies incorporation of CLA into phospholipids of cultured cells occurs in a manner that is similar to linoleate (66 , 74). When radiolabeled tracers were used to study the kinetics of ^{14}C-CLA uptake into keratinocytes or hepatoma cells, ^{14}C-CLA was incorporated to the same extent and at a similar rate as ^{14}C-linoleate. In addition, the level of incorporation of ^{14}C-CLA and ^{14}C-linoleate into epidermal phospholipid and neutral lipid fractions was similar (66). However, a recent study of rats fed a diet containing CLA-rich butter (and linoleate) showed that accumulation of CLA and linoleate into rat liver is not similar (3). In fact, CLA preferentially accumulated in neutral lipids (~79%) with less incorporation into phosphatydilcholine (~10%), the major phospholipid of liver cells. In contrast, linoleate accumulated preferentially in phosphatydilcholine (~50%), with less in neutral lipids (~17%).

Of the two main isomers studied, c9t11-CLA accumulates to a higher extent than t10c12-CLA in tissue phospholipids of liver (3, 8), skin (56), and bone (64) of experimental animals. Furthermore, in neutral lipids of mammary (44) and muscle (31), c9t11-CLA accumulates to a greater extent than the t1012-CLA. The higher level of c9t11-CLA may be due to either preferred incorporation into tissues and/or more rapid metabolism of the t10c12-CLA isomer. In support of the latter, we have found that human subjects supplemented with CLA (6.0 g/day; ~37% c9t11-CLA and ~39% t10c12-CLA) for 8 weeks show significantly higher accumulation of c6t10c12-CLA, the Δ6 desaturase metabolite of t10c12-CLA compared with the amount of metabolite formed from c9t11-CLA (e.g., c6c9t11-CLA) in serum (M.A. Belury & S. Banni, unpublished data).

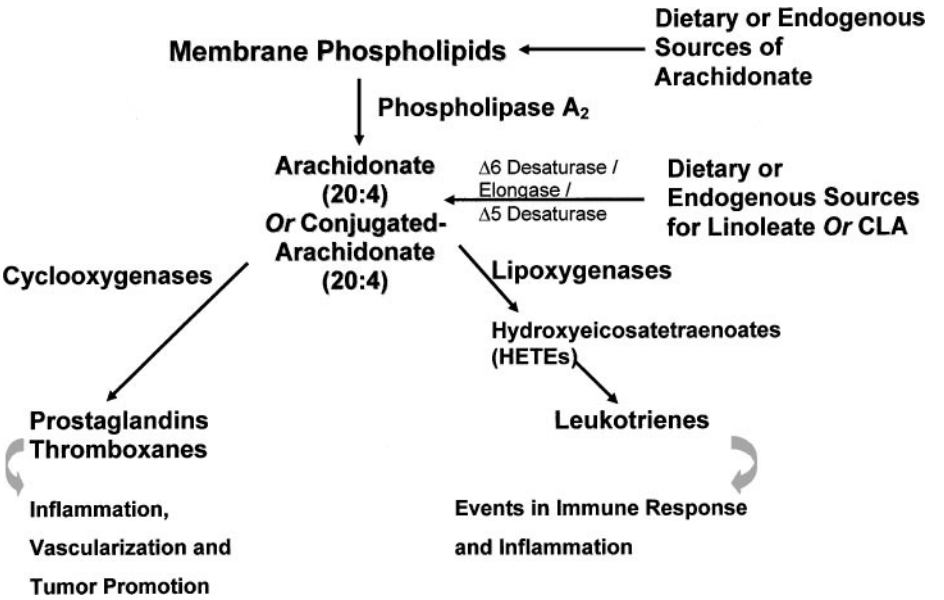

Figure 3 General schematic pathway for eicosanoid synthesis from arachidonate.

