Alterations in Lipid Metabolism in Mouse Tissues and Hepatic Cell Lines in Response to the *Trans*10, *Cis*12-18:2 Isomer of Conjugated Linoleic Acid

by

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Dairy Science

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(ABSTRACT)

Conjugated linoleic acid (CLA) reduces adipose mass in several species. Studies were conducted to determine: 1) the effect of dietary *trans*10,*cis*12-CLA on growth, tissue fatty acid profile, mRNA expression for stearoyl-CoA desaturase (SCD) in adipose and liver, and mRNA expression for fatty acid synthase (FAS) in adipose of mice, 2) the effect of a dietary combination of *trans*-vaccenic acid (TVA) and trans10,cis12-CLA on Δ^9- desaturation, and 3) the effect of *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA, and carnitine palmitoyltransferase-1 (CPT-1) inhibitors on expression of mRNA for CPT-1 and fatty acid profile in mouse hepatocytes (AML-12) and human hepatoma cells (HepG2). In the first study, male or female mice were fed diets containing 0, 0.15%, or 0.30% *trans*10,*cis*12-CLA for 6 wk. Epididymal adipose weights (males) and inguinal adipose weights (females) decreased by 81% and 52%, respectively, in response to 0.30% *trans*10,*cis*12-CLA. Dry carcass weights decreased from 4.75 $\pm$ 0.11 g for the control to 3.62 $\pm$ 0.11 g for mice fed 0.30% *trans*10,*cis*12-CLA and the decrease was due to a reduction in ether extract. Liver weights increased linearly from 0.55 $\pm$ 0.01 g (control) to 0.65 $\pm$ 0.01 g (0.30% *trans*10,*cis*12-CLA). Dietary *trans*10,*cis*12-CLA (0.30%) reduced FAS and SCD mRNA in adipose by 60 and 30 % respectively, compared with the control, suggesting reduced lipogenesis and desaturation might be primary factors responsible for reducing body fat. In the second study, adult male or female mice were fed diets containing 0.40% TVA in combination with 0, 0.15, or 0.30%

*trans*10,*cis*12-CLA for 10 d. Both TVA and trans10,cis12-CLA were incorporated into plasma, liver, adipose, muscle, and bone lipids proportional to their concentrations in the diets. Desaturation ratios were not affected in adipose, liver, and bone. However, ratios of 16:0 to 16:1 and 18:0 to 18:1 increased from 0.81 ± 0.01 to 0.86 ± 0.01 and 0.15 ± 0.01 to 0.19 ± 0.01 respectively, in response to dietary *trans*10,*cis*12-CLA (0.30%), suggesting inhibition of Δ^9 desaturation in muscle. In the third study, AML-12 or HepG2 cells were incubated with control media or media containing 15 μM etomoxir (ETM), 30 μM ETM, 15 μM hemipalmitoylcarnitinium (HPC), 30 μM HPC, 100 μM *cis*9,*trans*11-CLA, or 100 μM *trans*10,*cis*12-CLA for 24 h. Half the cells were harvested for analysis of fatty acids, mRNA for CPT-1, and cholesterol after 24 h. The remaining cells were incubated for an additional 24 h in control medium. Incorporation (% of total fatty acids) of *trans*10,*cis*12-CLA was greater than *cis*9,*trans*11-CLA in AML-12 (34 ± 0.7 vs 23.6 ± 0.7) and HepG2 (28 ± 0.6 vs 18 ± 0.3) cells. Cells incubated with *trans*10,*cis*12-CLA had higher ratios of 16:0 to 16:1, 18:0 to 18:1, and 18:2n6 to 20:4n-6, suggesting inhibition of Δ^9, Δ^5, and Δ^6 desaturation. *Cis*9,*trans*11-CLA also reduced ratio of 18:2n-6 to 20:4n-6 in both cell lines. *Trans*10,*cis*12-CLA increased mRNA for CPT-1 in both cell lines compared with the control, suggesting enhanced oxidation of fatty acids. In addition, *trans*10,*cis*12-CLA caused a 4-fold and 5-fold increase in free cholesterol content of AML-12 and HepG2 cells, respectively. Overall, results demonstrated that *trans*10,*cis*12-CLA modulated lipid metabolism in tissues *in vivo* and altered fatty acid metabolism, cholesterol synthesis, and CPT-1 mRNA in hepatic cell lines *in vitro*.

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DEDICATION

To my family for their love, support and constant encouragement

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CHAPTER 1

Literature Review

Over many years, dietary fat has been implicated as a factor detrimental to human health and is associated with cancer, obesity, diabetes, stroke, hypertension, and atherosclerosis. Ironically, however, during the past few years there has been a growing awareness that meat and dairy products contain some minor fatty acids that might benefit human health. These fatty acids were identified as conjugated dienoic derivatives of linoleic acid and denoted as conjugated linoleic acid (CLA). Conjugated linoleic acid is therefore a collective term that denotes 18-carbon fatty acids with conjugated double bonds, i.e. two double bonds separated by a single bond in between them, and exist as positional or geometrical isomers of octadecadienoic acid (18:2). While linoleic acid has *cis* double bonds at positions $\Delta^{9,12}$, double bonds in CLA might exist in *cis* or *trans* configuration at positions $\Delta^{7,9}$, $\Delta^{8,10}$, $\Delta^{9,11}$, $\Delta^{10,12}$, $\Delta^{11,13}$, or $\Delta^{12,14}$ along the carbon chain (1, 2, 3, 4, 5, 6). Thus CLA differs from linoleic acid in the position and configuration of the double bonds (**Figure 1-1**). Interestingly, this seemingly minor difference in structure affects their behavior in a number of biological systems (7, 8). Among the many isomers of CLA, the *cis*9,*trans*11 and the *trans*10,*cis*12 have received considerable attention due to their contribution to human health.

Occurrence and formation of CLA

Foods of ruminant origin such as beef and dairy products form the primary sources of CLA, with *cis*9,*trans*11 being the major isomer (5). Meat from ruminants generally contains more CLA than meat from non-ruminants. Because of its predominance in ruminants, *cis*9,*trans*11-CLA is also referred to as rumenic acid (9). Data on the CLA content of various food sources shows that the CLA content of lamb is the highest, averaging about 5.6 mg/g of fat, followed by dairy products and ground beef with values ranging from 4.3 to 5.5 mg/g of fat (5).

Early work by Bartlett and Chapman (10) indicated that CLA is formed as an intermediate in the microbial biohydrogenation of linoleic acid in the rumen. This

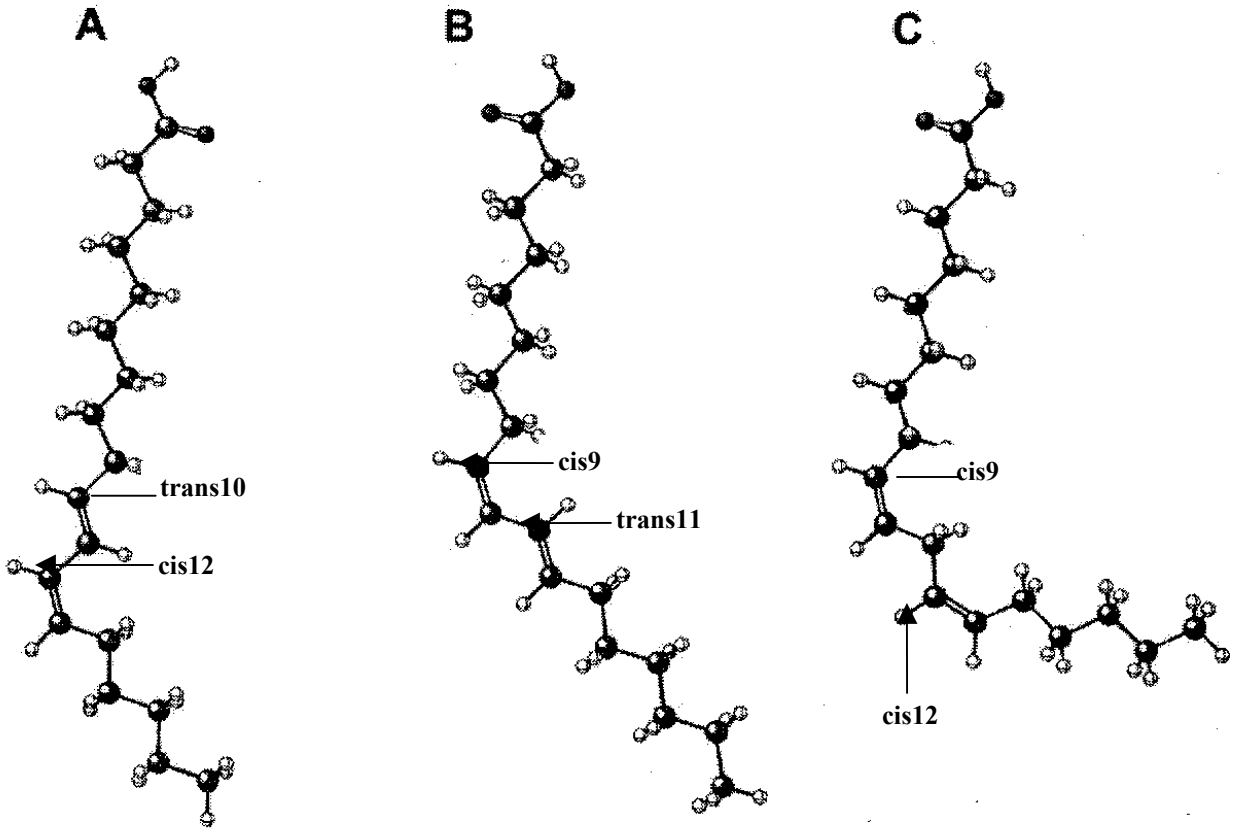


Figure 1-1. Structure of: A. *trans*10,*cis*12-CLA; B. *cis*9,*trans*11-CLA; and C. linoleic acid

conversion is facilitated by the rumen bacterium, Butyrivibrio fibrisolvens (11). The initial step in the biohydrogenation pathway is an isomerization reaction that converts the *cis*-12 double bond in unsaturated fatty acids such as linoleic acid (*cis*9,*cis*12-18:2) to a *trans*-11 isomer, thus forming *cis*9,*trans*11-CLA (12). Once the *trans*-11 bond in linoleic acid is formed by the action of an isomerase, hydrogenation of the *cis*-9 bond in linoleic acid occurs by a microbial reductase forming *trans*11-18:1 (12), also known as *trans*-vaccenic acid. Rumen conditions govern the extent of formation and accumulation of *trans*-vaccenic acid. For example, complete hydrogenation to stearic acid is promoted by the presence of cell-free ruminal fluid and feed particles (13) but is inhibited irreversibly by large amounts of linoleic acid (14). Pathways for the production

of *trans*-10 intermediates involve a specific *cis*9,*trans*10 isomerase in rumen bacteria with the formation of *trans*10,*cis*12-CLA as the first intermediate (15). Fellner et al. (16) demonstrated that *trans*10,*cis*12-CLA was one of the three major CLA isomers in rumen digesta obtained through continuous flow-through fermenters.

Cis-9,*trans*-11 CLA is also synthesized endogenously in tissues from the desaturation of *trans*-vaccenic acid by Δ^9-desaturase (17). Abomasal infusion of *trans*-vaccenic acid (12.5 g/d) for 3 d into cows, increased CLA content of milk fat by over 40% (18), indicating that lactating cows have the ability to endogenously synthesize CLA. Endogenous conversion of *trans*-vaccenic acid to CLA therefore, represents the predominant source of CLA in milk fat (17). Ip et al. (19) observed increased accumulation of CLA in tissues in rats fed butterfat containing *trans*-vaccenic acid and suggested that endogenous Δ^9-desaturase might be responsible for this conversion. Similar conversion of *trans*-vaccenic acid to *cis*9,*trans*11-CLA was observed in serum lipids of humans administered deuterated fatty acids (20). Concentrations of CLA and its metabolites in liver and mammary gland of rats increased proportionately to the amount of dietary *trans*-vaccenic acid, indicating Δ^9-desaturase mediated conversion (21). Similarly, Santora et al. (22) demonstrated desaturation of *trans*-vaccenic acid to *cis*9,*trans*11-CLA in carcass and tissues of mice fed 1% *trans*-vaccenic acid. Thus, endogenous synthesis of CLA facilitated by the desaturation of *trans*-vaccenic acid appears to be one of the major sources of CLA in tissues and milk fat.

Health benefits of CLA

Over the past 15 years, researchers have demonstrated that CLA possess the ability to inhibit or modulate disease processes, thus benefiting human and animal heath. The role of *cis*9,*trans*11-CLA in tumorigenesis, atherosclerosis, immune function, and diabetes will be discussed in this literature review. Emphasis will be laid on the lipid modulating effect of *trans*10,*cis*12-CLA in various species and an attempt will be made to discuss the underlying mechanisms of action. In this review, the abbreviation ‘CLA’ shall be used to represent mixtures of CLA isomers, while the pure isomers shall be referred to as ‘*cis*9,*trans*11-CLA’ or ‘*trans*10,*cis*12-CLA’.

Inhibition of carcinogenesis

Early work by Ha et. (7), demonstrated the efficacy of CLA in inhibiting epidermal tumors in mice. In their study, CLA or linoleic acid was applied to the skin of mice 7 d, 3 d, or 5 min prior to application of dimethylbenzanthracene (DMBA), a potent carcinogen. A significant reduction in the number of papillomas in CLA treated mice was observed compared to mice treated with linoleic acid or the controls. This was the first study to demonstrate the role of CLA in inhibition of carcinogenesis and opened the door for further exploration on the properties of this fatty acid.

In the follow-up study, Ha et al. (23) demonstrated the reduction of mouse forestomach neoplasms in mice treated with synthetic CLA prior to administration of benzo(a)pyrene. Ip et al. (24) observed that feeding as little as 0.05 g CLA/100 g of dietary DM to rats caused a reduction in the number of mammary tumors. Similar inhibition of mammary tumorogenesis by CLA was observed in other studies with rats (25, 26) and mice (27, 28). A 3-year Finnish study showed an inverse relationship between the amount of dietary CLA intake and the risk of breast cancer in postmenopausal women (29). Their findings suggested that a diet rich in CLA might decrease the incidence of breast cancer in postmenopausal women.

In vitro work also demonstrated that CLA inhibited growth of MCF-7 breast cancer cells (30), NMU rat mammary adenocarcinoma cells (31), human colorectal and prostate cancer cells (32), gastric cancer cells (33), and Caco-2 colon cancer cells (34). Feeding CLA to rats caused a significant reduction in azoxymethane-induced colonic cryptic foci in rats thus, suggesting a role for CLA in preventing colon tumorigenesis (35).

Efforts have been made to elucidate the mechanism of action of the CLA-mediated inhibition of carcinogenesis. Early work attributed the suppression of carcinogenesis by CLA to its antioxidant property (23). In rats, CLA reduced proliferation of the mammary epithelium (24), neoplastic progression (36), and

carcinogen-DNA adduct formation (37), thus preventing initiation of tumorigenesis. Rats fed CLA had a significant reduction in the number of mammary tumors along with reduced levels of arachidonic acid, a substrate for eicosanoid synthesis, in mammary lipids (26). The correlation between the incidence of mammary tumors and the levels of arachidonate suggests a possible role for eicosanoids in the inhibition of carcinogenesis (26).

Increased programmed cell death (apoptosis) as indicated by changes in nuclear morphology and induction of DNA laddering, was observed in mammary epithelial cells organoids (19), rat mammary adenocarcinoma cells (31), and human colon cells (38) treated with CLA. Dietary CLA also stimulated apoptosis in diemthylhydrazine-induced colon cancer in rats (39). Thus, increased apoptosis could potentially be a mechanism for the CLA-mediated suppression of carcinogenesis. Apoptosis could also be mediated by increased peroxisome-proliferator activated receptor (PPAR) activity promoted by PPAR agonists such as CLA (40).

More recently, Moon et al. (41) demonstrated that CLA decreased basic fibroblast growth factor (bFGF), a potent angiogenic factor expressed in many tumors, in a dose -dependent manner. Inhibition of bFGF induced cell proliferation by CLA could therefore be one of the mechanisms by which it suppresses tumor growth. Further elucidation of the mechanism of CLA in tumor prevention and suppression at the molecular level is required before it can be used as an aid in cancer therapy in humans.

Inhibition of atherosclerosis

Atherosclerosis is one of the major causes of stroke and heart attack, and occurs due to deposition of fatty substances, cholesterol and other products along the inner lining of an artery. Diets rich in cholesterol contribute to the development of atherosclerosis. With the discovery of CLA as an anticarcinogenic agent, researchers explored the possible beneficial effects of this fatty acid on other metabolic or systemic disorders including atherosclerosis and diabetes.

Rabbits fed a hypercholesterolemic diet containing 0.5 g / d CLA exhibited lower circulating total and low-density lipoprotein (LDL) cholesterol and triglycerides (42). A positive correlation exists between plasma LDL cholesterol levels and the incidence of coronary atherosclerosis. Examination of the aorta revealed decreased atherosclerosis in the rabbits fed CLA (42), thus establishing a protective role of CLA in coronary atherosclerosis. In a subsequent study in hamsters, dietary CLA reduced plasma total cholesterol, very low-density lipoproteins (VLDL), and VDL along with a 45% decrease in aortic plaque formation compared with the control animals (43). The authors suggested that alteration in oxidative susceptibility of LDL could be a possible mechanism of CLA-mediated reduction of atherosclerosis (43).

Kritchevsky et al. (44) demonstrated that CLA as low as 0.1% of the diet resulted in a 34% reduction in experimentally induced atherosclerosis in rabbits. In contrast, however, mice fed a atherogenic diet containing 5 g CLA / kg diet, had increased development of aortic fatty steaks (45), suggesting that the effect of CLA and its relative potency might vary across species (46). More evidence in other species, including humans, is necessary to establish the efficacy of CLA as an anti-atherogenic agent. Also, the mechanism by which CLA exerts its effect on the establishment and regression of atherosclerosis requires further investigation.

Effect on immune functions

Other effects of CLA include its ability to modulate the immune system. Addition of CLA to porcine *in vitro* lymphocyte cultures increased mitogen induced lymphocyte blastogenesis and lymphocyte cytotoxic activity (47). Diets containing 0.5% CLA diminished Escherichia coli lipoprotein polysaccharide (LPS)-mediated weight loss in both chicks and rats and enhanced phytohemagglutinin response and macrophage phagocytosis in rats (48).

Miller and co-workers (49) fed mice 0.5 % CLA, 0.5 % fish oil or a basal diet after endotoxin injection. After 72 h, dietary CLA prevented endotoxin-mediated weight loss

compared to animals fed fish oil or the basal diet. An increase in splenocyte blastogenesis due to CLA also was observed in this study. Enhancement in lymphocyte proliferation and stimulation of interleukin-2 production were observed in mice fed CLA for 3 or 6 wk (50). However, another study by the same research group demonstrated that porcine blood lymphocytes and murine peritoneal macrophages incubated with several concentrations of CLA, exhibited increased lymphocyte proliferation, lymphocyte cytotoxic activity, and macrophage bactericidal activity while it inhibited interleukin-2 production by lymphocytes and macrophage phagocytic activity (47).

Feeding diets containing 1% CLA to rats for 3 wk resulted in reduced leukotriene-B4 and leukotriene-C4 in spleen and lungs without any effect on the release of histamine (51). Concentrations of IgA, IgG, and IgM increased, but IgE decreased in the splenic and mesenteric lymph nodes in rats fed the 1% CLA diet, suggesting that CLA could play a potential role in alleviating food-induced allergic reactions (51). Hayek et al. (52) conducted a study to examine immune response in young and adult mice fed a diet containing 1% CLA for 8 wk. The authors reported greater splenocyte proliferation in both young and adult mice fed CLA diet compared with control animals. However, interleukin-2 production was increased only in young mice in response to dietary CLA, suggesting that the response to CLA varied with age. Dietary CLA also prevented an increase in the ratio of blood heterophils to lymphocytes and a decrease in food intake following immune stimulation by salmonella enteridis LPS injections in chicks (53).

Yamasakhi et al. (54) evaluated the effect of the pure *cis*9,*trans*11 and *trans*10,*cis*12 isomers or a CLA mixture on immune system functions in mice. Mice were fed with a control diet or a diet containing either 1% *cis*9,*trans*11-CLA, 1% *trans*10,*cis*12-CLA or 1% CLA (1:1 *cis*9,*trans*11-CLA:*trans*10,*cis*12-CLA) for 3 wk. While *trans*10,*cis*12-CLA increased IgA and IgM production from spleen lymphocytes following stimulation by a mitogen, the *cis*9,*trans*11-CLA had a higher concentration of tumor necrosis factor alpha in the lymphocytes, compared with the control group. Levels of CD8 (+) T cells were higher in mice fed the 1:1 mixture compared with the

controls. Their results indicated that both isomers of CLA modulate various aspects of immune responses.

Kelley et al. (55) conducted a study using women to determine if the immuno-modulating effects of CLA observed in animals could be seen in humans as well. Subjects were divided into two groups and were fed either a basal diet or a diet containing CLA (3.9 g/d) for 63 d. Dietary CLA did not affect any of the immune variables tested, including circulating lymphocytes, monocytes, and granulocytes, indicating that short –term CLA feeding does provide a beneficial immune response in humans. In a more recent study, the effect of *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA or a CLA mixture on immune status of healthy men following hepatitis B vaccination was tested (56). Results indicated approximately twice the number of subjects consuming the CLA mixture reached protective antibody titers to hepatitis B compared with the control group, thus suggesting that dietary CLA might be beneficial for enhancing the immune response following hepatitis B vaccination.

Role in Type 2 diabetes

Non-insulin dependent diabetes mellitus (Type 2) is the most common form of diabetes affecting adults. One of the many reasons behind the cause of Type 2 diabetes is obesity. Because of the link between diabetes and obesity and the findings that dietary CLA reduces adiposity in animals, as discussed later in the literature review, the role of CLA in Type 2 diabetes was explored.

Houseknecht et al. (57) provided the first evidence that CLA normalizes impaired glucose tolerance and improves hyperinsulinemia in the diabetic rat. In their study, rats fed CLA exhibited lower fasting plasma glucose and insulin levels compared with the control animals. The authors suggested that activation of PPAR gamma by CLA could be partly responsible for the insulin sensitizing effects. Similarly, pigs fed diets containing 1 % CLA exhibited 37% higher serum insulin concentrations than the controls (58).

Long-term feeding (8 mo) of 1 % CLA resulted in insulin resistance and lipodystrophy in C57BL/6J mice (59). Data therefore suggest that CLA improves insulin resistance only in diabetics, whereas it increases serum insulin levels with reduced insulin sensitivity in the normoglycemic state. Diets containing a 50:50 mixture of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA resulted in improved glucose tolerance in the Zucker diabetic fatty rat compared with rats fed a diet containing the *cis*9,*trans*11-CLA, suggesting that the *trans*10,*cis*12-CLA is responsible for the delayed onset of Type 2 diabetes (60).

In diabetic human subjects, CLA supplementation (6.0 g CLA/d) for 8 wk reduced fasting blood glucose without affecting serum insulin (61). Because the *trans*10,*cis*12-CLA reduces body fat, the effect of this isomer on insulin sensitivity in obese men was investigated (62). Abdominally obese men were treated daily with 3.4 g CLA mixture, *trans*10,*cis*12-CLA or a placebo for 12 wk. Although the CLA mixture did not affect glucose metabolism, *trans*10,*cis*12-CLA caused increased insulin resistance and hyperglycemia (62). Given the contradicting results on the role of CLA in glucose metabolism in humans, further trials, especially in obese and diabetic individuals are recommended to document its effects and clinical implications.

Modulation of lipid metabolism and gene expression by CLA

Fat intake averages between 30 to 40% of food energy in the western world. Most of the dietary fatty acids are long chain fatty acids (LCFA) and are absorbed from the intestinal tract into blood in the form of triglycerides. Once in the circulation, triglycerides are carried by chylomicrons and VLDL and hydrolyzed by the enzyme lipoprotein lipase (LPL) at the surface of the capillaries. Free fatty acids bound by albumin in circulation are rapidly taken up by tissues and used in various metabolic pathways. Although some LCFA diffuse through the cell membrane, the majority of fatty acid uptake by the tissues is facilitated by fatty acid transporters such as CD36 (63). Mice overexpressing CD36 had increased fatty acid oxidation in muscle in response to contraction (64), while CD36-null mice had a marked reduction in fatty acid oxidation in adipose tissue and muscle (65). Thus, transport proteins appear to be rate limiting in

the metabolism of fatty acids in some tissues, particularly muscle. Within the cell, fatty acids are bound to a fatty acid binding protein. Some of the fatty acids are oxidized within the mitochondria, while the remainder is esterified and stored.