Based on findings from a number of laboratories, it is likely that one mechanism for the ability of CLA to exert many of its physiological functions (e.g., carcinogenesis, diabetes, obesity, immunity, bone formation, and platelet aggregation) is by modulating the accumulation of arachidonate in phospholipids, resulting in a reduced arachidonate pool and reduced production of downstream eicosanoid products (Figure 3). In fact, the role of CLA to reduce cyclooxygenase products (e.g., prostaglandin-E2, prostaglandin F2alpha) has been demonstrated in vivo in bone and macrophages (64, 104) but not small intestine tissue from Min mice (87) or spleen from rats (104). In addition, we have found that phorbol-ester-induced prostaglandin-E2 is reduced in the epidermis of mice fed CLA (56) and in cultured keratinocytes in vitro (67). Furthermore, dietary CLA reduced accumulation of the lipoxygenase products leukotriene-B4 and leukotriene-C4 in spleen and lung (104) but not ^{14}C-hydroxyeicosatetraenoic acid (^{14}C-12-HETE) in cultured human platelets (107). It appears that CLA modulation of eicosanoid production is tissue specific.

The mechanism of how CLA reduces arachidonate-derived eicosanoids such as prostaglandin E2, prostaglandin F2alpha, leukotriene-B4, and leukotriene-C4 has been explained by at least three theories. First, it is theorized that CLA displaces arachidonate in phospholipids. In cultured keratinocytes, CLA reduced incorporation of ^{14}C-arachidonate (67). In addition, dietary CLA displaced the arachidonate precursor, linoleate, in a dose-responsive manner in livers of mice fed various doses of CLA (0–1.5%) in some studies (9, 73) but not others [(2, 64); M.S. Belury,

unpublished data]. Importantly, only one study has shown that dietary CLA, rumenic acid, or t10c12-CLA reduces phospholipid-associated arachidonate in liver (49). In contrast, the remaining studies have not found that phospholipid-associated arachidonate levels are altered significantly after feeding CLA.

A second explanation for the reduction of arachidonate-derived eicosanoids by CLA is through inhibition of the constitutive enzyme, cyclooxygenase-1, and/or the inducible form, cyclooxygenase-2, at the level of mRNA, protein, or activity. An in vitro activity assay showed that CLA or individual isomers inhibited the rate of oxygenation of arachidonate in the presence of cyclooxygenase-1 (18). However, whether CLA reduces the expression of cyclooxygenase (either the constitutive form, cyclooxygenase-1, or the inducible cyclooxygenase-2) is yet to be determined.

A third theory raises the possibility that CLA or elongated and desaturated products from CLA (e.g., conjugated arachidonate) may act as substrates or antagonists for cyclooxygenase, thereby reducing available enzyme for arachidonate. It seems unlikely that conjugated-eicosatetraenoate (20:4; c5c8c11t13) can act as a substrate for cyclooxygenase because cyclooxygenase requires the 1,4 methylene interruption be farther from the carboxyl end for efficient electron abstraction by prostaglandin synthase. More likely, CLA may act antagonistically to inhibit the activity of cylooxygenase. The antagonistic property of CLA in vivo may also be regulated by the formation and accumulation of the arachidonate analogue of CLA, conjugated-eicosatetraenaote (24:4) in phospholipids. The ability of downstream metabolites of CLA to interfere with cyclooxygenase activity and/or eicosanoid production deserves further attention.

Conjugated Linoleic Acid Modulates Lipid Metabolism and Gene Expression

Until recently, the influence of CLA on lipid metabolism and gene expression in the liver and extrahepatic tissues was largely unknown. Dietary CLA alters the levels of other (nonconjugated) 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 (85) and rats (3, 64, 73, 100). However, the ability of CLA to lower monounsaturated fatty acids seems to be somewhat specific for the strain and/or species of animal: We found that the monounsaturate-lowering effects of dietary CLA were more pronounced in male ZDF rats than lean (nondiabetic) littermates (M.A. Belury, unpublished data). In addition, in SENCAR mice a significant and dose-dependent increase of hepatic levels of oleic acid was observed in mice fed increasing doses of CLA (0.5–1.5%) for 6 weeks (8). The differential effects of CLA to lower monounsaturated fatty acid levels in the liver may be due to differences in isomeric compositions or dosages of CLA oils in the diet, duration of feeding, sources of non-CLA dietary fat, and/or species-, strain-, and/or metabolic status of animals (e.g., lean, diabetic).

It is possible that the altered levels of monounsaturated fatty acids such as palmitoleate and oleate may result from displacement of monounsaturated fatty acids by CLA, because CLA appears to be incorporated into similar lipid fractions as oleic acid in some studies (3, 9). In addition, CLA may alter enzymatic pathways responsible for altering fatty acid composition of lipid fractions. In fact, CLA has been shown to reduce the $\Delta 9$ desaturase index in mouse liver (61) and in cultured preadipocytes (21). In 3T3-LI preadipocytes it appears that t10c12-CLA is the most potent isomer for reducing stearoyl-CoA desaturase activity (85). The reduction of monounsaturated products of $\Delta 9$ desaturase (also called stearoyl-CoA desaturase) was at the level of reduced mRNA for stearoyl-CoA desaturase-1 regulation in liver (61). In contrast, t10c12-CLA reduction of stearoyl-CoA desaturase in cultured hepatic (HepG2) cells was not found to be associated with reduced stearoyl-CoA desaturase mRNA (22). Instead, it was proposed that CLA reduced the activity of stearoyl-CoA desaturase in cultured hepatic HepG2 cells by posttranslational modification of the protein. The finding that CLA regulates the activity of the enzyme, stearoyl-CoA desaturase, at multiple levels dependent, in part, on cell type suggests that CLA regulation of lipid metabolism and gene expression occurs through multiple signaling pathways.

CLA is readily metabolized by $\Delta 6$ desaturase to form numerous downstream products, but less is known about how CLA modulates metabolism of nonconjugated fatty acids via enzymatic systems such as $\Delta 6$ desaturase-elongase-$\Delta 5$ desaturase (Figure 3). The ability of CLA to alter levels of linoleate (18:2) and its desaturated and elongated product, arachidonate (20:4), has been observed in neutral lipid fractions of several tissues. Dietary CLA reduces arachidonate levels of mammary tissue (2), liver (8), and inguinal fat pads (88). In contrast, other studies have shown that CLA may have a modest enhancing effect on levels of neutral lipid-associated arachidonate in liver of rats (3) or epidermis of mice (56). Yet other studies show no effect of CLA on arachidonate levels of neutral lipids in fat pads (100), bone (63), liver (73), or small intestine (87). It appears that the ability of CLA to alter arachidonate levels is tissue and perhaps species dependent. Furthermore, the relevance of altered arachidonate levels in neutral lipids to modulate lipid metabolism and eicosanoid formation is not clear at the present time.

The role of CLA in modulating hepatic lipid metabolism is associated with modulating fatty acid composition. In addition, CLA appears to induce lipid accumulation of the liver in studies in which animals are fed longer than 6 weeks (8, 108) but not in shorter-term studies (2 weeks or less) (92). In fact, lipid accumulation occurs in a dose-dependent manner in female SENCAR mice fed diets with CLA (0.5–1.5%) for 6 weeks (8). Furthermore, we found that CLA-induced lipid accumulation is related to alterations in the expression of genes known to modulate lipid metabolism (9). In particular, we propose that 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) (Figure 4). In the liver, PPARα is a critical transcription factor