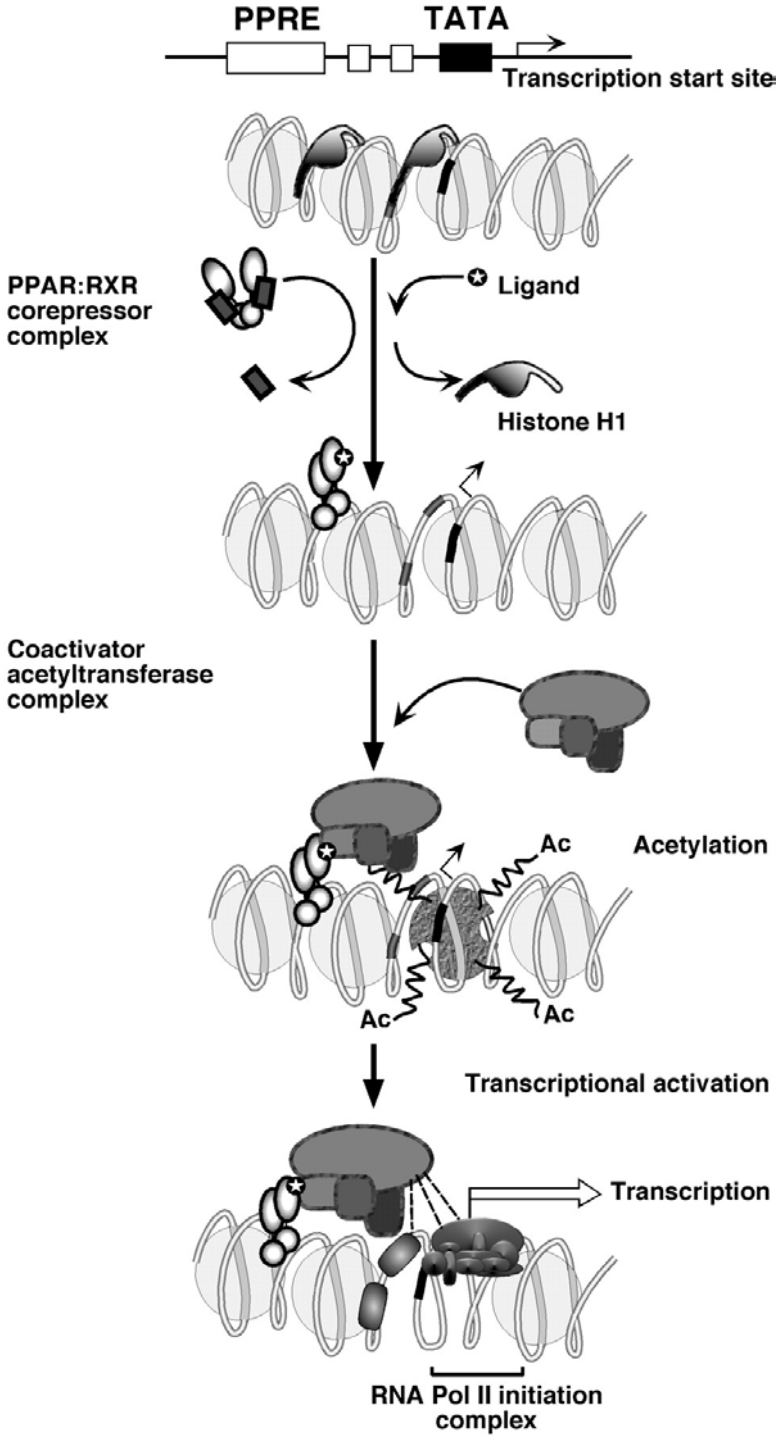
Adipose tissue is the main store of triacylglycerol in the body. Triglycerides in adipose tissue continually undergo breakdown (lipolysis) and re-esterification depending on nutritional status and hormones such as epinephrine and insulin. The extent of lipolysis or re-esterification determines the magnitude of the free fatty acid pool in the adipose and circulation. Fate of fatty acids in the liver also is dependent on the nutritional status. In conditions where energy fuels are abundant, re-esterification and triglyceride release into circulation in the form of VLDL is favored.

Fatty acids are also synthesized *de novo* in mammary, adipose, and liver from acetyl-CoA derived from the oxidation of pyruvate within the mitochondria. The majority of fatty acids synthesized via the lipogenic pathway are short or medium chain i.e. 8 to 14 carbon chains.

Flow of intermediates through most of the pathways described above is controlled by the activity of rate limiting enzyme systems. Some of the enzymes discussed in this review and the following chapters govern the extent of fatty acid synthesis, oxidation, or desaturation within tissues and are described in **Table 1-1**. Genes encoding these enzymes are in turn regulated by a group of nuclear hormone receptors known as peroxisome proliferator-activated receptors (PPAR). Three PPAR subtypes have been identified and named PPARα, PPARβ, and PPARγ. Because of their ability to bind to several ligands including unsaturated fatty acids, PPARs have been projected as potential therapeutic targets. These transcription factors form heterodimers with the cis9-retinoic acid receptor (RXR) and the complex is designated as PPAR:RXR. The general mechanism of PPAR-induced gene transcription is depicted in **Figure 1-2**.

Pathway	Enzyme	Function
Hydrolysis	Lipoprotein Lipase (LPL)	Located on the walls of blood capillaries and hydrolyzes triacylglycerol, releasing fatty acids that are incorporated into extrahepatic tissues for storage or utilization as fuels.
	Hormone-Sensitive Lipase (HSL)	Facilitates the hydrolysis of triacylglycerol into free fatty acids and glycerol in adipose tissue. Free fatty acids formed from lipolysis can either be reesterified within the tissue or diffuse into the plasma.
Lipogenesis	Acetyl-CoA Carboxylase (ACC)	Catalyzes the first rate-limiting step in the biosynthesis of fatty acids. Facilitates the conversion of acetyl-CoA to malonyl CoA in the presence of ATP and biotin
	Fatty Acid Synthase (FAS)	Multienzyme complex and exists as a dimer. Catalyzes a series of reactions resulting in the synthesis of fatty acids from acetyl and malonyl-CoA
Oxidation	Carnitine palmitoyltransferase 1 (CPT1)	Catalyzes the rate-limiting step in oxidation of long chain fatty acids in the mitochondria. Converts long-chain acyl-CoA to acylcarnitine, which is able to penetrate the inner mitochondrial membrane and gains access to the β-oxidation enzyme system.
Desaturation	Δ^9-stearoyl-CoA desaturase (SCD); also referred to as Δ^9-desaturase	Iron-containing microsomal enzyme that catalyzes the critical step in the biosynthesis of monounsaturated fatty acids by introducing a double bond at the Δ^9 position. For example, SCD catalyzes the conversion of stearic acid (18:0) to oleic acid (*cis*9-18:1).
	Δ^6 & Δ^5- desaturases	Key enzymes for the synthesis of highly unsaturated fatty acids such as arachidonic acid (20:4n-6) from linoleic acid (18:2n-6) and eicosapentaenoic acid (20:5n-3) from α-linolenic acid (18:3n-3). Conversions involve desaturation at cabonyl-6 position by Δ^6-desaturase followed by a 2-carbon elongation step, and finally desaturation at the carbonyl-5 position by Δ^5-desaturase.

Table 1-1. Enzyme pathways and functions



PPAR-responsive promoter in a linear form with a PPRE, two binding sites for transcription factors (small white boxes), the TATA box and transcription start site. The same region organized in a repressive chromatin structure is also shown. PPAR:RXR/corepressor complex is activated by a ligand that results in the dissociation of the corepressors from the ligand activated PPAR:RXR complex.

Activated PPAR:RXR complex binds to PPRE and causes histone H1 release. PPRE-bound PPAR:RXR targets a coactivator-acetyltransferase complex to the promoter.

Promoter chromatin at the transcription initiation site region is modified by the coactivator-acetyltransferase complex, which acetylates the histone tails (Ac) producing a transcriptionally competent structure.

Additional transcription factors and the basal transcriptional machinery, including the RNA Polymerase 2 initiation complex, are recruited to the acessible promoter and transcription of the DNA is initiated.

Figure 1-2. Transcriptional activation by PPARs [adapted from Desvergne & Wahli, (66)].

Research has demonstrated that CLA affects metabolism of fatty acids including lipogenesis, lipolysis, desaturation, and oxidation in tissues of different species. However, the potency and the effect of CLA varies across species and is also different between tissues. Tissue-specific effects of CLA and the probable mechanisms of action are therefore discussed in this review.

Mammary Gland

Initial research on the protective role of CLA in health was focussed on its effects on carcinogenesis. As more data on the anticarcinogenic property of CLA became available, researchers recommended increasing CLA intake in order to derive maximum health benefits. Because CLA is found abundantly in foods of ruminant origin as discussed earlier, a number of studies dealt with ways to maximize the CLA content of milk and meat.

Cows grazing permanent pasture had 500% higher CLA content in milk compared to cows fed preserved forage and grain in a 50:50 ratio (67). Feeding protected fats (68), plant oils such as soybean oil (69) and corn oil (70) or fat extruded soybeans and cottonseed (71) to dairy cows, increased the CLA content of milk. Similarly, abomasal infusions of CLA mixtures also increased the CLA content of milk fat (72, 73).

A marked reduction in milk fat content and yield was also noticed due to abomasal infusions of CLA (72, 73). In the study by Loor and Herbein (72), milk fat yield decreased by 34% from 24 to 72 h due to CLA along with an increased ratio of stearic to oleic acid in milk fat, when compared with control. Results indicated that CLA appeared to be a potent inhibitor of de novo fatty acid synthesis and desaturation in the mammary gland. Saturated fatty acids (SFA) increased while monounsaturated fatty acids (MUFA) decreased in milk of sows fed CLA, suggesting involvement of CLA in the de novo sythesis and desaturation of fatty acids within the mammary gland (74). Percentage and yield of milk fat were reduced by 52 and 55% in cows infused abomasally with 50 g of a CLA mixture (75).

To determine the CLA isomer responsible for milk fat depression, Baumgard et al. (76) infused either *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA into the abomasum of dairy cows. Their results showed that only the *trans*10,*cis*12-CLA reduced milk fat percentage and yield, thus suggesting that the *trans*10,*cis*12-CLA, and not the *cis*9,*trans*11-CLA, affects lipid metabolism. Similarly, daily intravenous infusions of 6 g *trans*10,*cis*12-CLA caused a 30 and 20% reduction in milk fat percentage and yield over a 5-d period (77).

Feeding 100 g calcium salts of CLA for 14 d to lactating Holstein cows caused a 34% reduction in milk fat (78). Feeding calcium salts of CLA to pregnant cows over the last 140 d of lactation cycle decreased the proportion of short and medium chain fatty acids secreted in milk as well as milk fat percent and yield (79). Similarly, dietary CLA decreased milk fat content without affecting milk output in lactating women (80).

To determine the mechanism of CLA-mediated depression in milk fat, Baumgard et al. (81) infused either skim milk (control) or 13.6 g *trans*10,*cis*12-CLA into the abomasum of lactating Holstein cows for 5 d. Infusions resulted in 42 and 48% reduction in milk fat percentage and yield. A decrease in mRNA expression for LPL, fatty acid binding protein (FABP), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD) was noted in the mammary tissue. The ratio of SFA to MUFA in milk also increased, consistent with mammary SCD mRNA data. The results indicated that *trans*10,*cis*12-CLA reduced lipid synthesis and inhibited desaturase activity within the mammary gland.

The *trans*10,*cis*12-CLA, but not the *cis*9,*trans*11-CLA, reduced milk fat and proportion of MUFA in the mammary gland of lactating mice (82). Dietary CLA also reduced mammary ACC and FAS mRNA abundance suggesting that this could be a potential mechanism for the observed reduction in milk fat (82).

Adipose

Given the alarming rise in the number of overweight and obese individuals in recent years, the fat-reducing ability of CLA has evoked tremendous interest and enthusiasm within the research community. In the past 5 years, there has been a plethora of data demonstrating the lipid modulating effect of CLA in various species including mice, rats, pigs, chicken, hamsters and humans. Park et al. (83) provided the first evidence that dietary CLA affects body fat in mice. In their study, male and female mice fed 0.5% CLA mixture, had 57% and 60% reduction in percentage body fat. In hamsters, 6.6 g *trans*10,*cis*12-CLA/kg of diet caused a 16% reduction in epididymal adipose weights (84). Dietary CLA increased gain to feed ratio and lean tissue deposition and decreased fat deposition in finisher pigs (85). Body fat content was lower in broilers fed greater than 2% CLA mixture (86). In exercising human subjects, CLA reduced body fat but not body weight (87).

Park et al. (88) demonstrated that the body fat reduction observed with CLA feeding was due to the *trans*10,*cis*12 isomer and not *cis*9,*trans*11-CLA. Purified *trans*10,*cis*12-CLA or *cis*9,*trans*11-CLA were fed to ICR mice for 4 wk. The mice fed *trans*10,*cis*12-CLA exhibited lower body fat than mice fed *cis*9,*trans*11-CLA or the control diet. *Trans*10,*cis*12-CLA, but not *cis*9,*trans*11-CLA, was inversely associated with body weight in subjects with Type 2 diabetes, indicating that *trans*10,*cis*12-CLA is the bioactive isomer modulating glucose and lipid metabolism (61).

Rahman et al. (89) studied the effect of 4-wk CLA supplementation in Otsuka Long-Evans Tokushima (OLETF) rats. The OLETF rat is characterized by mild obesity with visceral-fat accumulation. Dietary CLA enhanced CPT-1 activity in peri-renal adipose tissue compared with the control group. Feeding a diet containing 0.5% CLA for 5 wk to lean or obese Zucker rats showed that CLA reduced inguinal fat pad weights in only the lean rats. In obese rats, it had the opposite effect (90). Daily, intake of 4.2 g CLA resulted in a significant reduction in abdominal fat without affecting overall obesity in abdominally obese human males (91). Data therefore suggest that CLA is not able to

reduce body fat in established obesity conditions. Therefore its use as an anti-obesity agent requires further exploration.

Efforts have been made to explain the mechanisms by which the CLA- mediated reduction in body fat is brought about. Several mechanisms have been proposed with varying emphasis on energy expenditure, fatty acid uptake, desaturation, apoptosis, glucose utilization, lipogenesis, and fatty acid oxidation.

Increased metabolic rate without any change in intake was observed in mice fed a diet containing 1% CLA for 6 wk (92). Energy expenditure and energy lost in the excreta in CLA-fed mice were 74% and 26% higher than those in mice fed control diet (93). Expression of uncoupling protein 2 (UCP2), a mitrochondial transporter that dissipates the proton gradient and increases energy expenditure, was increased in white adipose tissue (WAT) and brown adipose tissue (BAT) in mice fed CLA (59, 94). Uncoupling proteins (UCP) belong to a class of transmembrane proteins found in the inner mitochondrial membranne and play a major role in whole-body energy balance (95). Results therefore suggest that the reduction in adipose tissue stores could partly be due to an increase in energy expenditure.

Reduced lipoprotein lipase (LPL) activity was observed in 3T3-L1 adipocytes supplemented with CLA (83). Pure *trans*10,*cis*12-CLA also reduced LPL activity in 3T3-L1 adipocytes (88). Adipose tissue in CLA-fed mice had lower LPL mRNA expression (59). These findings indicate that the CLA-mediated reduction in body fat is accomplished via a reduction in fatty acid uptake.

To determine the basis for the reduction in fat pads due to CLA, Azain et al. (96) fed female Sprague-Dawley rats diets containing 0, 0.25, or 0.5 g CLA/100g diet for 5 wk. Dietary CLA reduced mass of retroperitoneal fat pad and MUFA content. The reduction in fat due to CLA was accounted for by a reduction in the adipocyte size rather than number. Mouse 3T3-L1 adipocytes supplemented with *trans*10,*cis*12-CLA had decreased expression of SCD (97). The cells had smaller lipid droplets with

reduced levels of MUFA indicating that inhibition of desaturase activity could potentially be involved in mechanisms by which CLA reduces body fat. Dietary CLA increased SFA and decreased MUFA in the back fat of sows suggesting inhibition of desaturase activity (74). It also decreased subcutaneous fat (98) and increased the proportions of SFA, while reducing the proportions of polyunsaturated fatty acids (PUFA) in subcutaneous adipose tissue (99). Bellies of pigs fed a mixture of CLA were firmer and had a higher proportion of SFA than MUFA (100). Firmer bellies improve bacon slicing, while decreased unsaturated fatty acids decrease the problems associated with rancidity. Pigs fed CLA had decreased Δ^9-desaturase index and SCD enzyme activity suggesting that it could partly be responsible for the reduction in adiposity (101). In chickens fed CLA for 5 wk, abdominal fat decreased by 33% coupled with an increased ratio of SFA to MUFA (102).

In a long-term study (8 months), feeding CLA to female C57BL/6J mice resulted in ablation of BAT and a marked decrease in WAT from the parametrial, renal, retroperitoneal, abdominal and dorsal subcutanetous region (59). Mean diameters of adipocytes in the CLA-fed mice were 41% smaller than the controls, consistent with the studies discussed previously. However, increased apoptosis manifested by a decrease in adipocyte number was also noticed suggesting that both reduction in adipocyte size and decreased cell numbers contributed to the decrease in fat mass. Expression of tumor necrosis factor-α (TNF-α), a cytokine that leads to apoptosis of adipocytes, was increased in WAT and BAT. Apoptosis, measured by internucleosomal DNA degradation, was increased in retroperitoneal fat pads of mice fed 2% CLA for 2 wk (103). Similarly, pure *trans*10,*cis*12-CLA induced body fat loss due to increased apoptosis in the adipose tissue when fed to mice (104).

Dietary CLA also decreased adipose mRNA levels for glucose transporter 4 (GLUT4), FAS, ACC, and the expression of sterol-regulatory element binding protein (SREBP) and PPARγ (59). *Trans*10,*cis*12-CLA inhibited preadipocyte differentiation and reduced levels of PPARγ and FAS mRNA (105). Reductions in GLUT-2 and PPARγ mRNA in both WAT and BAT were observed in mice in response to CLA treatment (94).

Parametrial adipose tissue in CLA-fed mice had reduced incorporation of glucose into fatty acids (106). Triglyceride content decreased along with a decrease in glucose incorporation into total lipid in human pre-adipocytes supplemented with *trans*10,*cis*12-CLA (107). Reduction in uptake of glucose as a substrate for fatty acid synthesis and increased cell death could therefore contribute to decreased adipose mass.

Activity of carnitine palmitoyltransferase-1 (CPT-1) was increased by dietary CLA in the epididymal fat pad of mice (83). Male weanling Wistar rats fed 1% *trans*10,*cis*12-CLA also exhibited a 30% increase in CPT-1 activity in epididymal adipose compared to animals fed a control diet (108), suggesting that increased fatty acid oxidation by CLA might contribute to the reduction in fat mass.

Liver

The liver has a central role in the maintenance of fat homeostasis in the body, because it is involved in lipid processing, oxidation and distribution. Depending on the nutritional status of the animal, fatty acids in the liver can be stored as triglycerides, oxidized for fuel, or transported to other tissues in the form of VLDL. Although CLA reduces adipose stores as described earlier, it also appears have adverse effects on hepatic lipid metabolism including lipid accumulation and increased insulin resistance.

Belury et al. (109) evaluated the role of CLA in modulating lipid metabolism in liver of mice. Female SENCAR mice fed diets containing 1.5% CLA for 6 wk had higher liver lipid content compared to the control group. Similarly, increased lipid accumulation in the liver was observed in rats fed CLA containing diets (110). Hamsters fed CLA diets for 8 wk had increased liver weights but not increased liver lipid content (84). Liver histology revealed that the increase in weight was due to hypertrophy (84). Incubating human hepatoma cells (HepG2) with CLA resulted in increased triglyceride and cholesterol accumulation in the cells (111), consistent with *in vivo* data (112). Broiler chicks fed CLA for 21 d, however, had reduced triacylglycerol accumulation in the liver (113). Thus, the effect of CLA on the liver varied between species.

Mice fed a fat-free high carbohydrate or 5.0% corn oil diet containing 0.5% CLA had a 45% and 75% reduction in SCD mRNA in the liver (114). Consistent with the *in vivo* data, 150 μM CLA reduced SCD mRNA expression by 60% in mouse liver cells (114). Rats fed 10 g CLA/kg diet for 6 wk exhibited higher SFA and lower MUFA in their liver (115). Bretillon et al. (116) demonstrated that only the *trans*10,*cis*12-CLA inhibited desaturation of stearic acid into oleic acid in rat liver microsomes and suggested that this was the isomer responsible for altering the ratio of SFA to MUFA in liver of animals fed a CLA mixture. Addition of *trans*10,*cis*12-CLA to the incubation medium for 15 min decreased SCD activity in rat liver microsomes (117), indicating that the *trans*10,*cis*12-CLA could directly inhibit SCD enzymatic activity. Rats fed *trans*10,*cis*12-CLA for 6 wk had a 32% reduction in their liver triacylglycerol content along with an increased ratio of 18:0 to *cis*9-18:1, suggesting an alteration of Δ^9-desaturase activity (118). Thus, *trans*10,*cis*12-CLA appears to be the isomer responsible for the alteration in SFA and MUFA observed in the liver of animals fed a CLA mixture.

Reduction of Δ^5 and Δ^6 desaturation was noticed in HepG2 cells supplemented with 100 μM *trans*10,*cis*12-CLA, while *cis*9,*trans*11-CLA had no effect on the desaturation process in these cells (119). The Δ^5 and Δ^6 desaturation of 18:2n-6 to 20:4n-6 is a key step in the synthesis of eicosanoids (120). Because eicosanoids are involved in cancer, immune functions and cardiovascular diseases, *trans*10,*cis*12-CLA could potentially have an indirect role in the modulation of these health process through inhibition of Δ^5 and Δ^6 desaturation.

Liver CPT-1 activity was not influenced by dietary CLA in mice (83). Activities of liver acyl CoA oxidase, the rate-limiting enzyme for β-oxidation in peroxisomes, and CPT-1, were not influenced by *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA or a CLA mixture in male Wistar rats (108). However, liver of rats fed CLA for 2 wk produced more ketone bodies than the controls indicating that dietary CLA enhanced hepatic fatty acid oxidation (12). Enhanced CPT-1 activity was also noticed in the liver of OLETF rats fed CLA compared to animals fed the control diet (89). Specific activity of CPT-1 in liver of hamsters fed CLA was higher than the control group (122). A dietary CLA mixture

containing equal proportions of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA increased hepatic CPT-1 mRNA expression and activity in mice (111). Interestingly, mRNA levels and activity of ACC and FAS in livers also increased, indicating dietary CLA increases hepatic lipid synthesis. If the rate of triglyceride synthesis exceeds the capacity of the liver to oxidize lipid in mice fed CLA, the result may be net accumulation of fatty acids and enlarged livers

Clement et al. (123) provided the first demonstration that the *trans*10,*cis*12-CLA and not the *cis*9,*trans*11-CLA causes fatty liver and induces hepatic lipid changes in mice. In their study, female mice were fed 0.4% linoleic acid or highly purified *trans*10,*cis*12-CLA or *cis*9,*trans*11-CLA containing diets. Along with hepatic lipid accumulation, hyperinsulinemia and an increase in the expression of FAS, CD36, and PPARγ mRNA was observed in mice fed *trans*10,*cis*12-CLA. Hepatic overexpression of PPARγ gene is a specific characteristic of steatotic livers (124). Increases in FAS and CD36 indicated an increased rate of lipogenesis and fatty acid uptake by the liver, thus contributing to the development of fatty liver. Hyperinsulinemia along with fatty liver was also noticed in mice fed CLA for 8 months (59). Insulin upregulates PPARγ (124) and FAS (125) gene expression in the liver suggesting that the occurrence of hepatic steatosis by trans10,cis12-CLA is secondary to hyperinsulinemia.

Discrepancies among results from different studies may be due to differences in the combinations of the amount and quality of the CLA mixture, gender, age, duration of supplementation or species responsiveness. However, given the undesirable effects of *trans*10,*cis*12-CLA on the liver, further studies on its effects in humans are warranted prior to its recommended usage as an anti-obesity or a hypolipidemic agent.

Bone

The bone is a multifunctional organ that produces several regulatory factors including cytokines, prostaglandins and growth factors such as IGF-1. These factors orchestrate the various events associated with bone modeling and remodeling process. Prostaglandin E_2 (PGE_2) is derived from arachidonic acid and is associated with bone

resorption and metabolism (126). Because arachidonic acid metabolism is influenced by dietary CLA in various tissues, the role of CLA in bone is emerging as an interesting area of research.

Initial evidence for the potential role of CLA in bone formation was reported by Watkins et al. (127). Chicks fed butterfat had a reduced concentration of arachidonic acid in bone, *ex vivo* PGE_2 production, and increased bone formation rate (127). Because the beneficial effects of butter were attributed to its CLA content, further studies were undertaken to determine the effects of CLA on bone metabolism in rats.

Li & Watkins (115) examined the effect of feeding 10 g CLA / kg of diet to weanling rats for 6 wk. Dietary CLA lowered the concentrations of n-6 fatty acids in cortical bone, while in the bone marrow and periosteum, it decreased MUFA and PUFA and increased SFA concentrations. Concentrations of PGE_2 also were reduced due to CLA. Reduced ex vivo PGE_2 production due to CLA also was noticed in bone organ culture (128). Because PGE_2 stimulates bone formation at low concentration (129) and inhibits matrix formation at higher concentration (130), reduction in PGE_2 concentration by CLA might prove beneficial in bone modeling. Increased bone weight was reported for pigs fed 0.5 or 1.0% CLA (131), indicating that CLA increases bone formation. Unlike the bone changes observed in chicks, rats and pigs, supplementation with 6 g CLA daily in resistance-trained human subjects for 4 wk did not affect bone mass (132).

Few studies have examined the effect of CLA on bone fatty acids and mineral deposition. From the available data, it appears that CLA could have a beneficial effect on bone health. Further studies are therefore necessary to confirm the role of CLA in bone especially in regard to bone disorders.

Muscle

The muscle plays an important role in overall fuel balance and has the capacity to utilize carbohydrate or lipid fuels depending on the hormonal and nutritional status. Because the skeletal membrane has a large capacity for lipid oxidation, SFA are usually

oxidized. This allows accumulation of unsaturated fatty acids in the phospholipid fraction of the plasma membrane and provides fluidity and insulin resistance (133). Impairment in fatty acid metabolism in the muscle occurs in conditions such as obesity. The muscle, therefore, is an important tissue involved in the regulation of lipid metabolism.

Park et al. (83) were the first to demonstrate the effects of CLA in skeletal muscle of mice. Male ICR mice fed a diet containing CLA had increased β-oxidation in the skeletal muscle manifested by an increase in CPT-1 activity (83). Enhanced CPT-1 activity in gastrocnemius muscle also was noted in OLETF rats fed CLA for 4 wk (89).

Sprague-Dawley rats fed diets containing CLA for 5 wk had decreased proportions of palmitoleic, oleic, and arachidonic acids in the skeletal muscle (134). Piglets reared on sows fed CLA diets had increased total SFA and decreased MUFA in their skeletal muscle, suggesting down-regulation of Δ^9-desaturase activity (74). Similarly, lattisimus muscle of grower pigs fed diets containing 2% CLA had increased stearic acid and reduced oleic acid percentages (99). Oxidative stability of longissimus lumborum muscle was increased in rabbits fed diets containing 0.5% CLA (135). Because oxidative stability is a function of MUFA content, reduced oxidation would imply an increase in SFA with a reduction in the MUFA content as observed in other studies. Reductions in the proportions of MUFA and PUFA with increased SFA also were reported for the breast muscle of broiler chickens fed diets containing CLA for 6 wk (102). Diets enriched in *trans*10,*cis*12-CLA decreased proportions of MUFA in gastrocnemius muscle of male Wistar rats, while *cis*9,*trans*11-CLA had no such effect (136). Data therefore suggests that *trans*10,*cis*12-CLA downregulates Δ^9-desaturation in muscle and is therefore responsible for the altered fatty acid profile observed in animals fed a CLA mixture.

Because skeletal muscle is a primary site for insulin-stimulated glucose uptake, Ryder et al. (60) examined the effect of a CLA mixture on glucose transport in male Zucker Diabetic Fatty rats. Dietary CLA improved insulin-stimulated glucose transport

and glycogen synthase activity in the skeletal muscle, suggesting that the skeletal muscle could be a potential target for therapeutic intervention in diabetics. Obese Zucker rats fed either *trans*10,*cis*12-CLA or a CLA mixture for 3 wk had enhanced insulin-mediated glucose transport and reduced oxidative stress and triglyceride accumulation in soleus muscle (137). The *cis*9,*trans*11-CLA, however, did not affect glucose transport or lipid oxidation, indicating that the metabolic effects observed in the muscle can be attributed to the *trans*10,*cis*12-CLA (137).

Expression of UCP2 was upregulated in the skeletal muscle of CLA-fed rats (60). Skeletal muscle UCP-3 protein was higher in mice fed CLA (95). Because UCP are involved in thermoregulation and lipid metabolism, increased UCP resulting from dietary CLA could perhaps play a major role in the regulation of adiposity in these animals.

In summary, *trans*10,*cis*12-CLA reduces adipose mass by several mechanisms including inhibition of fatty acid uptake, reduced lipogenesis, increased apoptosis, or increased oxidation of fatty acids. However, liver weights and hepatic lipid content increase due to *trans*10,*cis*12-CLA, possibly though an increase in lipogenesis. Ratio of SFA to MUFA is reduced in adipose, liver, muscle, and bone in response to *trans*10,*cis*12-CLA through inhibition of SCD mRNA expression and activity. The response, however, is determined by the dose of CLA and the species in which the studies are conducted. In contrast, *cis*9,*trans*11-CLA has no affect on lipid metabolism. Therapeutic utility of *trans*10,*cis*12-CLA therefore needs to be exploited, especially in conditions such as diabetes and obesity.

Objectives of this study were:

1. To determine the effects of dietary *trans*10,*cis*12-CLA on lipid metabolism in adipose, liver, bone, and muscle in growing male and female mice.
2. To evaluate the effects of a dietary combination of *trans*10,*cis*12-CLA and *trans*-vaccenic acid on SCD desaturation in adipose, liver, bone and muscle of adult mice.

3. To determine the effects of cis9,trans11-CLA, trans10,cis12-CLA or CPT-1 inhibitors on CPT-1 mRNA expression and fatty acid profile in mouse hepatocytes (AML-12) and human hepatoma cells (HepG2)

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CHAPTER 2

Dietary *trans*10,*cis*12-conjugated linoleic acid (CLA) alters muscle and bone fatty acid profile in growing male and female mice

Abstract

The *trans*10,*cis*12 isomer of CLA increases liver weight and reduces mass of adipose depots in mice. To determine the long-term effects of the isomer on fatty acid profiles of bone and muscle, 63 male and 63 female mice (3 to 4 wk of age) were fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA. Seven mice per dietary group within gender were sacrificed after consuming the assigned diet for 2, 4, or 6 wk. Dietary *trans*10,*cis*12-CLA reduced carcass fat in a dose-dependent manner from 1.58 $\pm$ 0.7 g for the control group to 0.71 $\pm$ 0.7 g for the 0.30% *trans*10,*cis*12-CLA group without altering net protein content. Treatments did not affect plasma GH or IGF-1 concentrations. However, plasma IGF-1 concentration decreased with age and was higher in males (417 $\pm$ 8 ng/ml) than females (353 $\pm$ 8 ng/ml). Treatments caused linear increases in 16:0, 18:0, 20:4n-6, 22:4n-6, 22:5n-3 and 22:6n-3 in muscle, with corresponding decreases in 18:1, 18:2, and 18:3n-3. Ratios of 16:0 to 16:1 and 18:0 to 18:1 increased in muscle and bone, but ratio of 18:2n-6 to 20:4n-6 decreased. Fatty acid profile in bone and muscle was age-dependent. Changes included increases in *cis*9-16:1, 18:2n-6, 18:3n-3, 22:5n-3, and 22:6n-3 and decreases in 14:0, 16:0, 17:0, 18:0, 20:0 in muscle over 6 wk. In bone, monounsaturates such as *cis*9-16:1, *cis*9-18:1, and *cis*11-18:1 increased, but 18:2n-6, 18:3n-3, 20:0, 20:3-n5, 20:4n-6, 20:5n-3, and 20:6n-3 decreased with increasing age. Overall, results suggested *trans*10,*cis*12-CLA inhibited Δ^9 desaturase in muscle and bone while enhancing activity of Δ^5 and Δ^6 desaturase.

Key words: desaturase, fatty acids, IGF-1, GH

Introduction

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometrical isomers of linoleic acid with conjugate double bonds, i.e. two double bonds separated by a single bond, and are found in a variety of food sources including milk, fish, and meat. Interest in CLA, especially the *cis*9,*trans*11 and the *trans*10,*cis*12 isomers, has grown in the last decade, because of their beneficial role in suppression of carcinogenesis (1, 2, 3), reduction in atherogenesis (4), prevention of diabetes (5), and modulation of the immune system (6). Apart from the potential health benefits mentioned, CLA isomers also have profound effects on lipid metabolism. Dietary CLA reduced body fat in mice (7, 8), rats (5), and pigs (9, 10). Park et al. (7) reported that CLA exerts direct effects on adipocytes, which are principle sites of fat synthesis and storage, and skeletal muscle cells, which are principle sites of fat combustion. Despite reduction in adipose fat mass (5), dietary CLA did not influence body weight or weight gain in rats (11). Possibly, the reduction in fat mass by CLA is offset by an increase in weights of organs such as liver and intestines.

The metabolic interactions by which CLA exerts effects on cellular functions at the molecular level have not been clearly elucidated. Early work by Lee et al. (12) indicated CLA exerts its effects mainly by reducing the content of monounsaturated fatty acids in tissues, thus shifting the balance between monounsaturated and saturated fatty acids. Li and Watkins (11) demonstrated that dietary CLA decreased monunsaturates in the skeletal muscle, bone marrow, and periosteum in rats. A proper ratio of monounsaturated and saturated fatty acids is essential for maintaining membrane fluidity and disruption of this ratio could be one of the potential mechanisms by which CLA reduces body fat. Early studies (7, 10, 11) utilized a commercial CLA mixture which contained primarily *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA along with a variety of other CLA isomers at low concentrations. Recently, pure sources of either *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA became available for use as diet supplements. Park et al. (13) reported that the *trans*10,*cis*12-CLA isomer reduced body fat mass in mice, thus, implicating it as the isomer associated with lipid metabolism. Apart from reducing adipose tissue depots, *trans*10,*cis*12-CLA also inhibited stearoyl-CoA

desaturase (SCD) in mouse pre-adipocytes (14) and mammary tissue (15). The SCD enzyme catalyzes the conversion of saturated fatty acids)14:0, 16:0, and 18:0) to their cis9,isomer. In addition, SCD provides endogenous conversion of *trans*11-18:1 (*trans*-vaccenic acid) to *cis*9,*trans*11-CLA (16). Inhibition of this key enzyme, therefore, could potentially decrease the monounsaturated fatty acid content of tissues. However, the effects of the pure *trans*10,*cis*12 isomer of CLA on growth and muscle and bone fatty acid profiles in growing male and female mice have not been studied adequately.

Our primary objectives were to determine changes in body weight, carcass composition, tissue weights, and muscle and bone fatty acid profiles in growing male and female mice fed 0, 0.15, or 0.30% *trans*10,*cis*12-CLA over a 6 wk period. Because GH has a lipolytic effect and an anabolic effect mediated through IGF-1, we also evaluated the effect of the CLA isomer on plasma concentrations of GH and IGF-1.

Materials and Methods

Animals, Diets, and Sampling

All procedures involving animals were approved by the Virginia Polytechnic Institute and State University Animal Use and Care Committee. Mice were housed in polypropylene cages in the Laboratory Animal Resources facility with a 12-h light and 12-h dark cycle. Mice had access to drinking water at all times. Sixty-three male and 63 female, 3 to 4 wk old, CD-1 mice were used in a 6-wk feeding trial to evaluate growth, body composition, and fatty acid and hormonal profiles in response to dietary *trans*10,*cis*12-CLA. For 7 d before the start of the trial, mice were fed a Harlan Teklad (Harlan, Madison, WI) diet to which 3% (wt/wt) high-oleic sunflower oil was added. On d 1 of the trial, male and female mice were randomly assigned to receive 3.0 % high-oleic sunflower (control, **0%**), 2.85 % high-oleic sunflower oil + 0.15 % *trans*10,*cis*12-CLA (**0.15%**), or 2.70 % high-oleic sunflower oil + 0.30% *trans*10,*cis*12-CLA (**0.30 %**) (>95% purity, Natural Lipids, Hordebygda, Norway) diet on a wt/wt basis. Thus, CLA replaced a portion of the sunflower oil in the 0.15 % and 0.30 % treatment groups.

Mice were fed once a day at 16:00 h. Food intake was estimated by subtracting the weight of refusals from the weight of food offered (**see appendix figures A-1 and A-2**). Diet samples were collected every week and stored at 4 ^{o}C prior to fatty acid analysis (**Table 2-1**). Body weights were determined twice weekly and the day of sacrifice (**see appendix figures A-3 and A-4)**. Seven mice per dietary treatment group within each gender were sacrificed at wk 2, 4 , and 6 of the experimental period. Mice were anesthetized using metofane (Pitman-Moore, Inc, NJ). Blood was collected by heart puncture into 1.5-ml microfuge tubes. Plasma was harvested by centrifugation at 3000 x *g* for 15 min and stored at -20 ^{o}C prior to hormone assays. Viscera was exposed by dissecting the skin and the underlying membranes from the abdominal cavity through the neck. The head, skin, feet, stomach, intestinal tract, liver, lungs, and heart were removed. The small intestine was flushed thoroughly with water, blotted dry, and weighed. The liver also was weighed. The gastrocnemius muscle and the femur were excised from the left hind limb and stored at -20 ^{o}C prior to fatty acid analysis. The

remainder of the empty carcass was weighed and stored at -20 ^{o}C prior to dry matter and proximate analysis.

Carcasses were dried at 55 ^{o}C until weight was constant. Carcass was ground in a conventional coffee grinder. After grinding, crude protein content of the carcass was estimated by the Kjeldahl procedure (17). Ether extract was also determined (17) to estimate the total lipid content of the carcass.

Plasma IGF-1 and GH concentrations were determined by radioimmunoassay as outlined previously (18, 19). Mouse anti-IGF-1 (1st antibody), goat anti-mouse antiserum (2nd antibody) and radioiodinated IGF-1 were provided by Dr. R. M. Akers (Dairy Science, Virginia Tech.). Mouse GH antigen for iodination, and rat GH antiserum (monkey) were donated by Dr. A. F. Parlow (National Hormone & Peptide Program, Torrance, CA). Anti-monkey gamma globulin was purchased from Antibodies Inc. (Davis, CA). Briefly, plasma IGF-1 was extracted by mixing 100 μl of blood plasma with 900 μl of extraction mixture (87.5% ethanol + 12.5% 2N HCl) and centrifuged at 13,600 x *g* for 10 min in a Micromax microfuge (IEC, Needham Heights, MA). Supernatant was *trans*ferred to a 12 x 75-mm glass tube and 200 μl of 0.855M of Tris base was added to neutralize the sample. Samples were stored at –20 ^{o}C for 1 h, followed by centrifugation at 3,000 x *g* for 30 min (Beckman Instruments, Columbia, MD). Thirty microliters of standards or samples were suspended in assay buffer (30 mM sodium phosphate, 10 mM EDTA, 0.02% protamine sulfate, 0.05% Tween-20, pH 8.0) to a final volume of 500 μl. Subsequently, 100 μl of radiolabeled IGF-1 and 100 μl of 1st antibody were added to each tube and incubated for 24 h at 4 ^{o}C. Thereafter, 100 μl of 2nd antibody was added and incubated for 72 h at 4 ^{o}C. Phosphate buffer saline (PBS) was added to each tube before centrifugation at 1,660 x g for 30 min. Tubes were decanted and the radioactivity was measured by a gamma counter (Model # D5002, Perkin Elmer, Downers Grove, IL).

For GH assay, 100 μl plasma was added directly to the assay buffer to a final volume of 500 μl. The 1st antibody was added on d 1, followed by iodinated GH antigen

on d 2 and 2nd antibody on d 3. Incubations were carried out at room temperature on d 1 and d 2 and at 4 °C on d 3. Centrifugation and decanting were as described for the IGF-1 assay.

Lipids from diets, bone and muscle were extracted using the Folch procedure (20) with modifications (21). Bone samples were scraped carefully under a microscope to remove any adherent muscle or cartilage, weighed, and transferred to glass tubes containing 12 ml of 2:1 chloroform:methanol. A POLYTRON® (Binkmann Instruments Inc, Westbury, NY) homogenizer, fitted with a PTA-10 generator probe, at medium speed was used to homogenize a femur for 30 s. Another 3 ml of 2:1 chloroform:methanol were added and the tubes were stored at 4 °C for 24 h. Muscle samples were weighed and homogenized in 15 ml 2:1 chloroform: methanol with a Tissue-Tearor® (Biospec Products Inc., Racine WI) and incubated at room temperature for 1 h. Diet samples were weighed and mixed with 15 ml 2:1 chloroform: methanol to extract lipids.

Homogenate from either muscle or bone, or diet sample in chloroform-methanol, was filtered into clean 50 ml screw cap tubes using Whatman filter paper No. 1 (Whatman Inc, Clifton, NJ) and 5 ml of 0.88% NaCl was added. Tubes were shaken on a horizontal shaker for 10 min at high speed, then centrifuged at 960 x *g* for 5 min in a swinging bucket centrifuge (IEC, Needham Heights, MA). The top layer was removed by aspiration and the remaining solvent was evaporated under nitrogen in an N-Evap (Model # 112, Organomation Inc., South Berlin, MA) at 40 °C until approximately 2 ml remained. The solvent was transferred to a methylation tube and completely evaporated under nitrogen to obtain the pure lipid fraction. Methylation tubes were capped and stored at -80 °C.

Fatty acids were transesterified according to the method of Park and Goins (22). Briefly, 200 μl dichloromethane, 500 μl hexane, and 2 ml of 0.5 N NaOH in methanol were added. Undecenoic acid (NU-Check Prep, Elysian, MN) was used as an internal standard for peak quantification. The tubes were heated at 90 to 95 °C for 10 to 20 min.

After cooling the tubes to room temperature, 2 ml 14% boron trifluoride in methanol was added. Tubes were again heated and cooled as before. Deionized water (1 ml) and hexane (1ml) were added tubes were shaken on a platform shaker at high speed for 5 min, then tubes were centrifuged for 5 min at 480 x *g* (IEC, Needham Heights, MA). A small amount of dry sodium sulfate was added to a crimp vial and the top hexane layer in the tube was transferred to the vial. Vials were sealed and stored at -80 ^{o}C.

Methyl esters of fatty acids of diets, muscle and bone were separated on an Agilent 6890N gas chromatograph equipped with 7683 autosampler and a flame ionization detector (Agilent Technologies, Palo Alto, CA). Agilent ChemData software was used for data acquisition, integration and quantification.

Diet fatty acid profile was determined by split injection (70:1) onto a 100 m x 0.25 mm i.d. x 0.20 μm film thickness CP-SIL column (Chrompack, Raritan, NJ) fitted with a 2.5 m x 0.25 mm i.d. fused silica methyl deactivated retention gap. Hydrogen at constant pressure was used as the carrier gas and the injector and the detector temperature were 250 ^{o}C and 375 ^{o}C, respectively. A programmed temperature gradient controlled the column temperature. Initial column temperature was 70 ^{o}C (held for 1 min), increased to 100 ^{o}C at 5 ^{o}C per min (held for 2 min), followed by an increase at 10 ^{o}C per min to 175 ^{o}C (held for 40 min), and finally increased at 5 ^{o}C per min to 220 ^{o}C (held for 15 min). Total run time was approximately 81.5 min.

Muscle fatty acid profile was determined by split injection (50:1). Injector and detector temperature were 250 ^{o}C and 300 ^{o}C, respectively. A programmed temperature gradient controlled the column temperature. Initial column temperature was 70 ^{o}C (held for 1 min), increased to 100 ^{o}C at 5 ^{o}C per min (held for 3 min), followed by an increase at 10 ^{o}C per min to 175 ^{o}C (held for 42 min), and finally increased at 5 ^{o}C per min to 220 ^{o}C (held for 19 min). Total run time was approximately 87.5 min.

Methyl esters of fatty acids obtained from bone samples were run splitless using hydrogen as the carrier gas. Initial column temperature was 40 ^{o}C (purge valve closure time was 0.6 min), increased at 40 ^{o}C per min to 100 ^{o}C (held for 10 min), followed by an increase at 25 ^{o}C per min to 175 ^{o}C (held for 45 min), and finally increased at 10 ^{o}C per min to 220 ^{o}C (held for 25 min). Total run time for bone fatty acid quantification was 89.6 min.

Desaturation ratios were calculated based on the amounts (μg of fatty acids per mg of tissue extracted) of substrate and product according to the following equation:

$$\text{Ratio} = \frac{[\text{substrate}]}{[\text{substrate}] + [\text{product}]}$$

Statistical Analysis

Data were analyzed as a completely randomized design with factorial arrangement of treatments using the MIXED procedure of SAS according to the following model:

$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl}$

where Y_{ijkl} is the observation for the l^{th} mouse of the k^{th} gender, receiving the i^{th} treatment, at week j^{th}.

μ = grand mean,

α_i = effect of treatment i [i = 1 (control), 2 (0.15% *trans*10,*cis*12-CLA), or 3 (0.30% *trans*10,*cis*12-CLA)

β_j = effect of week j (j = 2, 4, or 6 wk)

γ_k = effect of gender k (k = male or female)

$(\alpha\beta)_{ij}$ = interaction of treatment and week,

$(\alpha\gamma)_{ik}$ = interaction of treatment and gender,

$(\beta\gamma)_{jk}$ = interaction of week and gender,

$(\alpha\beta\gamma)_{ijk}$ = interaction of treatment, gender, and week,

ε_{ijkl} = error effect associated with Y_{ijkl} (l = 1, ……7 mice).

All data are reported as least square means ± SEM. Pre-planned contrasts were performed to test the linear and quadratic effects of the dose of CLA. Interactions are reported only when significant. Significance level was specified at $P < 0.05$.

Results

Table 2-2 lists the effects of *trans*10,*cis*12-CLA dose on body weight, carcass composition, and tissue weights in male and female mice. Dietary *trans*10,*cis*12-CLA had no effect on body weight. Mean body weight of male and female mice increased irrespective of treatment from 27.2 ± 0.3 at wk 2 to 29.6 ± 0.3 g at wk 6. Overall dry carcass weights increased from 3.6 ± 0.1 at wk 2 to 4.2 ± 0.1 g at wk 6. However, dry carcass weights decreased linearly with increasing dose of *trans*10,*cis*12-CLA (**see appendix figures A-5 and A-6)**. A significant gender by treatment interaction ($P < 0.01$) indicated that females responded to the CLA isomer to a lesser extent than males. Dry carcass weights were 30% lower in males fed 0.30% *trans*10,*cis*12-CLA compared with controls (5.9 ± 0.2 vs. 4.1 ± 0.2g). In contrast, females exhibited only a 13% reduction in response to 0.30% *trans*10,*cis*12-CLA (3.6 ± 0.2 vs. 3.1 ± 0.2g).

Carcass analyses revealed a significant linear decrease in ether extract (EE) percentage and amount (g) in response to dietary *trans*10,*cis*12-CLA. A significant wk by treatment interaction was also noticed (**see appendix figures A-7 and A-8)**. Amount of EE (g) increased from 1.39 ± 0.12 g at 2 wk to 2.07 ± 0.12 g at 6 wk in the control mice, but decreased from 0.64 ± 0.12 g at 2 wk to 0.58 ± 0.12 g at 6 wk in mice fed diets containing 0.30% *trans*10,*cis*12-CLA. Although crude protein (CP) percentage increased linearly with dose of the isomer, the amount of CP in the carcass was not affected. A significant treatment by gender interaction ($P < 0.01$) was noted for the carcass CP percentage of mice. CP percentage was higher in females than in males. Amount of EE and CP was higher in males than females due to the greater weight of the male carcasses. However, EE percentage was similar in both males and females. Amount of EE and CP increased with age, but the percentages of EE and CP did not change (data not shown).

Weights of femur, liver and small intestine, but not muscle, increased linearly with dose of *trans*10,*cis*12-CLA. Tissue weights were higher in males than females and also increased with age in both male and female mice (data not shown).

Plasma IGF-1 and GH were not affected by *trans*10,*cis*12-CLA dose (**Table 2-3**). Males had lower GH and higher IGF-1 concentrations than females. Age related changes in males and females included a decrease ($P < 0.01$) in plasma IGF-1 concentration from 414 $\pm$ 10 at wk 2 to 352 $\pm$ 10 ng/ml at wk 6.