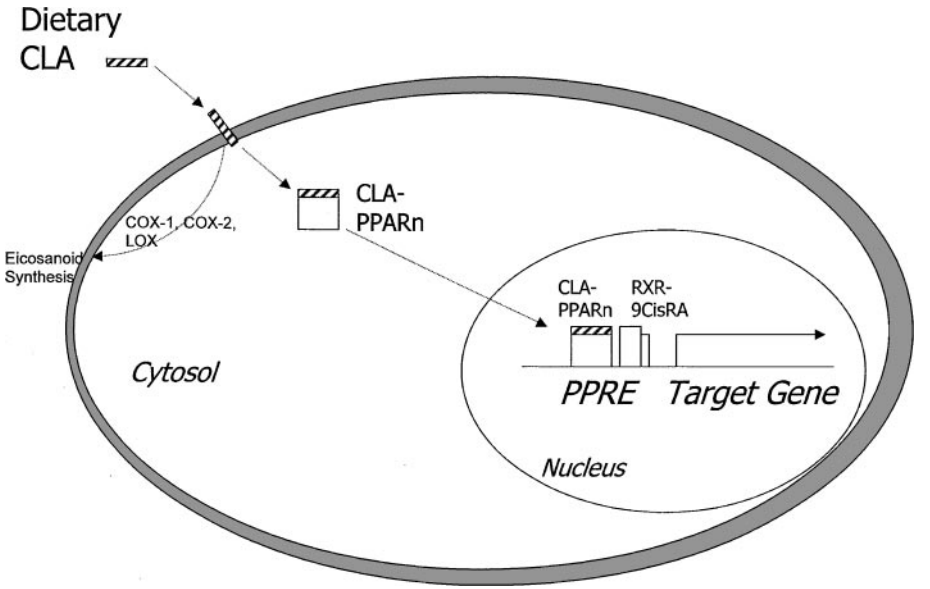

Figure 4 Schematic diagram of putative cellular and molecular mechanisms of CLA in modulating systemic conditions such as carcinogenesis, adiposity, diabetes, and cardiovascular disease.

for lipid metabolism, because several genes coding for enzymes involved with β-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, cytochromep 4504A, hepatic lipoprotein lipase, and others) (95). In fact, several isomers of CLA are high-affinity ligands and activators of PPARα (74).

Because synthetic ligands for PPARα (also known as peroxisome proliferators) act as nongenotoxic carcinogens in rodent liver (89), we determined the ability of CLA to induce peroxisome proliferation and mediate apoptosis and cell proliferation in diethylnitrosamine-induced hepatic tumor promotion in rat liver. When fed to male or female Sprague-Dawley rats for up to 6 weeks, dietary CLA (0.5–1.5%) had no effects on peroxisome proliferation (73). In contrast, the prototypical peroxisome proliferator, [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid (Wy-14,643) induced significantly more peroxisomal area in livers (as indicated by increases in both peroxisome size and number). In this same study both dietary CLA and WY-14,643 induced PPAR-responsive genes. In F344 rats initiated with diethylnitrosamine dietary CLA was associated with increased cell proliferation and apoptosis in precancerous foci areas of rat liver (68). In contrast, Wy-14,643 induced cell proliferation without inducing apoptosis. The data suggest that CLA does not act as a typical synthetic ligand for PPARα to support hepatic

tumor promotion in rats. However, the effects of CLA on hepatic tumor promotion is yet to be determined.

Whereas evidence from our laboratory suggests that PPARα plays a role in the ability of CLA to modulate lipid metabolism, recent data using the PPARα-null mouse suggest that PPARα may not be a pivotal transcription factor for the adipose-tissue lowering effect of CLA (86). In these studies, diets with (0.5%) or without CLA were fed to PPARα null mice or wild-type for 4 weeks. Compared with wild-type (expressing PPARα), PPARα null mice (not expressing PPARα but expressing PPARβ/PPARδ and PPARγ) fed a diet with CLA had similar responses: Dietary CLA reduced adipose tissue and induced some PPAR-responsive genes in liver. It is possible that CLA modulates lipid metabolism via PPAR-independent mechanisms and/or mechanisms involving other isoforms of PPAR such as PPARβ/PPARδ and PPARγ.

The PPARγ isoform is found in extrahepatic tissues such as adipose, prostate, colon, mammary gland, and others. It has been well established that PPARγ2 is a required transcription factor in adipose tissue differentiation (77). In addition, it is known that thiazolidinediones are high-affinity ligands for PPARγ. It is therefore likely that thiazolidinediones exert their antidiabetic actions via activation of this receptor (62). In terms of affinity for PPARγ, isomers of CLA have moderate affinity for binding to and activating PPARγ (10). However, dietary CLA appears to modulate transcription of genes responsive to PPARγ in adipose tissue (41, 91) in vivo. Our initial attempts to elucidate the ability of CLA to activate PPARγ have focused on downstream metabolites of Δ6 desaturase metabolism of c9t11-CLA or t10c12-CLA. We utilized approaches to block desaturase activity to determine whether reducing metabolites will alter activation of PPARγ (10). CV-1 cells transiently transfected with murine PPARγ, luciferase–peroxisome proliferator–responsive element reporter and β-galactosidase were treated with c9t11-CLA or t10c12-CLA and the activation of PPARγ was measured. Blocking Δ6 desaturase with the synthetic inhibitor SC-26196 (78) significantly reduced the ability of CLA isomers to activate PPARγ ($P < 0.05$). These data indirectly suggest that activation of PPARγ by CLA is increased by the formation of the Δ6-desaturated products from CLA, c6c9t11-CLA, or c6t10c12-CLA. However, the activation of PPARγ by these products is yet to be measured directly.