Fatty acid profiles of gastrocnemius muscle (**Table 2-4**) and bone (**Table 2-5**) revealed marked changes in mice fed the *trans*10,*cis*12-CLA containing diet. Total fatty acids per unit weight of muscle and bone decreased linearly with dose of the *trans*10,*cis*12-CLA. Proportions of most saturated fatty acids (16:0, 17:0, and 18:0) and long-chain polyunsaturated fatty acids (20:4n-6, 22:4n-6, 22:5n-3, and 22:6n-3) increased linearly with dose of the CLA isomer in both muscle and bone. Proportion of 14:0 however, increased in bone but not in muscle. Similarly, the proportion of 20:3n-6 in muscle increased, but not in bone, in the mice fed *trans*10,*cis*12-CLA. To compensate for the elevated percentages of saturated and long-chain polyunsaturated fatty acids, there was a linear decline in the proportion of monounsaturated (*cis*9-16:1 and *cis*9-18:1) and polyunsaturated fatty acids (18:2n-6 and 18:3n-3) in both tissues. Dietary *trans*10,*cis*12-CLA was incorporated into both tissues linearly with dose.

Males had higher proportion of unsaturated, but lower saturated fatty acids than females, in both muscle and bone. Ratios of 16:0 to 16:1 and 18:0 to 18:1 in muscle (**Figure 2-1**) and bone (**Figure 2-2**) increased, but ratio of 18:2n-6 to 20:4n-6 decreased linearly in response to dietary treatment.

Age related changes in fatty acid profile of muscle (**Table 2-6**) included increased percentages of *cis*9-16:1, 18:2n-6, 18:3n-3, 22:5n-3, and 22:6n-3 and decreased proportions of 14:0, 16:0, 17:0, 18:0, and 20:0 between wk 2 and 6. In bone (**Table 2-7**), increased percentages of monounsaturates (*cis*9-16:1, *cis*9-18:1, and *cis*11-18:1) and corresponding decreases in 18:2n-6, 18:3n-3, 20:0, 20:3n-6, 20:4n-6, 20:5n-3, and 20:6n-3 were evident between wk 2 and 6. Most of the changes in muscle and bone followed a quadratic pattern, with most of the change occurring between wk 2 and 4 of

the experimental period. Total fatty acid content (μg/mg) of muscle increased by approximately 70% between wk 2 and 4. In contrast, there was no significant change in total fatty acid content of femur during the 6-wk study.

Discussion

Previous studies dealt with the effects of a CLA mixture or pure CLA isomers on body composition and adipose stores. In this study, we focussed on responses to dietary *trans*10,*cis*12-CLA in muscle and bone of growing mice over a 6 wk period. Because dietary treatment groups included male and female mice, the gender effect and the interaction between gender and treatment also could be evaluated.

Food intake and body weight were not affected by *trans*10,*cis*12-CLA in the current study. Although some studies reported no reduction in energy intake (23, 24) or body weight (25, 26), others indicated a reduction in feed intake and body weight (8, 13) in mice fed CLA. Reasons for these discrepancies could be related to the dose of the CLA, the *trans*10,*cis*12-CLA content of the CLA mixture, the length of the treatment period, or the strain of mice as suggested by Hayman et al. (27). Because food intake was not influenced by dietary treatments in the present study, results suggest that trans10,cis12-CLA reduces body fat depot mass by mechanisms other than a reduction in energy intake.

Dry carcass weight decreased linearly in response to *trans*10,*cis*12-CLA and the decrease was attributed mainly to a reduction in the amount of carcass fat. Carcass fat content (g) decreased by 55% in males and females for the 0.30% *trans*10,*cis*12-CLA dose compared with the control group. Although, protein percentage increased linearly due to treatment, the amount (g) of carcass protein was not affected by *trans*10,*cis*12-CLA dose. Park et al. (13) reported an increase in carcass protein percentage of mice fed *trans*10,*cis*12-CLA and suggested this isomer increases lean mass. DeLany et al. (23) observed a significant reduction in carcass fat percentage and amount accompanied by an increase in carcass protein percentage in mice fed 1 % CLA. However, there was no change in the total amount of protein. Decreased carcass weight coupled with a reduction in carcass fat would result in an obligatory increase in protein on a percentage basis.

To explore the possibility that anabolic hormones might be involved in the CLA-mediated reduction in adiposity, plasma levels of GH and IGF-1 were measured. Growth hormone deficient mice are characterized by large adipose stores despite normal energy intake, suggesting importance of GH in lipolysis (30). Furthermore, enhanced bovine GH production in transgenic mice resulted in higher IGF-1 concentration and reduced adipose tissue weights (31). Our data indicated no effect of dietary *trans*10,*cis*12-CLA on plasma GH and IGF-1 concentrations during the 6-wk feeding period. Poulos et al. (32) observed no change in serum IGF-1 in Sprague-Dawley rats fed CLA. Likewise, West et al. (33) reported no differences in plasma GH in AKR/J mice fed 1% CLA compared with the control group. Whereas, Li et al. (34) reported an increase in serum IGF-1 in rats fed CLA, Buison et al. (35) reported a decrease.

The observed gender differences in the relative concentration of GH in the present study could be related to their adiposity. Males had higher carcass fat content and lower GH concentrations than females. Circulating IGF-1 concentration in males, however, was greater than in females. Reasons for the differences in plasma IGF-1 concentrations between males and females cannot be explained from the available data.

Both GH and IGF-1 concentrations decreased over the 6-wk experimental period. Luna et al. (40) observed that plasma GH and IGF-1 concentrations were the highest during puberty. Pubertal age in mice is approximately 6 weeks after birth (41). Mice in the current study were approximately 3 to 4 wk old at start of the 6-wk feeding period and 5 to 6 wk old by the second week of the feeding period. Thus, plasma GH and IGF-1 should be expected to decline during wk 4 through wk 6 of the present study.

Femur weight increased linearly in response to dietary *trans*10,*cis*12-CLA. Increased bone weight could possibly be a result of a decrease in the 20:4n-6 concentration of bone. This might reduce production of eicosanoid products including prostaglandin E_2 (28), which is essential for bone resorption and osteoclastic activity

(29). In the absence of osteoclastic activity, net bone turn over is low and may lead to increased bone forming rate.

Feeding *trans*10,*cis*12-CLA to rats decreased the concentrations 16:1, 18:1, 18:2n-6, 20:2n-6, 20:3n-6, 22:5 n-6 , 20:3 n-6, but increased the concentrations of 22:5n-3, 22:6n-3, total n-3, and saturated fatty acids in muscle, bone marrow, cortical bone and periosteum (11). An elevation in saturated fatty acids, accompanied by a decline in monounsaturated fatty acids, was reported for muscle of growing pigs fed CLA (42). The changes were attributed to a reduction in SCD (Δ^9-desaturase) activity. In the current study, periosteum from the femur was removed and the remaining portion, which included cortical bone and marrow, was analyzed for fatty acids. In bone and muscle, proportions of saturated fatty acids (16:0 and 18:0) increased, while monounsaturated fatty acids (*cis*9-16:1 and *cis*9-18:1) decreased in response to *trans*10,*cis*12-CLA. Ratio of 16:0 to 16:1 and 18:0 to 18:1 increased linearly in both tissues as dose of *trans*10,*cis*12-CLA increased, suggesting inhibition of Δ^9 desaturase activity as proposed by Lee et al. (12).

In addition, proportions of 18:2n-6 decreased while 18:3n-3, 22:5n-3 and 22:6n-3 increased linearly in bone and muscle. Similar observations were reported for rats (11). Increased concentrations of n-3 fatty acids could possibly be due to their conservation in the bone or an increased utilization of n-6 fatty acids (11). An increase in the proportion of 20:4n-6 in bone and muscle was noted in this study, but it was accompanied by a linear decrease in the ratio of 18:2n-6 to 20:4n-6. Similar increase in 20:4n-6 was observed in mammary tissue of mice fed CLA (43). The Δ^5 and Δ^6 desaturases are key enzymes for the synthesis of arachidonic acid (20:4n-6) (44). Conversion of 18:2n-6 to 20:4n-6 involves desaturation at the Δ^5 and Δ^6 positions in the carbon skeleton and a 2-carbon elongation step (45). Mice fed CLA diets exhibited increased Δ^5 and Δ^6 desaturase mRNA expression in the liver compared with the controls (46). Therefore, the decreased ratio of 18:2n-6 to 20:4n-6 observed in the current study could be the result of increased mRNA expression and activity of Δ^5 and Δ^6 desaturases in muscle and bone. Increased Δ^5 and Δ^6 desaturase activity might

possibly be a mechanism to compensate for the apparent decrease in Δ^9 desaturase activity.

Dietary *trans*10,*cis*12-CLA caused a linear decrease in total fatty acids in muscle in the present study. Mice fed a CLA mixture exhibited higher carnitine palmitoyltransferase-1 (CPT-1) activity in their skeletal muscle (7). Because CPT-1 is a key enzyme involved in mitochondrial fatty acid oxidation, enhancement of CPT-1 activity by CLA in muscle might account for the decrease in total fatty acids observed in the present study.

Dietary *trans*10,*cis*12-CLA also caused a decrease in the total fatty acid content of bone. Although the majority of fatty acids in bone reflect the fatty acid content of the diet, a few are formed by *de novo* synthesis within the bone (47). In experiments where lipid-modulating substances such as CLA are used, bone fatty acid profile also might depend on fatty acid metabolism by other tissues and the net flux of fatty acids from these tissues to bone. For example, liver total fatty acid content was increased in mice fed CLA (48, 49). Apart from increased lipogenesis and decreased oxidation of fatty acids, factors such as increased uptake and decreased secretion of fatty acids into the blood stream could also contribute to lipid accumulation in liver. Reduced fatty acid concentrations in circulation therefore might lead to decrease in the total amount of fatty acids taken up by the bone.

This was the first study to evaluate gender and age-related changes in muscle and bone fatty acid profiles in mice. Females had a greater percentage of saturated fatty acids in muscle and bone than males. In contrast, males had a higher proportion of monounsaturated fatty acids. The difference could be attributed to an increased lipid peroxidation rate in skeletal muscle of males (50). Enhanced lipid oxidation in muscle results in oxidation of saturated fatty acids and a build up of monounsaturated fatty acids in the phospholipid fraction of the plasma membrane (51).

Aging was associated with an increase in fatty acid uptake and elevated triglyceride content in skeletal muscle of rats (Tucker and Turcotte, 2003). Findings from the current study also indicate a linear increase in total fatty acid content (g) of the gastrocnemius muscle with age. An increase in the proportion of mono and polyunsaturated fatty acids with age was noticed in the muscle relative to saturated fatty acids. Endogenous production of unsaturated fatty acids could possibly cause a progressive dilution of saturated fatty acids (52), thus altering the ratio of saturated to unsaturated fatty acids. Total fatty acid content of bone was not influenced by age in the present study. However, monounsaturates increased in bone with age followed by a decrease in eicosanoids including 20:4n-6 over the 6-wk period.

Conclusions

Dietary *trans*10,*cis*12-CLA decreased carcass fat without affecting the amount of carcass protein. Body weight and feed intake were not influenced by the CLA isomer indicating that the reduction in body fat is achieved by mechanisms other than a reduction in energy intake. GH and IGF-1 do not appear to play a role in the CLA-mediated reduction in body fat. Plasma GH and IGF-1 concentrations, and muscle and bone fatty acid profiles, however, were influenced by gender and age.

*Trans*10,*cis*12-CLA decreased Δ^9-desaturase activity in muscle and bone while increasing activities of Δ^5 and Δ^6- desaturases in these tissues. Because muscle has a large capacity for oxidizing lipids, the effects of the CLA isomer on this tissue should be explored further. Also, the role of *trans*10,*cis*12-CLA in mediating inflammatory and immune responses via eicosanoid production by bone requires further investigation.

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Table 2-1. Fatty acid composition of diets fed to growing male and female mice for 6 wk

Fatty acid	Dietary *trans*10,*cis*12-CLA		
	0 %	0.15 %	0.30 %
	% total fatty acids		
10:0	0.02	0.02	0.02
12:0	0.04	0.05	0.05
14:0	0.12	0.13	0.12
16:0	9.26	9.41	9.18
*cis*9-16:1	0.18	0.20	0.19
18:0	2.49	2.65	2.52
*trans*11-18:1	0.02	0.02	0.02
*cis*9-18:1	34.41	33.09	31.77
18:2n-6	47.34	46.54	46.28
*cis*9,*trans*11-18:2	0.02	0.18	0.32
*trans*10,*cis*12-18:2	0.00	1.37	2.67
18:3n-3	3.72	3.57	3.77
20:0	0.39	0.39	0.38
20:4n-6	0.02	0.02	0.03
Total (μg/mg of diet)	44.07	44.76	46.14

Table 2-2. Average body weight, carcass composition and tissue weights in male and female mice fed *trans*10,*cis*12-CLA for 6 wk

	Dietary *trans*10,*cis*12-CLA								
	0 %	0.15 %	0.30 %	SEM	P <	Male	Female	SEM	P <
Body weight (g)	28.5	28.5	28.5	0.3	0.99	32.1	24.9	0.2	0.01
Dry carcass (g)	4.75	3.74	3.62	0.11	0.01*	4.77	3.31	0.59	0.01
• CP %	47.2	57.3	61.7	0.70	0.01*	54.38	57.70	0.57	0.01
• CP (g)	2.24	2.13	2.23	0.05	0.26	2.51	1.89	0.04	0.01
• EE %	32.16	23.85	19.26	1.18	0.01*	26.16	24.02	0.97	0.12
• EE (g)	1.58	0.90	0.71	0.07	0.01*	1.31	0.82	0.05	0.01
Gastrocnemius muscle (g)	0.17	0.17	0.18	0.004	0.24	0.20	0.15	0.003	0.01
Femur (mg)	60.60	66.12	64.33	1.11	0.01**	70.13	57.24	0.90	0.01
Liver (wet) (g)	1.77	1.95	2.07	0.04	0.01*	2.23	1.62	0.03	0.01
Liver (dry) (g)	0.55	0.61	0.65	0.01	0.01*	0.70	0.50	0.01	0.01
Small intestine (g)	1.60	1.66	1.74	0.02	0.01*	1.74	1.60	0.02	0.01

*Linear effect of dietary *trans*10,*cis*12-CLA
**Linear and quadratic effect of dietary *trans*10,*cis*12-CLA

Table 2-3. Average GH and IGF-1 concentrations in blood plasma of male and female mice fed *trans*10,*cis*12-CLA for 6 wk

	Dietary *trans*10,*cis*12-CLA								
	0 %	0.15 %	0.30 %	SEM	P <	Male	Female	SEM	P <
GH (ng/ml)	8.4	9.3	6.2	1.4	0.27	6.4	9.5	1.1	0.05
IGF-1 (ng/ml)	387	390	378	10	0.69	417	353	8	0.01

Table 2-4. Fatty acid composition of gastrocnemius muscle of male and female mice fed *trans*10,*cis*12-CLA for 6 wk

	Dietary *trans*10,*cis*12-CLA								
Fatty acid	0 %	0.15 %	0.30 %	SEM	P<	Male	Female	SEM	P<
	% total fatty acids					% total fatty acids			
14:0	0.87	0.84	0.73	0.03	0.01*	0.77	0.86	0.03	0.02
*cis*9-14:1	0.06	0.04	0.04	0.004	0.01**	0.05	0.05	0.003	0.67
16:0	19.4	21.2	22.8	0.29	0.01*	20.63	21.66	0.24	0.01
*cis*9-16:1	4.09	2.85	2.48	0.14	0.01**	3.33	2.95	0.12	0.02
17:0	0.21	0.26	0.28	0.01	0.01*	0.24	0.25	0.009	0.33
18:0	5.81	6.89	7.78	0.29	0.01*	6.53	7.12	0.23	0.07
*cis*9-18:1	36.14	33.77	28.59	0.69	0.01*	32.55	33.11	0.56	0.48
*trans*11-18:1	0.03	0.04	0.04	0.003	0.04	0.04	0.03	0.003	0.01
18:2n-6	20.95	18.45	17.99	0.40	0.01**	20.14	18.12	0.33	0.01
*cis*9,*trans*11-18:2	0.04	0.05	0.05	0.004	0.03*	0.04	0.05	0.003	0.01
*trans*10,*cis*12-18:2	0.009	0.36	0.59	0.02	0.01**	0.28	0.35	0.01	0.01
18:3n-3	0.54	0.39	0.38	0.01	0.01**	0.47	0.40	0.01	0.01
20:0	0.24	0.14	0.16	0.01	0.01**	0.18	0.18	0.01	0.67
20:3n-6	0.23	0.29	0.35	0.02	0.01*	0.33	0.25	0.01	0.01
20:4n-6	2.70	3.64	4.77	0.21	0.01*	3.81	3.59	0.17	0.36
22:4n-6	0.36	0.53	0.72	0.03	0.01*	0.56	0.51	0.02	0.22
22:5n-3	0.43	0.67	0.91	0.04	0.01*	0.75	0.59	0.03	0.001
22:6n-3	3.21	4.53	5.88	0.30	0.01*	4.28	4.80	0.24	0.13
Total (μg/mg of muscle)	21.69	13.50	11.26	0.89	0.01**	16.11	14.85	0.89	0.23

*Linear effect of dietary *trans*10,*cis*12-CLA
**Linear and quadratic effect of dietary *trans*10,*cis*12-CLA

Table 2-5. Fatty acid composition of femur of male and female mice fed *trans*10,*cis*12-CLA for 6 wk

Fatty acid	Dietary *trans*10,*cis*12-CLA			SEM	P<	Male	Female	SEM	P<
	0 %	0.15 %	0.30 %						
	% total fatty acids					% total fatty acids			
14:0	1.34	1.57	1.52	0.03	0.01**	1.42	1.54	0.02	0.01
16:0	21.78	22.67	23.92	0.39	0.01*	22.23	23.35	0.32	0.01
*cis*9-16:1	4.74	4.74	3.90	0.20	0.01*	4.84	4.08	0.17	0.01
17:0	0.28	0.29	0.33	0.01	0.01*	0.29	0.31	0.01	0.27
18:0	8.49	8.71	10.08	0.38	0.01*	8.53	9.66	0.23	0.01
*cis*9-18:1	34.18	32.84	30.26	0.62	0.01*	32.28	32.59	0.51	0.67
*trans*11-18:1	0.031	0.029	0.034	0.001	0.01**	0.03	0.03	0.001	0.14
18:2n-6	16.39	15.60	14.67	0.39	0.01*	16.30	14.80	0.32	0.01
*cis*9,*trans*11-18:2	0.05	0.06	0.09	0.02	0.24	0.05	0.09	0.01	0.05
*trans*10,*cis*12-18:2	0.08	0.20	0.39	0.05	0.01*	0.26	0.19	0.04	0.24
18:3n-3	0.30	0.26	0.22	0.01	0.01*	0.29	0.23	0.01	0.01
20:0	0.25	0.23	0.25	0.008	0.08	0.24	0.25	0.007	0.39
20:3n-6	0.27	0.27	0.28	0.01	0.72	0.29	0.26	0.01	0.07
20:4n-6	3.39	3.63	4.30	0.23	0.02*	3.95	3.60	0.19	0.20
22:4n-6	0.69	0.77	0.94	0.04	0.01*	0.82	0.77	0.04	0.42
22:5n-3	0.20	0.22	0.28	0.01	0.01*	0.25	0.21	0.01	0.03
22:6n-3	0.87	1.02	1.22	0.07	0.01*	1.05	1.02	0.06	0.79
Total (μg/mg of femur)	3.76	3.21	2.49	0.17	0.01*	3.33	2.98	0.14	0.08

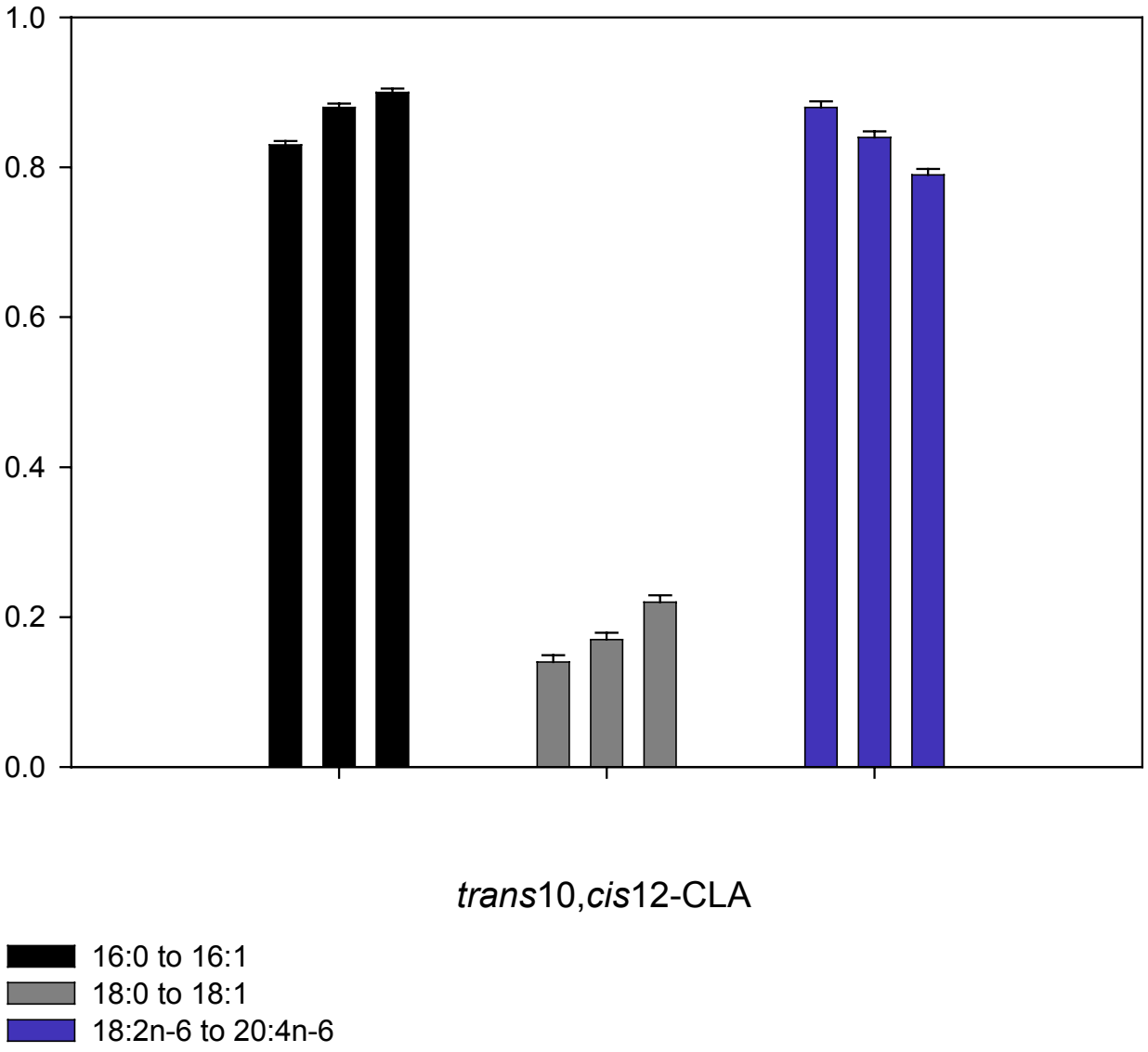
*Linear effect of dietary *trans*10,*cis*12-CLA
**Linear and quadratic effect of dietary *trans*10,*cis*12-CLA

Table 2-6. Fatty acid composition of gastrocnemius muscle of growing mice at 2, 4, and 6 wk of the experimental period

Fatty acid	Wk 2	Wk 4	Wk 6	SEM	P<
		% total fatty acids			
12:0	0.29	0.76	0.64	0.04	0.01
14:0	1.09	0.63	0.72	0.03	0.01
16:0	23.18	19.75	20.50	0.29	0.01
*cis*9-16:1	2.92	3.06	3.44	0.14	0.03
17:0	0.35	0.18	0.22	0.01	0.01
18:0	7.42	6.49	6.56	0.28	0.04
*cis*9-18:1	33.94	32.47	32.10	0.69	0.15
*trans*11-18:1	0.04	0.03	0.04	0.003	0.04
18:2n-6	16.55	20.42	20.41	0.40	0.01
*cis*9,*trans*11-18:2	0.06	0.05	0.04	0.003	0.01
*trans*10,*cis*12-18:2	0.29	0.34	0.33	0.02	0.08
18:3n-3	0.35	0.47	0.49	0.01	0.01
20:0	0.20	0.19	0.16	0.01	0.05
20:3n-6	0.32	0.28	0.27	0.01	0.05
20:4n-6	3.67	3.95	3.49	0.21	0.30
22:4n-6	0.55	0.57	0.49	0.03	0.20
22:5n-3	0.53	0.76	0.74	0.04	0.01
22:6n-3	3.17	5.39	5.06	0.30	0.01
Total (μg/mg of muscle)	10.54	18.72	17.19	0.89	0.01

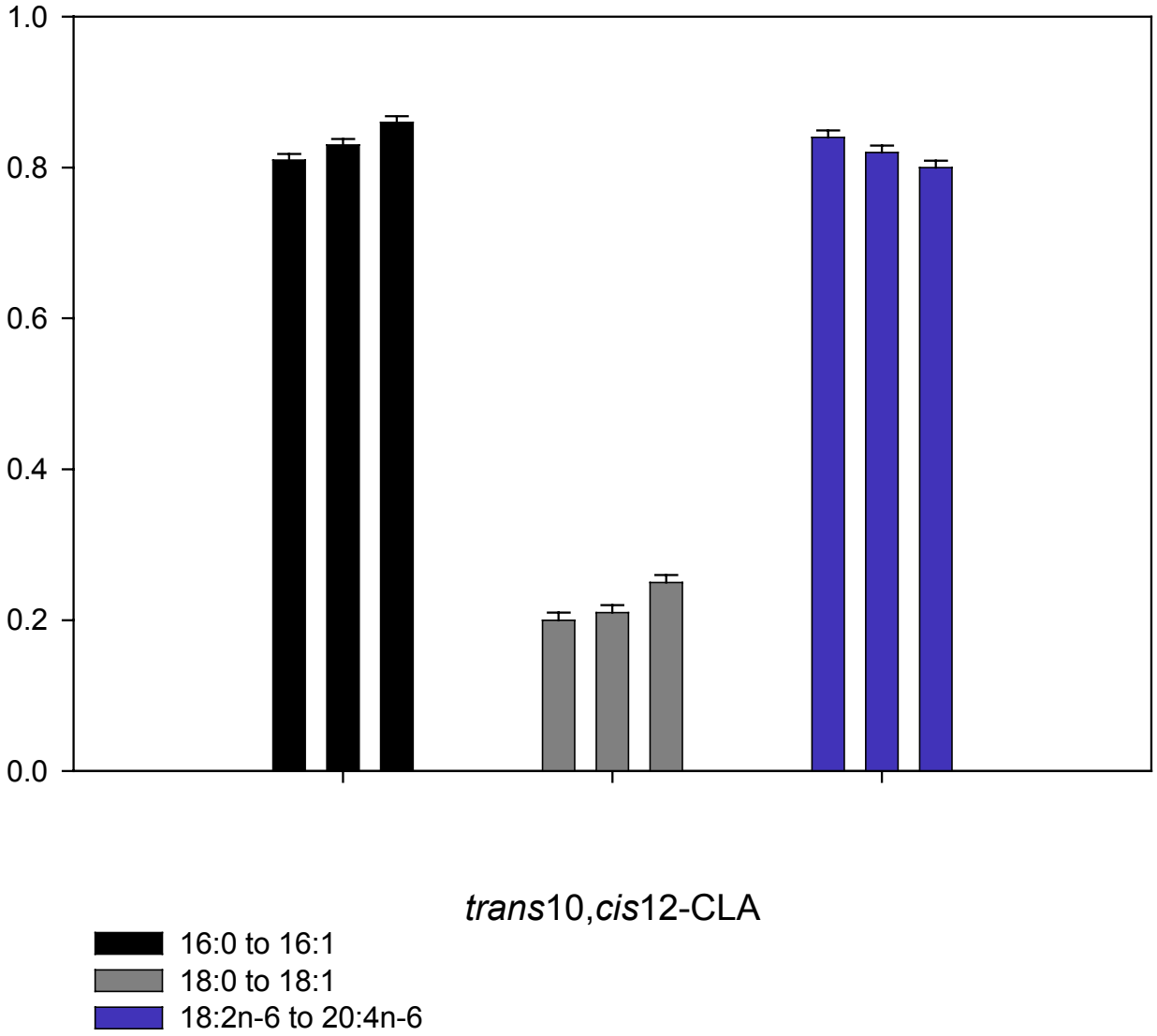
Table 2-7. Fatty acid composition of femur of growing mice at 2, 4, and 6 wk of the experimental period

Fatty acid	Wk 2	Wk 4	Wk 6	SEM	P<
		% total fatty acids			
14:0	1.36	1.62	1.45	0.03	0.01
16:0	22.37	23.41	22.59	0.39	0.15
*cis*9-16:1	3.56	4.92	4.91	0.21	0.01
17:0	0.30	0.30	0.30	0.01	0.92
18:0	9.61	8.87	8.81	0.38	0.27
*cis*9-18:1	27.51	34.69	35.10	0.62	0.01
*cis*11-18:1	2.04	2.18	2.24	0.03	0.01
*trans*11-18:1	0.031	0.035	0.028	0.001	0.01
18:2n-6	16.84	14.66	15.15	0.40	0.01
*cis*9,*trans*11-18:2	0.10	0.05	0.05	0.01	0.03
*trans*10,*cis*12-18:2	0.32	0.19	0.17	0.05	0.09
18:3n-3	0.32	0.23	0.23	0.01	0.01
20:0	0.26	0.23	0.24	0.008	0.03
20:3n-6	0.42	0.22	0.19	0.01	0.01
20:4n-6	6.36	2.41	2.54	0.24	0.01
22:5n-3	0.34	0.15	0.20	0.01	0.01
22:6n-3	1.83	0.60	0.68	0.07	0.01
Total (μg/mg of femur)	3.41	3.07	2.98	0.17	0.18



- Linear effect for all ratios ($P < 0.05$)

Figure 2-1. Muscle fatty acid ratios in response to dietary *trans*10,*cis*12-CLA



- Linear effect for all ratios (P < 0.05)

Figure 2-2. Bone fatty acid ratios in response to dietary *trans*10,*cis*12-CLA

CHAPTER 3

*Trans*10,*cis*12-CLA reduces FAS and SCD mRNA expression in the adipose and alters liver fatty acid profile in growing mice

Abstract

Dietary conjugated linoleic acid (CLA) mixtures alter adipose and liver weights and lipid metabolism in mice. The objectives of this study were to determine changes in liver weight and fatty acid profile due to dietary *trans*10,*cis*12-CLA, the effect of this CLA isomer on fatty acid synthase (FAS) mRNA expression in adipose, and the effect of this CLA isomer on stearoyl-CoA desaturase (SCD) mRNA expression in adipose and liver. Sixty three male and 63 female mice (3 to 4 wk of age) were fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA. Seven mice per dietary group within gender were sacrificed after 2, 4, or 6 wk. Dietary *trans*10,*cis*12-CLA caused linear reductions in adipose tissue weights in both males and females but the effect was more pronounced in males (81% vs. 52%). Expression of FAS mRNA and SCD mRNA in adipose decreased linearly with dose of the CLA isomer. Dry liver weights increased linearly due to dietary *trans*10,*cis*12-CLA from 0.55 $\pm$ 0.01 in the control mice to 0.65 $\pm$ 0.01 g in mice fed diets containing 0.30% *trans*10,*cis*12-CLA. Total fatty acids in liver increased by 32% for the groups fed 0.30% *trans*10,*cis*12-CLA compared with the controls. The proportion of *cis*9-18:1 in liver increased linearly, while the proportion of 18:0 decreased linearly with dose of dietary *trans*10,*cis*12-CLA. Thus, the ratio of 18:0 to 18:1 decreased. Although the amount of liver SCD mRNA was not affected by *trans*10,*cis*12-CLA, results suggest an increase in Δ^9-desaturase activity. The ratio of 18:3n-3 to 20:5n-3 in liver increased due to treatment, suggesting reduction in Δ^5 and Δ^6-desaturation in the liver. Reduced FAS expression and lipogenesis in adipose may be primary factors inhibiting body fat deposition in mice fed *trans*10,*cis*12-CLA, but primary factors responsible for excess hepatic fatty acid deposition remain to be determined.

Key words: *trans*10,*cis*12-CLA, mice, desaturation, lipogenesis

Introduction

Mixtures of conjugated linoleic acid (CLA) isomers may have beneficial health effects in mice, rats, hamsters, and humans. One of the suggested benefits may be a reduction in the incidence or development of obesity. Diets containing a CLA mixture with nearly equal amounts of the *cis*9,*trans*11-CLA and *trans*10,*cis*-12-CLA caused a significant reduction in adipose tissue stores in pigs (1), mice (2), rats (3), hamsters (4) and humans (5). Hayman et al. (6) reported that diets containing greater than 0.5% CLA elicited a significant response in terms of body fat reduction in growing mice. Park et al. (7) demonstrated that *trans*10,*cis*12-CLA is the specific isomer responsible for decreasing body fat. Reductions in mass of white adipose tissue of mice fed a 1% CLA mixture for 5 months were observed by Tsuboyama- Kasaoka et al. (8). However, a significant enlargement of the liver was noted in their study. DeLany and West (9) also reported increased liver weights in mice fed a high dose of CLA.

The observed reduction in body fat was attributed to inhibition of lipoprotein lipase (2), increased apoptosis (8), decreased PPAR-gamma mRNA expression (10) in white adipose tissue, and an increase in energy expenditure (11). Choi et al. (12) demonstrated reduction in stearoyl-CoA desaturase gene expression in 3T3-L1 adipocytes by the *trans*10,*cis*12-CLA without an effect on other lipogenic enzymes such as fatty acid synthetase (FAS). Inhibition of SCD in adipocytes also resulted in an increase in the ratio of saturated to monounsaturated fatty acids. A dietary CLA mixture increased glucose-6-phosphate dehydrogenase and malic enzyme activities in the fat tissue of weaned pigs but did not affect FAS activity (13). However, lactating dairy cows showed a marked reduction in mRNA expression of FAS and SCD in response to CLA treatment (14, 15). Information on the effects of diets containing small amounts of the pure *trans*10,*cis*-12 CLA on expression of FAS and SCD mRNA and fatty acid profiles of liver and white adipose of mice is not available. Thus, the present study therefore, focussed on the effects of feeding 0, 0.15, or 0.30% *trans*12,*cis*12-CLA on the liver and adipose of growing male and female mice over a 6-wk period.

Materials and Methods

Animals, Diets, and Sampling

All procedures involving animals were approved by the Virginia Polytechnic Institute and State University Animal Use and Care Committee. Mice were housed according to the procedures outlined in Chapter 2. Sixty-three male and 63 female (3 to 4 wk old) CD-1 mice (same mice as in Chapter 2) were used in a 6 wk feeding trial to evaluate liver and adipose responses to dietary *trans*10,*cis*12-CLA. Mice were assigned to diets containing 0, 0.15 or 0.30 % *trans*10,*cis*12-CLA as described in the previous chapter.

Seven mice per treatment group within each gender were sacrificed at wk 2, 4 , and 6 of the experimental period. All instruments used for sacrifice and tissue removal were treated with RNase Zap (Ambion, Inc. TX) to remove contaminating RNAses. Mice were anesthetized using Metofane® (PITMAN-MOORE, INC, NJ). Viscera was exposed by dissecting the skin and the underlying membranes from the abdominal cavity through the neck. Liver was collected, cleaned with 1:1000 diethyl pyrocarbonate (DEPC) (Sigma, St. Louis, MO) treated water, and weighed. A portion of the liver was cut and frozen immediately in liquid nitrogen for mRNA analysis. It was later stored at -80^{o}C. The remainder was stored at -20 ^{o}C and used for dry matter and fatty acid analysis. Adipose tissue from the inguinal region in females and the epididymal region in males was collected and treated similar to the liver. For estimating dry matter, liver samples were dried at 55 ^{o}C until they maintained a constant weight.

Fatty acid analyses

Lipids from liver were extracted using the Folch procedure (16) with modifications (17) and the resulting fatty acids were transesterified according to the method of Park and Goins (18) as described in Chapter 2. Methyl esters of fatty acids of liver were separated on an Agilent 6890N gas chromatograph equipped with 7683 autosampler and a flame ionization detector (Agilent Technologies, Palo Alto, CA). Agilent ChemData software was used for data acquisition, integration and quantification. Liver

fatty acid profile was determined by split injection (70:1) onto a 100 m x 0.25 mm i.d. x 0.20 μm film thickness CP-SIL column (Chrompack, Raritan, NJ), fitted with 2.5 m x 0.25 mm i.d. fused silica methyl deactivated retention gap, using a programmed temperature gradient method. Hydrogen at constant pressure was used as the carrier gas and the injector and the detector temperatures were 250 ^{o}C and 300 ^{o}C, respectively. Undecenoic acid (NU-Check Prep, Elysian, MN) was used as an internal standard. Initial column temperature was 70 ^{o}C (held for 1 min), increased to 100 ^{o}C at 10 ^{o}C/min (held for 3 min), followed by an increase at 10 ^{o}C/min to 175 ^{o}C (held for 40 min), and finally increased at 5 ^{o}C/min to 220 ^{o}C (held for 17 min). Total run time was approximately 80.5 min. Deaturation ratios were calculated as described in Chapter 2.

RNA, DNA, and Protein

Adipose and liver RNA were extracted using the TRI REAGENT® (MRC, Cincinnati, OH). Briefly, tissue samples were weighed and homogenized in TRI REAGENT (1 ml/50-100 mg tissue) using a Tissue-Tearor® (Biospec Products Inc., Racine WI) at high speed for 30 s. Homogenate was stored for 5 min to permit complete dissociation of nucleoprotein complexes. Next, 0.2 ml chloroform was added to the tubes, vortexed, stored for 5 min, and centrifuged at 12,000 x *g* for 15 min at 4 ^{o}C. Following centrifugation, the aqueous phase was transferred to a new tube and RNA was precipitated by mixing with 0.5 ml isopropanol and centifugation at 12,000 x g for 8 min at 4 ^{o}C. The interphase and the lower organic phase were stored at 4 ^{o}C for subsequent DNA and protein isolation. Supernatant was removed and the RNA pellet was washed by vortexing with 75% ethanol and centrifugation at 7,500 x *g* for 5 min. After the ethanol was removed, the RNA pellet was dissolved in FORMAzol® (MRC, Cincinnati, OH) and stored at -80 ^{o}C. Absorbance (A) of RNA was measured on a spectrophotometer (Model # 121-0002, Hitachi Instruments Inc. San Jose, CA) at a wavelength of 260 nm and the concentration was determined by the following equation:

$$\text{RNA } (\mu g/\mu l) = \frac{A_{260} * \text{dilution factor} * 40}{1000}$$

where A_{260} = absorbance at 260 nm,

40 = absorbance unit for RNA

1000 = conversion factor from ng to μg.

DNA was extracted from the interphase and the organic phase with ethanol. Briefly, 3 ml of 100 % ethanol was added per 1 ml of TRI REAGENT used for initial homogenization. Samples were stored at room temperature for 3 min and centrifuged at 2,000 x *g* for 5 min at 4 ^{o}C. The phenol-ethanol supernatant was removed and stored at 4 ^{o}C for subsequent protein isolation. The DNA pellet obtained was washed twice in 1 ml of solution containing 0.1 M sodium citrate in 10 % ethanol. Tubes were then centrifuged at 2,000 x *g* for 5 min at 4 ^{o}C. The DNA pellet was suspended in 75% ethanol, stored for 20 min and centrifuged at 2,000 x g for 5 min. The pellet was air-dried and dissolved in 8 mM NaOH. Absorbance was determined on a spectrophotometer. Concentration was calculated using the following equation:

$$\text{DNA } (\mu g/\mu l) = \frac{A_{260} * \text{dilution factor} * 50}{1000}$$

where A_{260} = absorbance at 260 nm,

50 = absorbance unit for DNA

1000 = conversion factor from ng to μg.

Proteins were isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol. Briefly, 1.5 ml of isopropanol was added to the solution. Samples were stored for 10 min and centrifuged at 12,000 x *g* for 10 min at 4 ^{o}C. Supernatant was removed and the protein pellet was washed thrice in 2 ml of a solution containing 0.3 M guanidine hydrochloride in 95 % ethanol. The protein pellet was then mixed with 2 ml 100% ethanol and precipitated by centrifuging at 7,500 x *g* for 5 min at 4 ^{o}C. The pellet was dissolved in 1 % sodium dodecyl sulfate to extract soluble proteins. Insoluble material was precipitated by centrfugation at 10,000 x *g* for 10 min at 4 ^{o}C. Proteins in the supernatant were assayed using the BCA Protein Kit (Pierce, Rockford, IL) and the concentration was determined using a microplate spectrophotometer (Bio-Tek Intsruments Inc, Winooski, VT) at 560 nm.

Plasmid amplification and isolation of cDNA probes

The pmFAS/CR11 plasmid containing the FAS fragment was obtained from Dr. Hitoshi Shimano (Department of Internal Medicine, University of Tsukuba, Japan). The pkk160 plasmid containing the SCD fragment was donated by Dr. James Ntambi (Department of Biochemistry, University of Wisconsin-Madison, WI). The pDrive cloning vector containing the rat β-actin was given by Dr. William Huckle (Department of Biomedical Sciences and Pathobiology, Virginia Tech.).

HB101 E.coli competent cells were used for transformation and amplification of plasmids to generate sufficient quantities of the vector containing inserts (Promega corporation, Madison, WI). A small quantity of plasmid DNA (10 to 50 ng) was added to 100 μl of competent cells in a microfuge tube and incubated on ice for 10 min. Cells were subjected to heat-shock by placing the tube in a 42 ^{o}C water bath for 90 s and immediately cooling in ice for 2 min. Cold SOC medium (900 μl) was added to each transformation reaction and incubated for 1 h at 37 ^{o}C without shaking. The SOC is a highly nutritious media and aids in the growth of transformed bacterial cells. One hundred microliters of the transformed cells were plated on LB agar plates containing 100 μg/ml amplicillin. Ampicillin was the antibiotic of choice because the plasmids carried the gene for ampicillin resistance. Therefore, only the bacterial cells transformed with the plasmid grew in the presence of ampicillin. Plates were placed upside down in a 37 ^{o}C incubator for 12 to16 h. A single colony was picked from the plate using a flamed inoculating loop, swirled into 3 ml LB broth containing 100 μg/ml ampicillin, and incubated at 37 ^{o}C with shaking (220 rpm) for 8 to 12h. Cells were pelleted by centrifugation at 4,000 x *g* for 5 min at 4 ^{o}C.

Plasmid DNA was purified using the QIAprep® Spin Miniprep Kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. Insert cDNA was isolated by digesting the plasmid with restriction enzymes corresponding to the restriction sites flanking the insert and gel electrophoresis on a 1 % agarose gel at 100 V for 4 h. The DNA bands stained with ethidium bromide were visualized on an UV transilluminator

(Fisher Scientific Instruments, Pittsburgh, PA). The band corresponding to the cDNA of interest was carefully ex*cis*ed from the gel and DNA was extracted using the QIAquick® gel extraction kit (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. The DNA obtained was further purified using phenol-chloroform. Briefly, 100 μl of 1:1 phenol-chloroform solution saturated with Tris was added to the tube containing DNA suspended in 100 μl Tris-EDTA (TE) buffer, vortexed, and spun at 10,000 x *g* for 1 min. Supernatant was transferred to a new tube and 100 μl of 100 % chloroform was added to the tube and centrifuged at 10,000 x *g* for 1 min. The top layer was again transferred to a new tube and 10 μl of 3M sodium acetate and 275 μl of ethanol were added and mixed. The tube was stored overnight at -20 ^{o}C and the DNA was pelleted by centrifuging at 10,000 x g for 30 min. Pellet was washed with 70 % ethanol and suspended in 50 μl TE buffer. Concentration of DNA was determined as described previously.

Northern analysis for detection of SCD and FAS mRNA

Total RNA was separated by electrophoresis on a 1% agarose gel containing 0.66 M formaldehyde. Approximately, 25 μg of total RNA was denatured by heating at 55 ^{o}C for 15 min and loaded into each well of the gel, and the gel was run at 120 V for approximately 4 h. Ethidium bromide stained 18S and 28S bands were visualized on an UV transilluminator. The RNA was transferred onto a Magna Charge nylon membrane (MSI, Westborough, MA) by downward capillary blotting for 4 h, using an alkaline transfer buffer (5X SCC, 10 mM NaOH, and distilled de-ionized water). Thereafter, the RNA was crosslinked to the membrane by UV irradiation at 1200 mW/cm^2 for 30 seconds.

Specific mRNA bands on the nylon membrane were detected using the DIG High Prime DNA Labeling and Detection Starter Kit 2 (Roche Diagnostics Corporation, Indianapolis, IN) (19). Modifications were made where necessary to obtain higher quality exposure and low background levels. The principle behind the DIG labeling and detection procedure involves labeling of cDNA probes with digoxigenin (DIG), a steroid hapten. DIG dUTP incorporates between every 20 to 25 nucleotides in the newly

synthesized DNA. DIG labeled cDNA probes are then hybridized to the corresponding mRNA on the membrane and the hybrids subsequently detected by enzyme immunoassay.

The DIG-High Prime labeling reaction mixture contained a 5X concentrated mixture of random hexamers, dNTP mix containing alkali-labile Digoxigenin (DIG), labeling grade Klenow enzyme, and an optimized labeling buffer. Autoclaved, double distilled water was added to 100 ng of the probe in a microfuge tube to a total volume of 16 μl and heated in a boiling water bath for 10 min followed by rapid cooling to denature the DNA. DIG-High Prime labeling mix (4 μl) was added to the DNA, mixed and incubated at 37 ^{o}C for 16 h. The labeling reaction was stopped by adding 2 μl 0.2 M EDTA and the tubes were stored at -20 ^{o}C until used. Efficiency of the labeling reaction and the quantity of labeled probe obtained was determined by analyzing a series of dilutions of the labeled probe against a known quantity of control DNA.

The nylon membrane containing RNA bands separated by electrophoresis was pre-hybridized with 30 ml of DIG Easy Hyb (Roche Diagnostics Corporation, Indianapolis, IN) at 50 ^{o}C for 30 min prior to incubation in the hybridization solution. DIG-labeled cDNA probe (denatured by boiling and rapid), was added to 25 to 30 ml of prewarmed DIG Easy Hyb at a concentration of 50 ng/ml to make up the hybridization solution. The nylon membrane was incubated in the hybridization solution for 16 h in a 50 ^{o}C water bath. The membrane was washed in ample 2X SSC and 0.1% SDS at room temperature for 5 min, then washed twice in 0.5X SSC and 0.1% SDS at 66 ^{o}C for 15 min with shaking to remove the unbound probe. Washes were followed by incubation in 100 ml 1X Blocking solution (Roche Diagnostics Corporation, Indianapolis, IN) and 25 ml Antibody solution (1:10,000 Anti-Digoxigenin-Alkaline Phosphatase in Blocking solution) for 30 min each at room temperature under constant agitation. The nylon membrane was equilibrated in 30 ml detection buffer (0.1 M Tis-HCl, 0.1 M NaCl, pH 9.5) for 5 min prior to detection with the chemiluminescent substrate CSPD® (Roche Diagnostics Corporation, Indianapolis, IN). Detection involved incubation in 15 ml of 1:14 CSPD® and detection buffer for 5 min, sealing in a plastic bag, and exposing with

an X-ray film for approximately 20 h. Intensity of the bands was measured using the GelWorks® 1D Intermediate software, version 4.01 (UVP Inc., Upland, CA), and were normalized to the β-actin bands.

Statistical Analysis

Data were analyzed as a completely randomized design with factorial arrangement of treatments using the MIXED procedure of SAS according to the following model:

$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl}$

where Y_{ijkl} is the observation for the l^{th} mouse of the k^{th} gender, receiving the i^{th} treatment, at week j^{th}.

μ = grand mean,

α_i = effect of treatment i [i = 1 (control), 2 (0.15% *trans*10,*cis*12-CLA), or 3 (0.30% *trans*10,*cis*12-CLA)

β_j = effect of week j (j = 2, 4, or 6 wk)

γ_k = effect of gender k (k = male or female)

$(\alpha\beta)_{ij}$ = interaction of treatment and week,

$(\alpha\gamma)_{ik}$ = interaction of treatment and gender,

$(\beta\gamma)_{jk}$ = interaction of week and gender,

$(\alpha\beta\gamma)_{ijk}$ = interaction of treatment, gender, and week,

ε_{ijkl} = error effect associated with Y_{ijkl} (l = 1, ……7 mice).

All data are reported as least square means ± SEM. Pre-planned contrasts were performed to test the linear and quadratic effects of the dose of CLA. Interactions are reported only when significant. Significance level was specified at $P < 0.05$.

Results

Dietary *trans*10,*cis*12-CLA increased liver weights (male and female), but decreased inguinal (female) or epididymal (male) adipose tissue weights linearly (**Table 3-1**). Males had higher dry liver weights than females. In males, epididymal adipose weights decreased by 81% in groups fed diets containing 0.30% *trans*10,*cis*12-CLA compared with the controls. Similarly, inguinal adipose weights in females decreased by 52% in groups fed diets containing 0.30% *trans*10,*cis*12-CLA compared with those fed the control diet. Overall, epididymal adipose weights were not affected by age (data not shown), but inguinal adipose increased from 0.17 $\pm$ 0.03 at 2 wk to 0.31 $\pm$ 0.03 at 6 wk. A significant treatment by week interaction ($P < 0.01$) was noted for epididymal adipose but not for inguinal adipose weights. The interaction was caused by epididymal adipose weights increasing with age for the control mice but decreasing with age from 0.35 $\pm$ 0.07 at wk 2 to 0.18 $\pm$ 0.07 at wk 6 for the group fed 0.15% *trans*10,*cis*12-CLA, and from 0.23 $\pm$ 0.07 at wk 2 to 0.10 $\pm$ 0.07 at wk 6 for the group fed 0.30% *trans*10,*cis*12-CLA.