In addition to evidence showing that CLA may induce PPARγ-responsive genes in vivo, CLA may induce the level of PPARγ itself (33). Because PPARγ2 is thought to be one of several transcription factors required for adipose tissue differentiation (77) and new evidence suggests that activators of PPARγ are protective against cancers arising in the mammary gland, colon, and prostate [reviewed in (102)], it is possible that some of the molecular mechanisms of action of CLA on obesity, diabetes, and carcinogenesis are mediated by PPARγ. Perhaps the ability of PPARγ to mediate effects of CLA occurs through increased levels of PPARγ protein (21) and/or through activation of PPARγ by downstream metabolites of CLA (e.g., desaturase and elongase products) (10).

SUMMARY: POTENTIAL ROLE OF DIETARY CONJUGATED LINOLEIC ACID IN HUMAN HEALTH

Some show that CLA-rich dairy products are associated with reduced breast cancer risk, whereas others show either no effects or even enhanced risk [reviewed in (58)]. It is estimated that the level of CLA consumed by a healthy population in the northwestern region of the United States is ∼150 mg/day for women and ∼200 mg/day for men (90). Based on food duplication data, most of the CLA consumed was rumenic acid (c9t11-CLA). In Canada, estimates from 7-day diet records indicated levels of intake of rumenic acid to be lower, ∼94.9 + 40.6 mg/day (32). Importantly, among the 22 free-living subjects there was a wide range of intake (15–174 mg/day), and this range may be even higher in breastfeeding women in the United States (49–659 mg/day; 82). In the future it may be possible that the levels in the food supply will be amplified by feed and other biotechnological strategies. Because such small amounts of CLA (0.5% of diet) have been shown to alter the expression of genes and impact conditions such as carcinogenesis, obesity, diabetes, and atherosclerosis in experimental animals, it is possible that small amounts consumed over a prolonged period of time may exert similar beneficial effects in human beings.

Thus far, studies using supplements in humans have shown that supplementation with CLA for short periods of time (up to 12 weeks), reduces body weight and body fat in some studies (15, 101, 105). However, at least one study has found that supplementation with CLA results in elevated levels of the lipid peroxidation product, 15-keto-dihydro-prostaglandin-$F_{2\alpha}$, in urine (5). Understanding the role of CLA in modulating events associated with macronutrient metabolism suggests that CLA may be a healthy dietary component with the potential for impacting human health in the areas of cancer, obesity, diabetes, and cardiovascular disease. However, more work is needed to fully elucidate the safety and efficacy of isomers and doses that are required for exerting this breadth of potential beneficial effects. It is hoped that with improved understanding of the doses and isomers required, improvements in recommendations may be made to people regarding the intakes of CLA to improve health.

One last note concerns the role of CLA in the health of subpopulations (e.g., children, the elderly, and women during pregnancy and lactation.) During lactation the content of CLA in human breast milk is sensitive to the consumption of foods rich in CLA (35). Specifically, women who are breastfeeding and consuming a rumenic acid–rich diet produce milk with significantly higher levels of rumenic acid (13.5 μmol/g) than when consuming a low–rumenic acid diet (8.2 μmol/g) (82). In experimental animals it was recently shown that pregnant rats consuming the synthetic mixture of dietary CLA (containing both rumenic acid and t10c12-CLA at a level of 0.5%) birthed pups that had significantly longer tail lengths (as a measure of skeletal growth), heavier gastrocnemius and soleus muscles, and similar adipose mass but smaller adipocyte size (88). These data suggest that CLA in the diet may be beneficial for some parameters of growth, especially through in

utero or early availability in breast milk. However, the safety and efficacy of CLA for growing animals and subgroups in the population who have special physiological needs requires further attention.

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