Liver composition data indicated no change in the RNA, DNA, or protein concentration in response to dietary *trans*10,*cis*12-CLA (**Table 3-2**). However, total protein in liver increased linearly with dietary *trans*10,*cis*12-CLA. Liver RNA, DNA, or protein concentrations were not influenced by gender. Total RNA and protein in liver, however, were higher in males than females.

Fatty acid profile of liver (**Table 3-3**) revealed significant changes in mice fed diets containing *trans*10,*cis*12-CLA. Dietary *trans*10,*cis*12-CLA was incorporated into liver lipids in concentrations proportional to those in the diet. Percentages of *trans*11-16:1, *cis*9-18:1, *cis*11-18:1, 18:2n-6, and *cis*9,*trans*11-CLA increased, while 17:0, 18:0, *cis*15-18:1, and *trans*11-18:1 decreased linearly with dose of the CLA isomer. Males had higher percentages of 16:0, *cis*9-16:1, *trans*11-16:1, *cis*15-18:1, *trans*11-18:1, 18:3n-3 and 20:0, whereas females had a higher proportion of 18:0 in their liver. Total fatty acids per unit of liver weight increased linearly with treatment and were higher in males than females.

Ratios of 18:0 to 18:1 and *trans*11-18:1 to *cis*9,*trans*11-CLA in liver decreased in response to dietary *trans*10,*cis*12-CLA (**Table 3-4**). In contrast, ratio of 18:3n-3 to 20:5n-3 in liver increased linearly with dose of the CLA isomer.

Age related changes in fatty acid profile of liver (**Table 3-5**) included accumulation of 18:2n-6, *trans*10,*cis*12-CLA, 20:4n-6, and 20:6n-3. Accumulation of these fatty acids resulted in lower percentages of 14:0, 16:0, 17:0, 18:0, 18:1, 18:3n-3 and 20:0 over the 6 wk period. Total fatty acids expressed per unit of liver weight also increased with age.

Northern analysis of adipose revealed a linear decrease in SCD (**Figure 3-1**) and FAS (**Figure 3-2**) mRNA expression in both male and female mice in response to dietary *trans*10,*cis*12-CLA. However, liver SCD mRNA expression was not affected by treatment (**Figure 3-3**).

Discussion

The inhibition of body fat deposition by dietary CLA mixtures in several species has received considerable attention and has become an area of intense research over the last decade. As pure isomers of CLA became available, the lipid modulating property of CLA mixtures was traced to the *trans*10,*cis*12 isomer (7). While the observed reduction in adipose stores seems desirable, recent studies have demonstrated an increase in liver weights in animals fed dietary *trans*10,*cis*12-CLA (21). This study was conducted to test the effect of two dietary concentrations of *trans*10,*cis*12-CLA on lipid metabolism in liver and adipose of growing mice over a 6-wk period. Differences due to gender also were evaluated in this study.

Dietary *trans*10,*cis*12-CLA caused a significant reduction in epididymal and inguinal adipose weights in this study. Similar reductions in adipose depot weights of mice were observed (7, 20, 21). However, previous studies using the pure *trans*10,*cis*12-CLA were conducted using either male or female mice. Reduction in adipose weights over time was more pronounced in males (significant treatment by week interaction) than females, thus suggesting that males were more responsive to *trans*10,*cis*12-CLA. This is in contrast to the report by Park et al. (2), which indicated that females fed a dietary CLA mixture had a greater reduction in whole body fat than males. Differences could be attributed to the purity of the *trans*10,*cis*12-CLA (>95% pure *trans*10,*cis*12-CLA vs. CLA mixture) fed to mice as well as the adipose sites (inguinal or epididymal adipose vs. whole body fat) used for analysis.

Several studies evaluated possible causes of body fat reduction due to a CLA mixture or the pure *trans*10,*cis*12-CLA (7, 21). Decrease in adipose mass is associated with an increased oxidation of fatty acids, breakdown of triglycerides, or apoptosis of adipocytes. Additionally, a reduction in fat synthesis could also potentially lead to a reduction in fat depots.

An increase in the activity of carnitine palmitoyltransferase-1, the rate-limiting enzyme in β-oxidation of fatty acids, was observed in adipose tissue of mice fed a CLA

mixture, suggesting increased oxidation of fatty acids (2). Increased lipolysis was noted in 3T3-L1 adipocytes treated with CLA (2). In addition, dietary CLA mixture also increased apoptosis (programmed cell death) of adipocytes, thus reducing adipose tissue mass (8).

Dietary CLA mixtures decreased expression of the glucose transporter, GLUT4, in adipose tissue of mice, thus reducing the uptake of glucose as a substrate for fatty acid synthesis by adipocytes (10). Intake of diets containing a CLA mixture inhibited lipoprotein lipase (LPL) in mouse adipose tissue thus reducing uptake of fatty acids for triglyceride synthesis within the adipose tissue (22). Similar inhibition in LPL activity noted in 3T3-L1 adipocytes treated with a CLA mixture (2)

Mice fed CLA mixtures for 5 months exhibited decreased FAS mRNA expression in their white adipose tissue (8). *Trans*10,*cis*12-CLA also decreased expression of lipogenic enzymes in mammary tissue of cows (15). In contrast, some studies have shown no effect of CLA on FAS expression in the adipose tissue. Expression of SCD mRNA was decreased while FAS mRNA remained elevated in mouse 3T3-L1 preadipocytes supplemented with *trans*10,*cis*12-CLA (12). Inhibition of SCD results in reduced accumulation of monounsaturated fatty acids such as oleic and palmitoleic, leading to fat cells with smaller lipid droplets and may thus contribute to the mechanisms of CLA-mediated fat reduction in mice (12). *De novo* fatty acid synthesis was not altered in AKR/J mice fed CLA (9). Because of the lack of data on the effect of the pure *trans*10,*cis*12-CLA in mice, this study was conducted to determine its effect on the expression of FAS and SCD mRNA in mouse adipose.

Our data showed linear decreases in expression of FAS and SCD mRNA in response to dietary *trans*10,*cis*12-CLA in both male and female mice, thus suggesting that this could potentially be one of the mechanisms involved in the reduction of body fat. Males had lower FAS and SCD mRNA in adipose than females, resulting in lower adipose accumulation, which is in agreement with the adipose weight data. Overall reductions in FAS and SCD mRNA were 60% and 30% respectively for the mice fed

0.30% *trans*10,*cis*12-CLA compared with the control, suggesting that inhibition of lipogenesis and desaturation could be major mechanisms by which *trans*10,*cis*12-CLA reduces body fat.

Male and female mice fed pure *trans*10,*cis*12-CLA exhibited a linear increase in dry liver weight over the 6-wk period in the present study. Liver enlargement was noted for mice fed CLA mixtures (23, 24, 9, 8) or pure *trans*10,*cis*12-CLA (20). Mice fed a CLA mixture for 5 months exhibited enlarged and pale livers suggestive of fat accumulation (8). In the current study, total liver fatty acids increased linearly with dose of the CLA isomer, indicating that the observed hepatomegaly in mice fed *trans*10,*cis*12-CLA was due to lipid accumulation. Increased lipid accumulation in the liver possibly could be due to increased uptake of fatty acids, increased lipogenesis, decreased oxidation of fatty acids, or a combination of one or more factors.

Fatty acid transport across membranes and their subsequent activation to acyl CoA in the liver are facilitated through the induction of a fatty acid transport (FAT) protein (25) and the upregulation of genes encoding acyl-CoA synthase (26). Both genes are under the control of the transcription factor, peroxisome proliferator receptor alpha (PPARα) (25, 27). Conjugated linoleic acid mixtures were a potent ligand of PPARα in FaO hepatoma cells (28). A marked upregulation of genes encoding the membrane protein, fatty acid transporter (FAT) was noted for mice fed 0.4% *trans*10,*cis*12-CLA (20). Overexpression of this transporter would result in increased uptake of fatty acids by the liver, thus serving as one of the factors associated with hepatic lipid deposition.

Apart from increased FAT expression, an increase in the expression of FAS was also observed (20), suggesting an increased rate of lipogenesis in the liver. Increase in levels of lipogenic enzymes were noted for mice fed a CLA mixture indicating enhanced lipogenesis also could be a mechanism for the increased liver lipid content (10). In addition to uptake and synthesis, a reduction in hepatic secretion of triglycerides and oxidation of fatty acids could be a determinant in hepatic lipid accumulation in response

to *trans*10,*cis*12-CLA. Liver DNA, RNA, and protein concentrations were not affected by dietary *trans*10,*cis*12-CLA in the current study, indicating that the enlargement of livers was due to hypertrophy rather than hyperplasia.

Marked alterations in liver fatty acid profile were observed for mice fed the *trans*10,*cis*12-CLA. These included increases in the proportions of 18:1 and18:2n-6 followed by a decrease in 18:0. In the liver and most other tissues, SCD, catalyzes the conversion of 18:0 to *cis*9-18:1 and 16:0 to *cis*9-16:1 with the introduction of a double bond at the Δ^9 position in the carbon chain (29). Thus, SCD also causes the conversion of *trans*11-18:1 to *cis*9,*trans*11-18:2 (30). In the current study, linear decreases in the ratios of 18:0 to *cis*9-18:1 and *trans*11-CLA to *cis*9,*trans*11-CLA were observed in the *trans*10,*cis*12-CLA fed mice suggesting enhancement of Δ^9-desaturase activity. Expression of SCD mRNA in the liver, however, was not affected by dietary *trans*10,*cis*12-CLA. Choi et al. (12) suggested that *trans*10,*cis*12-CLA probably alters SCD gene transcription or its mRNA stability. We speculate that *trans*10,*cis*12-CLA also could act partly at the translational level, thereby decreasing the amount of SCD synthesized from SCD mRNA. In contrast to the results obtained in the current study, a decrease in hepatic SCD mRNA expresssion was observed in mice fed a CLA mixture (0.5% of diet) for 2 wk (31). A similar reduction in liver desaturase activity was noted in rats fed 1% *trans*10,*cis*12-CLA for 6 wk (32). Activity of SCD in liver, however, was not affected by dietary CLA in lactating mice (33). Variation in SCD mRNA expression and activity may be attributed partly to the amount and source of the *trans*10,*cis*12-CLA in the diet (a mixture of isomers versus pure *trans*10,*cis*12-CLA) and the species used to evaluate responses. For example, Lee et al. (31) used a commercial CLA mixture, and rats were the model used in the study by Sebedio et al. (32). Moreover total liver lipid content of rats was not affected by dietary *trans*10,*cis*12-CLA (32), whereas hepatic fatty acid content of mice increased linearly with dose in the current study.

A linear increase in the ratio of 18:3n-3 to 20:5n3 was seen in liver of mice fed *trans*10,*cis*12-CLA in the present study. Highly unsaturated fatty acids such as 20:4n-6 and 20:5n-3 are formed from 18:2n-6 or 18:3n-3 by Δ^5 and Δ^6 desaturation (34).

Although liver Δ^5 and Δ^6 desaturase mRNA levels were not measured in the current study, the observed increase in the ratio of 18:3n-3 to 20:5n-3 suggest a reduction in the expression or activities of these two enzymes. Inhibition of Δ^5 and Δ^6 desaturases was observed in HepG2 cells supplemented with *trans*10,*cis*12-CLA (34). However, mice fed CLA mixtures exhibited higher levels of Δ^5 and Δ^6 desaturase mRNA in their liver when compared with a control group (23). Again, the CLA used in the study by Takahashi et al. (23) was a mixture of several isomers compared with the pure *trans*10,*cis*12-CLA used in our study.

Major age-related changes in the liver due to dietary *trans*10,*cis*12-CLA included a decrease in the proportion of the saturated fatty acids and 18:3n-3 accompanied by increases in the proportions of 18:1, 18:2n-6, 20:4n-6 and 22:6n-3. Results indicate significant shifts in the levels of n-3 and n-6 polyunsaturated fatty acids with aging similar to the findings of Engler et al. (35).

Conclusions

This was the first study to determine the effects of pure *trans*10,*cis*12-CLA on adipose depots in male and female mice. We demonstrated that reduction in adipose depot weight was more pronounced in males, and the reduction was associated with lower FAS and SCD mRNA in adipose.

Liver fatty acid content and proportions of those fatty acids also were influenced by dietary *trans*10,*cis*12-CLA. Enhancement of Δ^9-desaturase activity may have caused an elevation in the proportion of monounsaturated fatty acids, thus resulting in larger lipid droplets in hepatocytes. The speculated decrease in Δ^5 and Δ^6 -desaturase activity may be a compensation for an increase in Δ^9-desaturase activity. Further studies are need to determine the effect of the *trans*10,*cis*12-CLA on liver desaturases and the mechanisms involved in the reduction of adipose depots.

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Table 3-1. Average liver and adipose weights (g) of male and female mice fed *trans*10,*cis*12-CLA for 6 wk

	Dietary *trans*10,*cis*12-CLA								
	0 %	0.15 %	0.30 %	SEM	P <	Male	Female	SEM	P <
Liver (wet)	1.77	1.95	2.07	0.04	0.01*	2.23	1.62	0.03	0.01
Liver (dry)	0.55	0.61	0.65	0.01	0.01*	0.70	0.50	0.01	0.01
Inguinal Adipose	0.34	0.20	0.16	0.03	0.01*				
Epididymal Adipose	0.73	0.26	0.14	0.04	0.01**				

*Linear effect of dietary *trans*10,*cis*12-CLA
**Linear and quadratic effect of dietary *trans*10,*cis*12-CLA

Table 3-2. Liver DNA, RNA and Protein in mice fed *trans*10,*cis*12-CLA for 6 wk

	Dietary *trans*10,*cis*12-CLA								
	0 %	0.15 %	0.30 %	SEM	P <	Male	Female	SEM	P <
DNA (μg/mg dry liver)	24.7	21.7	21.5	2.4	0.56	21.8	23.5	1.9	0.53
Total DNA in dry liver (mg)	13.8	13.1	13.3	1.5	0.94	14.9	12.0	1.2	0.09
RNA (μg/mg dry liver)	8.8	9.6	8.9	0.7	0.65	9.8	8.4	0.6	0.08
Total RNA in dry liver (mg)	4.8	5.9	5.6	0.5	0.21	6.8	4.1	0.4	0.01
Protein (mg/mg dry liver)	0.36	0.39	0.37	0.008	0.06	0.36	0.37	0.007	0.10
Total protein in dry liver (mg)	196	231	237	6.7	0.01*	254	188	5.5	0.01

Table 3-3. Fatty acid composition of liver of male and female mice fed *trans*10,*cis*12-CLA

Fatty acid	Dietary *trans*10,*cis*12-CLA			SEM	P<	Male	Female	SEM	P<
	0%	0.15 %	0.30 %						
	% total fatty acids					% total fatty acids			
14:0	0.25	0.27	0.26	0.01	0.51	0.27	0.25	0.01	0.09
16:0	22.63	21.91	21.66	0.71	0.61	23.09	21.04	0.58	0.01
*cis*9-16:1	0.56	0.50	0.53	0.04	0.54	0.66	0.40	0.03	0.01
*trans*11-16:1	0.44	0.56	0.66	0.04	0.01*	0.67	0.44	0.03	0.01
17:0	0.40	0.35	0.32	0.02	0.01*	0.36	0.36	0.01	0.73
18:0	22.55	19.46	16.76	0.75	0.01*	16.95	22.24	0.61	0.01
*cis*9-18:1	20.31	20.29	22.67	0.73	0.03*	21.59	20.58	0.60	0.23
*cis*11-18:1	1.15	1.07	1.45	0.08	0.01*	1.32	1.12	0.07	0.04
*cis*15-18:1	0.32	0.28	0.23	0.01	0.01*	0.32	0.23	0.01	0.01
*trans*11-18:1	0.07	0.06	0.05	0.003	0.01*	0.06	0.05	0.002	0.01
18:2n-6	16.61	20.00	20.68	0.79	0.01*	19.42	18.77	0.65	0.47
*cis*9,*trans*11-18:2	0.02	0.03	0.03	0.004	0.01*	0.02	0.03	0.004	0.20
*trans*10,*cis*12-18:2	0	0.15	0.31	0.009	0.01*	0.15	0.15	0.007	0.98
18:3n-3	0.51	0.51	0.48	0.03	0.61	0.56	0.44	0.023	0.01
20:0	0.35	0.29	0.32	0.02	0.14	0.51	0.13	0.02	0.01
20:4n-6	7.33	7.47	7.04	0.64	0.89	7.30	7.26	0.52	0.95
20:5n-3	0.12	0.10	0.07	0.01	0.01*	0.09	0.10	0.01	0.20
22:6n-3	3.49	4.04	3.87	0.42	0.64	3.67	3.92	0.35	0.61
Total (μg/mg of dry liver)	36.57	44.11	54.31	1.94	0.01*	47.56	42.43	1.59	0.02

*Linear effect of dietary *trans*10,*cis*12-CLA

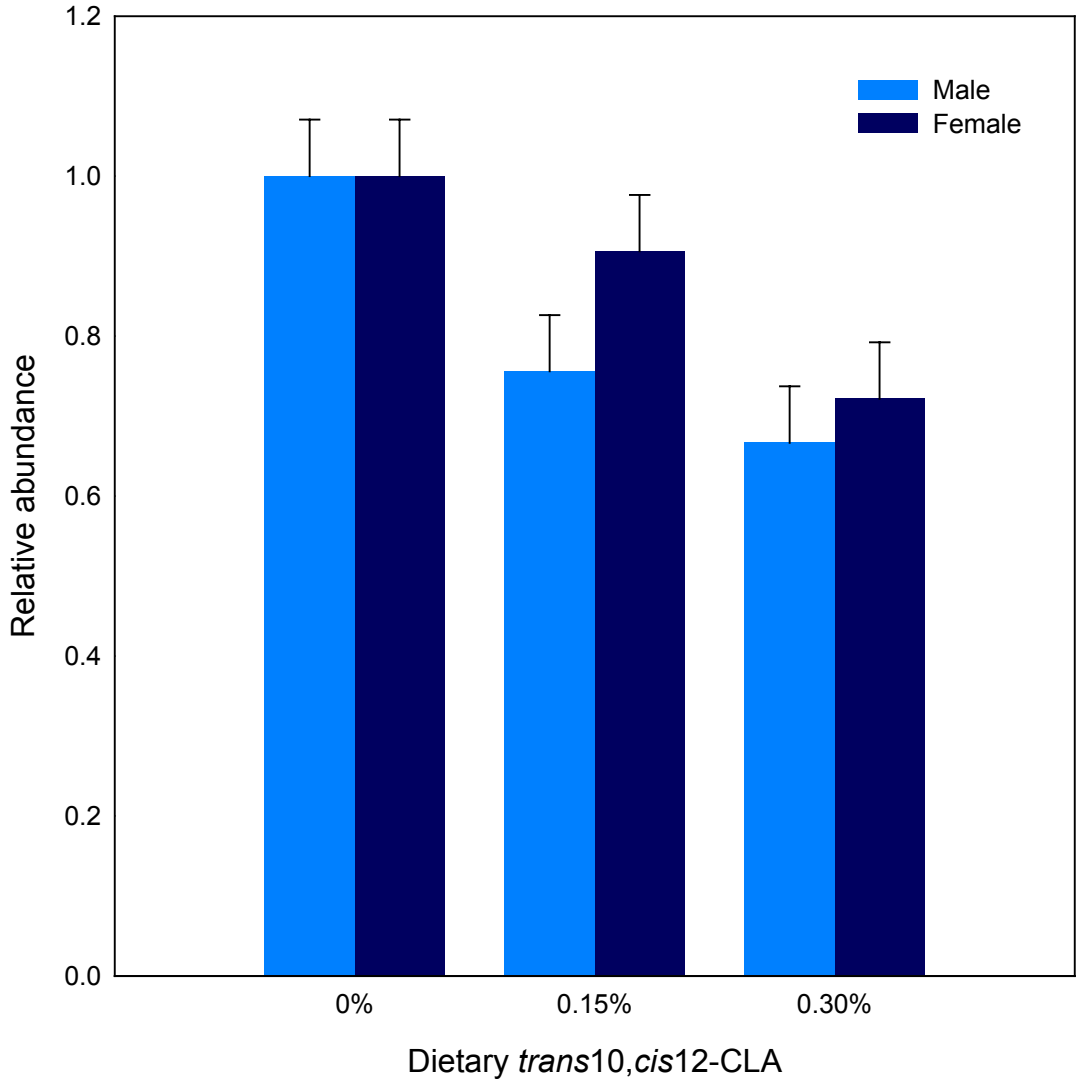
Table 3-4. Liver fatty acid ratios of male and female mice fed *trans*10,*cis*12- CLA

	Dietary *trans*10,*cis*12-CLA								
	CON	1X	2X	SEM	P <	Male	Female	SEM	P <
16:0 to 16:1	0.98	0.98	0.98	0.001	0.49	0.98	0.97	0.001	0.01
18:0 to 18:1	0.52	0.48	0.42	0.01	0.01*	0.44	0.51	0.01	0.01
t11C18:1 to c9,t11-CLA	0.87	0.71	0.63	0.03	0.01*	0.77	0.70	0.03	0.07
18:2n-6 to 20:4n-6	0.75	0.76	0.77	0.01	0.26	0.76	0.76	0.01	0.85
18:3n-3 to 20:5n-3	0.82	0.85	0.88	0.01	0.01*	0.87	0.83	0.01	0.02

*Linear effect of dietary *trans*10,*cis*12-CLA

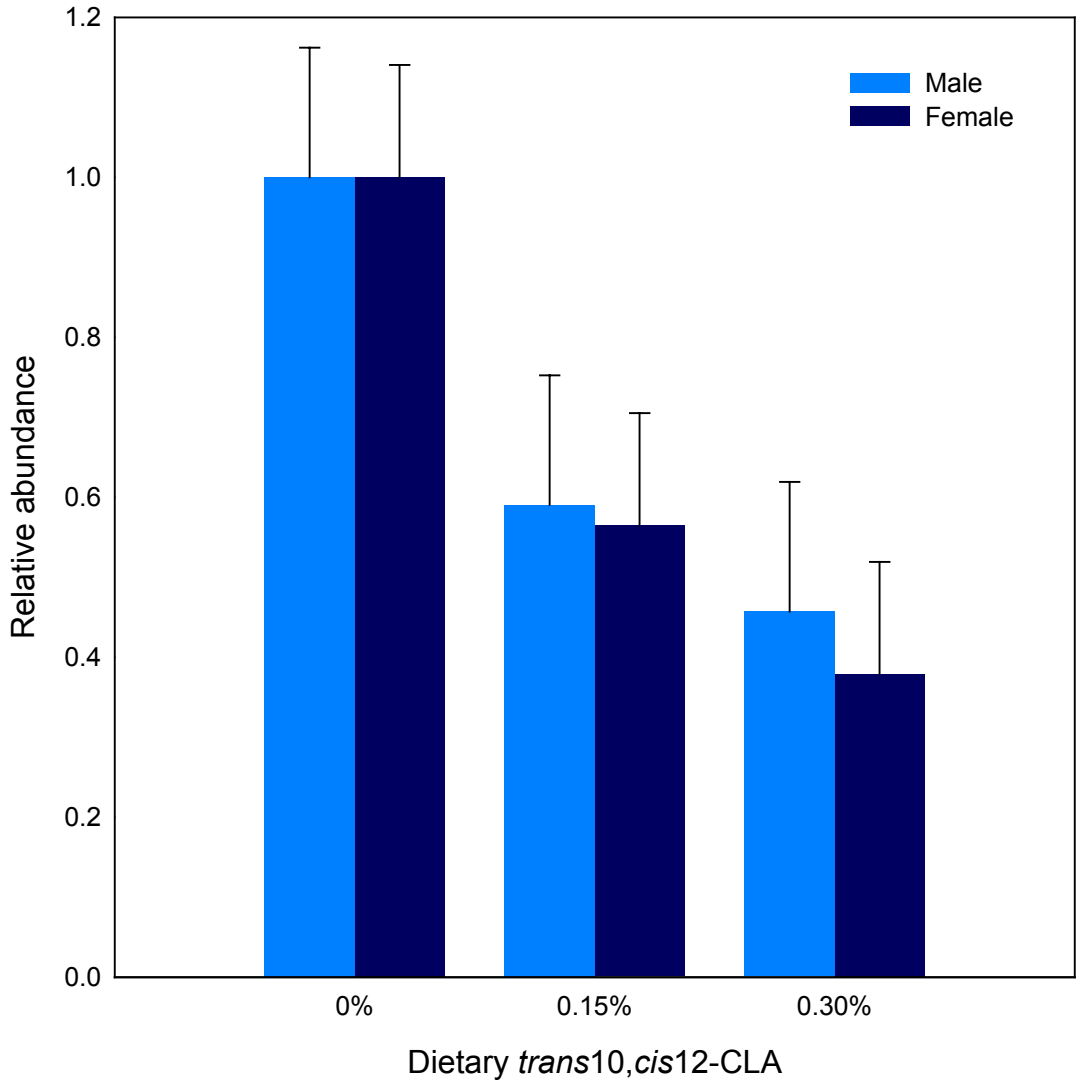
Table 3-5. Fatty acid composition of liver of growing mice at 2, 4, and 6 wk of the experimental period

Fatty acid	Wk 2	Wk 4	Wk 6	SEM	P<
	% total fatty acids				
14:0	0.32	0.26	0.21	0.01	0.01
16:0	27.21	21.83	17.16	0.71	0.01
*cis*9-16:1	0.56	0.58	0.46	0.04	0.07
*trans*11-16:1	0.58	0.54	0.54	0.04	0.72
17:0	0.45	0.36	0.27	0.02	0.01
18:0	24.71	19.49	14.58	0.75	0.01
*cis*9-18:1	23.08	21.49	18.69	0.73	0.01
*cis*11-18:1	1.45	1.23	0.99	0.08	0.01
*cis*15-18:1	0.33	0.28	0.22	0.01	0.01
*trans*11-18:1	0.07	0.06	0.05	0.003	0.01
18:2n-6	12.56	20.37	24.36	0.79	0.01
*cis*9,*trans*11-18:2	0.04	0.02	0.02	0.004	0.07
*trans*10,*cis*12-18:2	0.10	0.16	0.18	0.009	0.01
18:3n-3	0.60	0.46	0.44	0.03	0.01
20:0	0.40	0.31	0.25	0.02	0.01
20:4n-6	3.34	6.66	11.84	0.64	0.01
20:5n-3	0.15	0.12	0.02	0.01	0.01
22:6n-3	1.39	3.19	6.82	0.42	0.01
Total (μg/mg of dry liver)	35.32	43.21	56.45	1.94	0.01



- Linear effect ($P < 0.05$) for male and female

Figure 3-1. SCD mRNA expression in adipose of male and female mice fed *trans*10,*cis*12-CLA



- Linear effect ($P < 0.05$) for male and female

Figure 3-2. FAS mRNA expression in adipose of male and female mice fed *trans*10,*cis*12-CLA

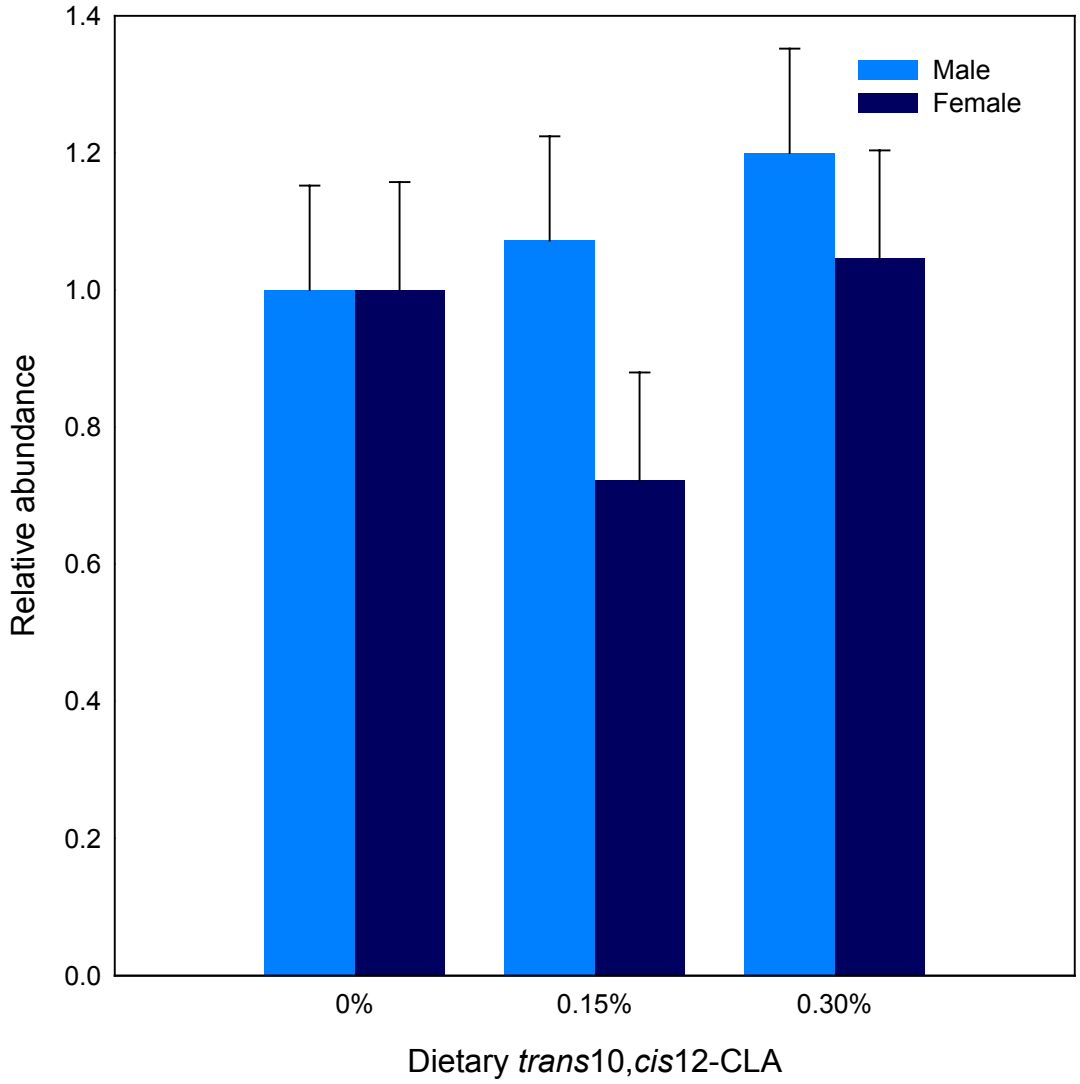


Figure 3-3. SCD mRNA expression in liver of male and female mice fed *trans*10,*cis*12-CLA

CHAPTER 4

Effect of dietary *trans*-vaccenic acid (TVA) and *trans*10,*cis*12-conjugated linoleic acid (CLA) on plasma and tissue fatty acid profile in adult mice

Abstract

The *trans*10,*cis*12 isomer of conjugated linoleic acid (CLA) inhibits desaturation of *trans*-vaccenic acid (TVA) to *cis*9,*trans*11-CLA in mammary tissue, but the effects of *trans*10,*cis*12-CLA on TVA desaturation in other tissues have not been evaluated. Male and female mice were randomly assigned to diets containing 0% TVA, 0.4% TVA, 0.4% TVA + 0.15% *trans*10,*cis*12-CLA, or 0.4% TVA + 0.30% *trans*10,*cis*12-CLA. Five mice per dietary group within sex were sacrificed after 10 d. Food intake, body weight, liver, femur, and gastrocnemius muscle weight, and, epididymal (males) and inguinal (females) adipose weights were similar for all treatments. However, dry carcass weights decreased linearly in response to dietary *trans*10,*cis*12-CLA from 5.20 $\pm$ 0.26 g for the 0.40% TVA group to 4.22 $\pm$ 0.26 g for mice fed diets containing 0.40% TVA + 0.30% *trans*10,*cis*12-CLA groups, respectively. *Trans11*-18:1 and *trans*10,*cis*12-CLA were incorporated into plasma, liver, adipose, muscle, and bone lipids proportional to those in the diets. Desaturation ratios in liver, adipose, and bone were similar for all groups fed TVA. Expression levels of mRNA for SCD in adipose and liver were not influenced by dietary treatments suggesting Δ^9-desaturation was not affected by *trans*10,*cis*12-CLA. However, ratios of 16:0 to 16:1 and 18:0 to 18:1 in muscle increased linearly due to dietary *trans*10,*cis*12-CLA. Results indicate potential inhibition of Δ^9-desaturation in muscle by *trans*10,*cis*12-CLA.

Key words: fatty acids**,** desaturation, SCD

Introduction

Trans-vaccenic acid (TVA) is an 18-carbon fatty acid with one double bond in the *trans* configuration at the Δ^{11} position. Interest in TVA has grown after it was proposed that it served as a substrate for the formation of *cis*9,*trans*11-CLA in tissues of ruminants (1). Initially, biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens* in the rumen results in formation of *cis*9,*trans*11-CLA as an intermediate product (2). Most of the *cis*9,*trans*11-CLA is further hydrogenated to TVA before being absorbed from the small intestine. *Trans* 18:1 monoenes, especially TVA, comprise the majority of the *trans* fatty acids found in ruminant tissues and milk (3). TVA can be converted to *cis*9,*trans*11-CLA within mammalian tissues by the enzyme stearoyl-CoA Δ^9-desaturase (SCD) (4, 5). Endogenous synthesis of *cis*9,*trans*11-CLA from TVA in mammary tissue represents the predominant source of the *cis*9,*trans*11-CLA in milk fat (4). Similarly, conversion of TVA to *cis*9,*trans*11-CLA by SCD might explain the presence of *cis*9,*trans*11-CLA in many tissues other than the mammary gland.

Approximately, 49% of the TVA consumed by mice during a 2-wk feeding period was desaturated in tissues to *cis*9,*trans*11-CLA (5). More recently, Loor et al. (6) demonstrated that lactating mice fed diets containing 1% TVA had increased concentrations of *cis*9,*trans*11-CLA in tissues and milk fat. Feeding rats diets containing 2% TVA increased liver and mammary *cis*9,*trans*11-CLA content and reduced intraductal proliferation of terminal end buds following carcinogen administration (7). This suggests that a potential anti-cancer effect of TVA was mediated by its endogenous conversion to *cis*9,*trans*11-CLA via SCD. Similarly, 19% of the TVA fed to human subjects was converted to *cis*9,*trans*11-CLA, indicating that dietary TVA content should be considered when predicting the whole body *cis*9,*trans*11-CLA status of an individual (8).

Previous reports indicated *trans*10,*cis*12-CLA decreases SCD mRNA expression and activity in liver (9, 10) and mammary tissue (11, 12, 13). Also, *trans*10,*cis*12-CLA downregulates SCD gene expression in pre-adipocytes (14). Inhibition of SCD by

*trans*10,*cis*12-CLA, therefore, could decrease concentrations of *cis*9,*trans*11-CLA in tissues, leading to accumulation of SCD substrates. Because TVA is a substrate for the synthesis of *cis*9,*trans*11-CLA, and *trans*10,*cis*12-CLA inhibits SCD gene expression, the following study was conducted to evaluate the interaction of *trans*10,*cis*12-CLA and TVA on the formation of *cis*9,*trans*11-CLA and other products of SCD activity in plasma, liver, adipose, bone, and muscle of adult male and female mice. Also, the effect of dietary treatments on SCD mRNA expression in the liver and adipose was investigated.

Materials and Methods

Animals, Diets, and Sampling

All procedures involving mice were approved by Virginia Polytechnic Institute and State University Animal Use and Care Committee. Mice were housed in polypropylene cages in the Laboratory Animal Resources facility, which was maintained at room temperature with a 12 h light and 12 h dark cycle. Mice had access to water at all times. Twenty male and 20 female, 8-wk old CD-1 mice were used in a 10-d feeding trial to evaluate growth and tissue fatty acid profile in response to dietary TVA or TVA plus *trans*10,*cis*12-CLA. Mice were fed a Harlan Teklad (Harlan, Madison, WI) diet containing 3% (wt/wt) high-oleic sunflower oil (control) for 5 days before the start of the trial. On d 1 of the trial, 5 mice per treatment group within gender were randomly assigned to receive 3.0 % high-oleic sunflower (**CON**), 2.60 % high-oleic sunflower oil + 0.40% TVA (Nu Check Prep Elysian, MN) (**TVA**), 2.45% high-oleic sunflower oil + 0.40% TVA + 0.15% *trans*10,*cis*12-CLA (>95% purity, Natural Lipids, Hordebygda, Norway) (**TVA + 1X**), or 2.30% high-oleic sunflower oil + 0.40% TVA + 0.30% *trans*10,*cis*12-CLA (**TVA + 2X**). Thus, TVA replaced a portion of the sunflower oil in the TVA diet, while TVA and *trans*10,*cis*12-CLA replaced sunflower oil in the diets fed to the TVA + 1X and TVA + 2X treatment groups.

Mice were fed daily at 1600 h. Feed intake was measured by subtracting the weight of the refusals from the amount of feed offered (**see appendix figures A-9 and A-10**). Diets were sampled twice and stored at 4 ^{o}C for fatty acid analysis (**Table 4-1**). Body weight was determined on d 1, 5, and 10 of the trial. On d 10 of the experimental period, mice were anaesthetized using Metofane (Pitman-Moore, INC, NJ). Blood was collected by heart puncture and the plasma was harvested by centrifugation at 3000 x *g* for 15 min and stored at -20 ^{o}C for fatty acid analysis. Viscera was exposed by dissecting the skin and the underlying membranes from the abdominal cavity through the neck. Inguinal or epididymal adipose, liver, femur, gastrocnemius muscle and small intestine were removed and weighed. A portion of the liver and adipose were frozen immediately in liquid nitrogen and later stored at -80 ^{o}C for RNA analysis. The

remainder of the adipose and liver along with the muscle and bone were stored at -20 ^{o}C for fatty acid analysis.

Lipids from diet, plasma, liver, bone, muscle, and adipose were extracted using the Folch procedure (15) with modifications (16) and the resulting fatty acids were transesterified according to the method of Park and Goins (17) as described in Chapter 2. Methyl esters of fatty acids were separated on an Agilent 6890N gas chromatograph as described previously. Split ratio and injection volume for adipose were 50:1 and 2.5 μl, respectively. Temperature programming for the column was similar to that described for liver fatty acids in Chapter 3. Methyl esters of fatty acids obtained from plasma were run splitless with an injection volume of 1 μl, using programming conditions similar to those described for the bone in Chapter 2. Desaturation ratios were calculated as described in Chapter 2.

Adipose and liver DNA, RNA, and protein were extracted as described in Chapter 4. Plasmid isolation, cDNA probe synthesis, RNA gel electrophoresis, Northern blotting, and DIG labeling and detection of SCD mRNA bands from liver and adipose were performed as described in Chapter 4. Intensity of the bands was measured using the GelWorks® 1D Intermediate software, version 4.01 (UVP Inc., Upland, CA), and SCD bands were normalized to β-actin bands.

Statistical Analysis

Data were analyzed as a completely randomized design with factorial arrangements using the MIXED procedure of SAS according to the following model:

$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$

where Y_{ijk} is the observation for the k^{th} mouse of the j^{th} gender, receiving the i^{th} treatment.

μ = grand mean,

α_i = effect of treatment i [i = 1 (control), 2 (0.40% TVA), 3 (0.40% TVA +0.15% *trans*10,*cis*12-CLA), or 4 (0.40% TVA + 0.30% *trans*10,*cis*12-CLA)]

β_j = effect of gender j (k = male or female)

$(\alpha\beta)_{ij}$ = interaction of treatment and gender,

ε_{ijk} = error effect associated with Y_{ijk} (k = 1, ….5 mice).

All data are reported as least square means $\pm$ SEM. Non-orthogonal contrasts were performed to compare CON vs TVA, TVA vs TVA + 1X, and TVA vs TVA + 2X and were tested for significance using the Bonferroni procedure. Linear and quadratic contrasts were performed to determine the effects of 0, 0.15% and 0.30% *trans*10,*cis*12-CLA. Interactions are reported only when significant. Significance was specified at $P < 0.05$.

Results

Dietary TVA or TVA + *trans*10,*cis*12-CLA had no effect on body weight and tissue weights of adult male or female mice (**Table 4-2**). Dry carcass weights however, decreased linearly in response to dietary *trans*10,*cis*12-CLA. Males had higher body weights than females. Dry carcass, liver, muscle, and bone weights were higher in males compared with female mice. Liver DNA, RNA, and protein content were unaffected by dietary treatments, but liver of females had a higher protein concentration but lower total protein than liver of males (**Table 4-3**).

Total fatty acids in the plasma increased linearly in mice fed diets containing *trans*10,*cis*12-CLA and were higher in the TVA + 2X group compared with the TVA mice (**Table 4-4).** Concentration of *trans*11-18:1 was higher in all TVA groups compared with CON. Dietary *trans*10,*cis*12-CLA caused a linear increase in concentration of *trans*10,*cis*12-CLA. Concentration of *cis*9,*trans*11-CLA increased linearly with dietary *trans*10,*cis*12-CLA and was higher in mice fed diets containing TVA than the control mice. Males had a higher concentration of total fatty acids, resulting in higher concentrations of most of the individual fatty acids.

Liver total fatty acids were not affected by dietary treatments (**Table 4-5**). Percentages of *trans*11-18:1 and *cis*9,*trans*11-CLA were higher in liver of mice fed diets containing TVA compared with those of control mice. *Trans*10,*cis*12-CLA content of liver increased linearly in mice fed TVA + 1X and TVA + 2X diets. Percentages of *trans*9-16:1 and *trans*9-18:1 increased linearly in mice fed diets containing *trans*10,*cis*12-CLA. *Trans*9-16:1 was higher in liver of mice fed TVA compared with CON. The livers of males had higher 14:0, 16:0, *cis*9-16:1, and 20:0, but lower 18:0, compared with females.

Fatty acid profile of adipose revealed a decrease in total fatty acids in mice fed diets containing *trans*10,*cis*12-CLA (**Table 4-6**). Mice fed TVA had corresponding increases in the percentages of *trans*11-18:1 and *cis*9,*trans*11-CLA in adipose. Percentages of *trans*10,*cis*12-CLA increased linearly in TVA + 1X and TVA + 2X mice.

Mice fed diets containing TVA had higher percentages of *trans*9-16:1 compared with the controls. Males had higher *cis*9-16:1, *cis*11-18:1, and 18:2n-6, but lower *trans*9-16:1, 18:0, *trans*11-18:1, *cis*9,*trans*11-18:2, 20:0, and 20:4n-6, than females.

Total fatty acids in femur were not affected by dietary treatments (**Table 4-7**). Percentages of TVA and *cis*9,*trans*11-18:2 were higher in the TVA group compared with CON. Mice fed diets containing *trans*10,*cis*12-CLA exhibited linear increases in the percentages of *trans*9-16:1 and *trans*10,*cis*12-CLA. Males had higher percentages of *cis*9-16:1, *cis*11-18:1, 18:2n-6, and 18:3n-3, but lower 16:0, 18:0, *trans*11-18:1, and *trans*10,*cis*12-18:2 than females.

Total fatty acids in gastrocnemius muscle were not affected by dietary treatments (**Table 4-8**). Mice fed diets containing TVA had higher *trans*11-18:1, *cis*9,*trans*11-CLA, and *trans*9-16:1 compared with CON. Dietary *trans*10,*cis*12-CLA resulted in linear increases in percentages of *trans*9-16:1, *trans*11-18:1, and *trans*10,*cis*12-18:2. Males had higher *cis*9-16:1, but lower *trans*11-18:1, than females.

Ratios of 16:0 to 16:1, 18:0 to 18:1, trans11-18:1 to cis9,trans11-CLA, 18:2n-6 to 20:4n-6, and 18:3n-3 to 20:5n-3 in liver were not affected by dietary treatments (**Table 4-9**). However, mice fed diets containing TVA had a higher ratio of *trans*11-18:1 to *cis*9,*trans*11-CLA in adipose (**Table 4-10**), bone (**Table 4-11**), and muscle (**Table 4-12**), compared with controls. In addition, muscle ratios of 16:0 to 16:1 and 18:0 to 18:1 increased linearly in mice fed diets containing *trans*10,*cis*12-CLA (**Table 4-12**).

Adipose tissue of females had higher 16:0 to 16:1, 18:0 to 18:1, *trans*11-18:1 to *cis*9,*trans*11-18:1, but lower 18:2n-6 to 20:4n-6, than males (**Table 4-10**). Similarly, ratios of 16:0 to 16:1 and 18:0 to 18:1 in bone were higher in females than males (**Table 4-11**). In muscle, females had higher ratios of 16:0 to 16:1 and *trans*11-18:1 to *cis*9,*trans*11-CLA than males (**Table 4-12**).

Expression of SCD mRNA was not affected in liver (**Figure 4-1**) or adipose (**Figure 4-2**) due to dietary treatments in adult male and female mice. Males (0.98 $\pm$ 0.01) had higher SCD mRNA in liver compared with females (0.94 $\pm$ 0.01). However, mRNA expression was not affected by gender in the adipose.

Discussion

Endogenous conversion of TVA to *cis*9,*trans*11-CLA is catalyzed by the enzyme SCD (4). The *trans*10,*cis*12-CLA inhibits SCD resulting in accumulation of TVA and reduced production of *cis*9,*trans*11-CLA (9). However, the combined effect of dietary TVA and *trans*10,*cis*12-CLA on *cis*9,*trans*11-CLA synthesis in tissues has not been investigated. This study, therefore, focussed on fatty acid responses to a dietary mixture of TVA and *trans*10,*cis*12-CLA.

Although food intake and body weight were not affected by dietary treatments, dry carcass weight decreased linearly in response to *trans*10,*cis*12-CLA. A similar reduction in dry carcass weights was observed in growing mice fed 0.15% and 0.30% *trans*10,*cis*12-CLA for 6 wk and this decrease was attributed to the reduction in carcass fat content (Chapter 2). Mice fed a diet containing *trans*10,*cis*12-CLA (18) or a 1% CLA mixture (19) had reduced carcass fat content. Because inguinal or epididymal adipose weights were not affected by *trans*10,*cis*12-CLA in this 10-d study, it appears that subcutaneous adipose was more responsive to *trans*10,*cis*12-CLA than the other adipose depots.

Feeding diets containing TVA to mice increased plasma concentrations of *trans*11-18:1 and *cis*9,*trans*11-CLA. Similar elevations in plasma *cis*9,*trans*11-CLA and *trans*11-18:1 were reported in lactating mice fed 1% TVA (6). Increased *cis*9,*trans*11-CLA concentration in blood plasma was also reported in humans provided a diet containing TVA (8). *Trans-vaccenic* acid is rapidly incorporated into plasma phospholipids (20), thereby increasing its concentration in blood plasma.

*Trans*10,*cis*12-CLA reduced triglyceride content in 3T3-L1 preadipocytes (21). In mice, dietary CLA increased fatty acid oxidation in the adipose (22). Mice fed diets containing *trans*10,*cis*12-CLA had reduced LPL activity, thereby leading to decreased uptake of free fatty acids by adipose for triglyceride synthesis (18). In the current study, dietary *trans*10,*cis*12-CLA caused a linear decrease in total fatty acids in the adipose tissue, possibly due to a reduction in LPL activity or increased β-oxidation.

Dietary TVA caused elevated percentages of TVA in the liver, adipose, femur, and gastrocnemius muscle in adult male and female mice. Plasma TVA is readily cleared from the plasma and incorporated into tissues and esterified (23). Increased levels of TVA in carcass, liver, and mammary gland were reported for lactating mice fed diets containing 1% TVA (6). Similarly, carcass TVA content was increased in mice fed a diet containing 1% TVA (5).

Cultures of liver microsomes desaturated TVA to *cis*9,*trans*11-CLA (24). *Trans*-vaccenic acid was desaturated to *cis*9,*trans*11-CLA in the carcass of mice fed 1% TVA (5). Similarly, an increase in *cis*9,*trans*11-CLA content of mammary tissue was reported for lactating mice fed TVA (6). In the current study, percentages of *cis*9,*trans*11-CLA increased 60-fold in the liver, 7-fold in adipose, 3-fold in muscle, and 2.5-fold in bone in response to dietary TVA, suggesting desaturation of TVA by SCD. However, ratio of *trans*11-18:1 to *cis*9,*trans*11-CLA in liver, adipose, bone and muscle was higher in mice fed diets containing TVA compared with the controls. This is probably due to the high amount of the substrate (TVA) available for desaturation to *cis*9,*trans*11-CLA relative to the quantity of SCD enzyme present within the cell. Therefore, only a portion of the available TVA is desaturated to *cis*9,*trans*11-CLA, while the remainder accumulates within the tissue.

A CLA mixture (containing equal proportions of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA) or the pure *trans*10,*cis*12-CLA, decreased SCD mRNA expression and activity in liver (25, 9), adipose (14, 26), and mammary tissue (11, 13). Decreased SCD mRNA expression and activity resulted in elevated ratios of 16:0 to 16:1 and 18:0 to 18:1 (14). In the present study, however, ratios of 16:0 to 16:1, 18:0 to 18:1, and TVA to *cis*9,*trans*11-CLA, in liver, adipose, and bone were not affected by dietary *trans*10,*cis*12-CLA. Apparently, SCD was not inhibited by *trans*10,*cis*12-CLA in the current study. Also, expression of SCD mRNA in the liver and adipose was not affected by dietary treatments. In muscle, however, dietary *trans*10,*cis*12-CLA cause a linear increase in ratios of 16:0 to 16:1, and 18:0 to 18:1 suggesting inhibition of SCD in this

tissue. Feeding diets containing 1% CLA to pigs increased the ratio of 18:0 to 18:1 in the skeletal muscle (27). The reasons for the preferential inhibition of SCD in muscle, compared with the other tissues, is not clearly understood from the available data.

*Trans*10,*cis*12-CLA inhibited Δ^5 and Δ^6 desaturases in HepG2 cells (Eder et al., 2002) and mouse liver (Chapter 3). The ratios of 18:2n-6 to 20:4n-6 and 18:3n-3 to 20:5n-3, are indicators of Δ^5 and Δ^6 desaturation (28). Because these ratios in liver, adipose, muscle and bone were not affected by dietary treatments, it can be concluded that *trans*10,*cis*12-CLA did not inhibit Δ^5 and Δ^6 desaturation in the current study.

*Trans*11-18:1 was retroconverted (shortening of fatty acid chain length by 2 carbons) to *trans*9-18:1 in Swiss mouse fibroblasts (29) and mouse mammary epithelial cells (30). In the present study, retroconversion of *trans*11-18:1 could be responsible for the increased percentages of palmitelaidic acid (*trans*9-16:1) observed in liver, adipose, muscle, and bone in mice fed diets containing *trans*11-18:1.

Conclusions

This was the first study to report the effect of a dietary combination of TVA and *trans*10,*cis*12-CLA on plasma, liver, adipose, bone, and muscle fatty acid profile and SCD mRNA expression in liver and adipose. Dietary TVA increased the incorporation of TVA into liver, adipose, bone, and muscle. In addition, it also increased *cis*9,*trans*11-CLA content of tissues. The *trans*10,*cis*12-CLA did not inhibit desaturation in the liver, adipose, or bone during the 10-d feeding trial. However, it appeared to inhibit SCD in the muscle. Additional studies are needed to evaluate the effect of TVA and *trans*10,*cis*12-CLA on the desaturation process in tissues. Also, muscle SCD mRNA expression and activity in response to dietary *trans*10,*cis*12-CLA requires further investigation.

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Table 4-1. Fatty acid composition of diets[1] fed to adult male and female mice

Fatty acid	CON	TVA	TVA + 1X	TVA +2X
	% total fatty acids			
10:0	0.02	0.02	0.01	0.02
12:0	0.04	0.05	0.04	0.06
14:0	0.12	0.11	0.11	0.11
16:0	9.26	9.18	9.18	9.04
*cis*9-16:1	0.18	0.17	0.17	0.17
18:0	2.49	2.45	2.40	2.33
*trans*11-18:1	0.02	4.22	4.24	4.17
*cis*9-18:1	34.41	31.34	30.20	28.78
18:2n-6	47.34	46.54	46.21	45.83
*cis*9,*trans*11-18:2	0.02	0.02	0.17	0.32
*trans*10,*cis*12-18:2	0.00	0.02	1.12	2.55
18:3n-3	3.72	3.82	3.78	3.80
20:0	0.39	0.36	0.36	0.35
20:4n-6	0.02	0.03	0.02	0.02
Total (μg/mg of diet)	44.07	46.48	50.18	44.58

Table 4-2. Body and tissue weights (g) in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

	CON	TVA	TVA +1X	TVA +2X	SEM	P<	Male	Female	SEM	P <
Body weight	32.1	32.1	31.8	31.7	0.77	0.98	35.9	27.9	0.54	0.01
Dry carcass weight	5.20	5.21	4.52	4.22	0.26	0.03*,c	5.78	3.79	0.19	0.01
Liver (wet)	1.87	1.98	1.90	2.00	0.06	0.40	2.18	1.69	0.04	0.01
Liver (dry)	0.58	0.62	0.59	0.63	0.02	0.35	0.69	0.52	0.02	0.01
Small intestine	1.72	1.82	1.67	1.73	0.06	0.35	1.71	1.76	0.04	0.37
Gastrocnemius muscle	0.19	0.18	0.18	0.19	0.007	0.56	0.21	0.16	0.005	0.01
Femur	0.11	0.10	0.11	0.11	0.004	0.82	0.11	0.097	0.003	0.01
Epididymal Adipose	0.86	0.82	0.74	0.68	0.14	0.81				
Inguinal Adipose	0.45	0.36	0.23	0.26	0.07	0.15				

*Linear effect of treatment; [c]TVA vs TVA + 2X

Table 4-3. Liver DNA, RNA and protein in mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

	CON	TVA	TVA + 1X	TVA + 2X	SEM	P <	Male	Female	SEM	P <
DNA (μg/mg dry liver)	21.4	13.3	12.2	17.6	4.4	0.44	12.6	19.6	3.1	0.12
Total DNA in dry liver (mg)	11.5	8.3	7.4	10.9	2.5	0.61	8.6	10.5	1.8	0.45
RNA (μg/mg dry liver)	10.3	11.8	11.8	12.8	1.3	0.60	12.1	11.2	0.91	0.52
Total RNA in dry liver (mg)	6.1	7.6	7.0	8.0	0.9	0.49	8.4	5.9	0.6	0.01
Protein (mg/mg dry liver)	0.38	0.39	0.34	0.40	0.02	0.06	0.35	0.41	0.01	0.01
Total protein in dry liver (mg)	221	241	201	250	13	0.06	244	213	9.2	0.02

Table 4-4. Fatty acid composition of plasma in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

Fatty acid	CON	TVA	TVA + 1X	TVA + 2X	SEM	P <	Male	Female	SEM	P <
				μg fatty acids per ml of plasma						
14:0	0.44	0.41	0.39	0.43	0.05	0.84	0.46	0.38	0.03	0.08
16:0	34.74	30.92	29.13	33.73	2.24	0.31	37.34	26.92	1.60	0.01
*cis*9-16:1	1.42	1.02	0.96	1.05	0.17	0.25	1.45	0.77	0.12	0.01
*trans*11-16:1	0.01	0.22	0.27	0.32	0.02	0.01[*,a,c]	0.22	0.18	0.01	0.03
17:0	0.61	0.55	0.52	0.59	0.04	0.39	0.65	0.48	0.03	0.01
18:0	33.21	28.03	26.98	30.70	1.97	0.14	31.49	27.98	1.41	0.09
*cis*9-18:1	48.22	39.92	36.30	43.35	4.15	0.25	47.00	36.89	2.97	0.02
*cis*11-18:1	3.51	2.90	2.68	3.17	0.36	0.43	3.66	2.47	0.26	0.01
*trans*11-18:1	0.09	5.42	4.49	5.92	0.48	0.01[a]	4.53	3.43	0.34	0.03
18:2n-6	95.8	86.3	107.9	122.9	9.5	0.05[*,c]	122.9	83.6	6.7	0.01
*cis*9,*trans*11-18:2	0.05	0.24	0.29	0.41	0.05	0.01[*,a]	0.28	0.22	0.04	0.25
*trans*10,*cis*12-18:2	0.04	0.04	0.43	1.64	0.15	0.01[*,c]	0.56	0.51	0.11	0.76
18:3n-3	0.94	1.00	1.64	1.98	0.19	0.01[*,c]	1.62	1.16	0.14	0.02
20:0	2.23	1.55	1.61	1.96	0.21	0.11	2.72	0.95	0.15	0.01
20:4n-6	81.0	64.1	93.6	102.6	12.9	0.20	98.3	72.4	9.3	0.06
20:5n-3	2.35	1.85	3.39	3.16	0.49	0.13	3.29	2.08	0.35	0.02
22:5n-3	1.22	1.09	2.78	3.26	0.42	0.01[*,b,c]	2.84	1.34	0.30	0.01
22:6n-3	24.28	17.57	44.69	44.52	6.18	0.01[*,b,c]	37.73	27.80	4.38	0.12
Total (μg/ml of plasma)	343	294	373	417	35	0.11[*]	414	300	25	0.01

[*]Linear effect of treatment; [a]CON vs TVA; [b]TVA vs TVA + 1X; [c]TVA vs TVA + 2

Table 4-5. Fatty acid composition of liver in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

Fatty acid	CON	TVA	TVA + 1X	TVA + 2X	SEM	P <	Male	Female	SEM	P <
				% total fatty acids						
14:0	0.37	0.36	0.36	0.39	0.02	0.85	0.40	0.34	0.01	0.01
16:0	35.34	35.59	34.28	35.02	1.24	0.90	36.36	33.75	0.89	0.05
*cis*9-16:1	0.99	0.90	0.88	0.79	0.10	0.57	1.09	0.68	0.07	0.01
*trans*9-16:1	0.06	0.26	0.30	0.33	0.02	0.01[*,a]	0.25	0.23	0.02	0.45
*trans*11-16:1	0.06	0.26	0.30	0.33	0.02	0.01[*,a]	0.25	0.23	0.02	0.45
17:0	0.45	0.47	0.46	0.46	0.03	0.95	0.47	0.45	0.02	0.43
18:0	21.83	21.70	20.09	19.92	1.25	0.59	19.12	22.65	0.89	0.01
*cis*9-18:1	21.91	21.75	19.66	21.20	1.19	0.56	21.21	21.05	0.85	0.89
*trans*9-18:1	0.08	0.07	0.04	0.04	0.008	0.01[*]	0.05	0.06	0.006	0.20
*trans*11-18:1	0.10	1.86	1.79	1.87	0.11	0.01[a]	1.39	1.42	0.08	0.77
18:2n-6	9.2	8.0	11.7	11.0	1.26	0.18	9.68	10.26	0.91	0.65
*cis*9,*trans*11-18:2	0.003	0.18	0.16	0.15	0.01	0.01[a]	0.13	0.12	0.01	0.35
*trans*10,*cis*12-18:2	0.00	0.005	0.10	0.18	0.008	0.01[*,b,c]	0.07	0.07	0.005	0.90
18:3n-3	0.15	0.11	0.16	0.17	0.02	0.12	0.14	0.15	0.01	0.64
20:0	0.41	0.39	0.39	0.41	0.04	0.94	0.66	0.14	0.03	0.01
20:4n-6	2.83	2.29	3.30	2.35	0.54	0.55	2.53	2.86	0.38	0.55
20:5n-3	0.02	0.04	0.04	0.05	0.01	0.33	0.03	0.04	0.01	0.45
22:6n-3	1.33	0.99	1.52	1.09	0.27	0.55	1.15	1.32	0.19	0.54
Total (μg/mg of liver)	25.0	23.4	24.2	26.9	2.0	0.65	24.5	25.2	1.4	0.72

[*]Linear effect of treatment; [a]CON vs TVA; [b]TVA vs TVA + 1X; [c]TVA vs TVA + 2X

Table 4-6. Fatty acid composition of adipose in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

Fatty acid	CON	TVA	TVA + 1X	TVA + 2X	SEM	P<	Male	Female	SEM	P<
					% total fatty acids					
14:0	0.59	0.59	0.58	0.52	0.03	0.23	0.55	0.59	0.02	0.22
16:0	13.08	13.24	13.75	12.33	0.63	0.53	12.86	13.34	0.44	0.45
*cis*9-16:1	2.51	2.61	2.06	2.07	0.21	0.19	2.67	1.95	0.15	0.01
*trans*9-16:1	0.06	0.11	0.13	0.13	0.01	0.01[a]	0.09	0.12	0.01	0.04
17:0	0.12	0.12	0.14	0.14	0.005	0.04[*,b,c]	0.13	0.13	0.003	0.15
18:0	2.13	2.30	2.17	2.04	0.24	0.90	1.69	2.63	0.17	0.01
*cis*9-18:1	39.7	39.3	39.1	39.1	0.50	0.77	39.2	39.4	0.36	0.77
*cis*11-18:1	1.35	1.40	1.33	1.38	0.04	0.74	1.42	1.30	0.03	0.01
*trans*9-18:1	0.08	0.02	0.02	0.01	0.01	0.01[a]	0.03	0.03	0.01	0.64
*trans*11-18:1	0.10	0.92	1.06	1.06	0.14	0.01[a]	0.46	1.11	0.09	0.01
18:2n-6	36.8	35.6	35.9	37.1	0.60	0.27	37.4	35.3	0.43	0.01
*cis*9,*trans*11-18:2	0.06	0.43	0.40	0.31	0.05	0.01[a]	0.20	0.40	0.04	0.01
*trans*10,*cis*12-18:2	0.03	0.05	0.35	0.65	0.09	0.01[*,b,c]	0.19	0.34	0.06	0.11
18:3n-3	1.50	1.33	1.43	1.46	0.07	0.37	1.45	1.42	0.05	0.66
20:0	0.16	0.21	0.16	0.12	0.03	0.33	0.12	0.21	0.02	0.01
20:4n-6	0.15	0.18	0.12	0.16	0.01	0.14	0.12	0.18	0.01	0.01
20:5n3	0.01	0.01	0.01	0.01	0.002	0.87	0.01	0.01	0.001	0.08
22:5n-3	0.009	0.011	0.008	0.015	0.001	0.04	0.01	0.01	0.001	0.73
Total (μg/mg of adipose)	845	965	540	559	99	0.01[*,b,c]	776	679	70	0.34

[*]linear effect of treatment; [a] CON vs TVA; [b]TVA vs TVA + 1X; [c] TVA vs TVA + 2X

Table 4-7. Fatty acid composition of femur in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

Fatty acid	CON	TVA	TVA + 1X	TVA + 2X	SEM	P <	Male	Female	SEM	P <
				% total fatty acids						
14:0	1.50	1.45	1.60	1.62	0.04	0.03[*,c]	1.44	1.64	0.03	0.01
16:0	23.27	23.17	23.54	23.63	0.95	0.98	22.12	24.68	0.67	0.01
*cis*9-16:1	6.52	6.00	6.32	5.27	0.45	0.24	7.10	4.95	0.32	0.01
*trans*9-16:1	0.03	0.12	0.15	0.17	0.009	0.01[*,a,c]	0.11	0.13	0.006	0.07
17:0	0.29	0.31	0.30	0.31	0.02	0.74	0.29	0.32	0.01	0.10
18:0	7.98	8.52	8.01	8.45	0.70	0.92	7.15	9.33	0.49	0.01
*cis*9-18:1	40.40	39.73	39.35	38.39	0.97	0.53	39.93	39.00	0.69	0.35
*cis*11-18:1	2.64	2.68	2.54	2.54	0.06	0.34	2.69	2.51	0.05	0.01
*trans*9-18:1	0.12	0.11	0.09	0.09	0.006	0.01	0.10	0.11	0.004	0.24
*trans*11-18:1	0.04	0.83	0.87	0.95	0.06	0.01[a]	0.56	0.79	0.05	0.01
18:2n-6	11.46	11.04	11.02	11.95	0.72	0.77	12.35	10.39	0.51	0.01
*cis*9,*trans*11-18:2	0.09	0.22	0.22	0.20	0.02	0.01[a]	0.18	0.19	0.02	0.65
*trans*10,*cis*12-18:2	0.04	0.05	0.10	0.20	0.009	0.01[**,b,c]	0.09	0.12	0.006	0.01
18:3n-3	0.16	0.14	0.13	0.13	0.02	0.53	0.16	0.12	0.01	0.01
20:0	0.24	0.26	0.23	0.22	0.02	0.56	0.22	0.25	0.01	0.15
20:4n-6	0.54	0.57	0.60	0.74	0.11	0.56	0.65	0.57	0.08	0.41
20:5n-3	0.10	0.12	0.11	0.10	0.01	0.79	0.09	0.12	0.01	0.18
22:6n-3	0.16	0.18	0.19	0.22	0.02	0.10	0.17	0.20	0.01	0.09
Total (μg/mg of femur)	3.55	3.29	3.08	2.63	0.43	0.50	3.67	2.60	0.31	0.02

[*]Linear effect of treatment; [**]linear and quadratic effect of treatment; [a] CON vs TVA; [b]TVA vs TVA + 1X; [c] TVA vs TVA + 2X

Table 4-8. Fatty acid composition of gastrocnemius muscle in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

Fatty acid	CON	TVA	TVA + 1X	TVA + 2X	SEM	P <	Male	Female	SEM	P <
				% total fatty acids						
14:0	1.14	1.13	1.13	1.18	0.06	0.92	1.16	1.14	0.04	0.73
16:0	23.0	22.9	23.4	26.7	1.4	0.19	23.5	24.6	1.0	0.45
*cis*9-16:1	5.34	5.10	4.16	4.06	0.29	0.01[*,c]	5.53	3.81	0.20	0.01
*trans*9-16:1	0.06	0.17	0.24	0.28	0.02	0.01[*,a,c]	0.17	0.20	0.01	0.15
17:0	0.31	0.37	0.36	0.42	0.04	0.41	0.36	0.37	0.03	0.78
18:0	6.74	6.62	6.46	8.39	0.66	0.16	6.70	7.41	0.47	0.29
*cis*9-18:1	39.57	41.98	39.15	36.67	1.58	0.13	38.65	39.54	1.12	0.58
*cis*11-18:1	1.92	2.00	1.79	1.99	0.08	0.25	1.96	1.89	0.06	0.41
*trans*9-18:1	0.06	0.06	0.05	0.05	0.005	0.29	0.05	0.05	0.003	0.90
*trans*11-18:1	0.04	1.10	1.44	1.42	0.11	0.01[*,a,c]	0.75	1.25	0.08	0.01
18:2n-6	13.52	12.21	14.43	9.19	2.28	0.40	12.72	11.96	1.61	0.74
*cis*9,*trans*11-18:2	0.08	0.25	0.28	0.21	0.02	0.01[a]	0.19	0.22	0.01	0.24
*trans*10,*cis*12-18:2	0.003	0.01	0.17	0.20	0.03	0.01[*,b,c]	0.08	0.11	0.02	0.32
18:3n-3	0.29	0.25	0.30	0.20	0.06	0.62	0.28	0.24	0.04	0.46
20:0	0.18	0.20	0.17	0.18	0.02	0.81	0.16	0.20	0.01	0.04
20:4n-6	1.46	0.51	1.41	1.46	0.52	0.50	1.39	1.03	0.37	0.49
20:5n-3	0.16	0.15	0.15	0.16	0.01	0.93	0.14	0.16	0.01	0.39
22:6n-3	1.66	0.48	1.51	1.78	0.67	0.52	1.39	1.32	0.48	0.92
Total (μg/mg of muscle)	13.51	14.61	11.57	9.07	2.17	0.31	11.50	12.88	1.54	0.53

[*]Linear effect of treatment; [a] CON vs TVA; [b]TVA vs TVA + 1X; [c] TVA vs TVA + 2X

Table 4-9. Liver fatty acid ratios in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

	CON	TVA	TVA +1X	TVA +2X	SEM	P<	Male	Female	SEM	P <
16:0 to *cis*9-16:1	0.97	0.97	0.97	0.98	0.003	0.74	0.97	0.98	0.002	0.01
18:0 to *cis*9-18:1	0.50	0.50	0.50	0.48	0.03	0.95	0.47	0.52	0.02	0.10
*trans*11-18:1 to *cis*9,*trans*11-CLA	0.97	0.91	0.91	0.92	0.02	0.06	0.93	0.93	0.01	0.90
18:2n-6 to 20:4n-6	0.78	0.80	0.79	0.82	0.02	0.42	0.80	0.80	0.01	0.87
18:3n-3 to 20:5n-3	0.91	0.83	0.88	0.77	0.05	0.20	0.88	0.82	0.04	0.23

Table 4-10. Adipose fatty acid ratios in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

	CON	TVA	TVA +1X	TVA +2X	SEM	P<	Male	Female	SEM	P <
16:0 to *cis*9-16:1	0.84	0.84	0.87	0.86	0.01	0.16	0.83	0.87	0.01	0.01
18:0 to *cis*9-18:1	0.05	0.05	0.05	0.05	0.005	0.89	0.04	0.06	0.004	0.01
*trans*11-18:1 to *cis*9,*trans*11-CLA	0.31	0.68	0.70	0.75	0.04	0.01[a]	0.56	0.66	0.03	0.01
18:2n-6 to 20:4n-6	0.996	0.995	0.997	0.996	0.001	0.17	0.997	0.995	0.0003	0.01
18:3n-3 to 20:5n-3	0.992	0.993	0.992	0.993	0.001	0.87	0.993	0.992	0.001	0.55

[a] CON vs TVA

Table 4-11. Femur fatty acid ratios in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

	CON	TVA	TVA +1X	TVA +2X	SEM	P<	Male	Female	SEM	P <
16:0 to *cis*9-16:1	0.78	0.79	0.79	0.82	0.02	0.43	0.76	0.83	0.01	0.01
18:0 to *cis*9-18:1	0.17	0.18	0.17	0.18	0.02	0.90	0.15	0.19	0.01	0.01
*trans*11-18:1 to *cis*9,*trans*11-CLA	0.37	0.78	0.78	0.82	0.03	0.01[a]	0.66	0.70	0.02	0.17
18:2n-6 to 20:4n-6	0.96	0.95	0.95	0.94	0.006	0.37	0.95	0.95	0.004	0.97
18:3n-3 to 20:5n-3	0.62	0.55	0.55	0.59	0.06	0.81	0.63	0.53	0.04	0.10

[a] CON vs TVA

Table 4-12. Gastrocnemius muscle fatty acid ratios in male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

	CON	TVA	TVA +1X	TVA +2X	SEM	P<	Male	Female	SEM	P <
16:0 to *cis*9-16:1	0.81	0.82	0.85	0.86	0.01	0.01[*,c]	0.81	0.86	0.01	0.01
18:0 to *cis*9-18:1	0.15	0.14	0.14	0.19	0.01	0.04[*,c]	0.15	0.16	0.01	0.41
*trans*11-18:1 to *cis*9,*trans*11-CLA	0.39	0.81	0.83	0.85	0.02	0.01[a]	0.69	0.75	0.01	0.01
18:2n-6 to 20:4n-6	0.92	0.96	0.93	0.92	0.02	0.42	0.93	0.94	0.01	0.75
18:3n-3 to 20:5n-3	0.60	0.54	0.61	0.50	0.07	0.62	0.58	0.54	0.05	0.52

[*] Linear effect of treatment; [a] CON vs TVA; [c] TVA vs TVA + 2X

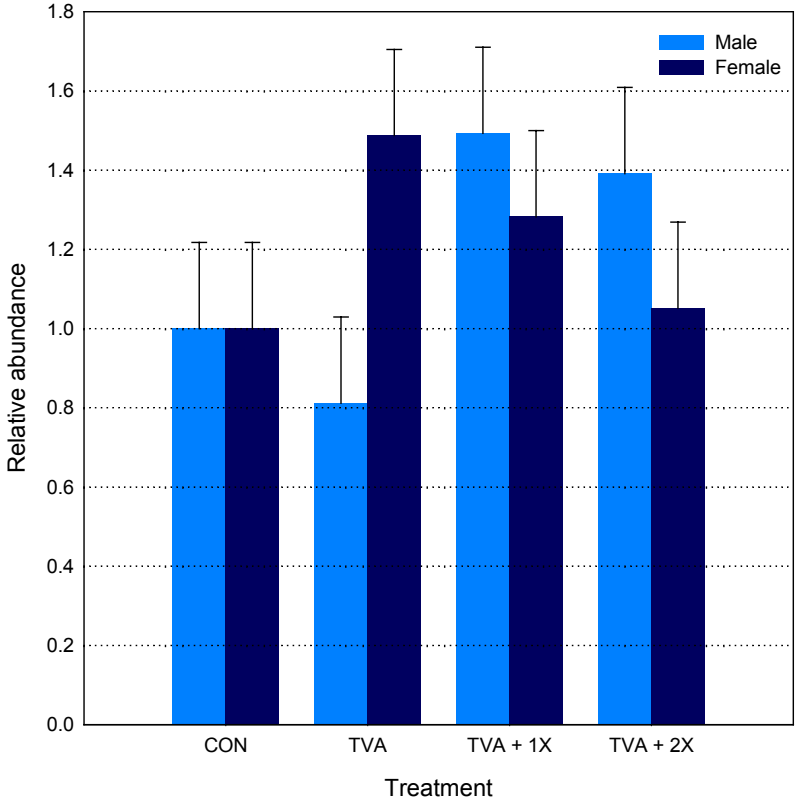

Figure 4-1. SCD mRNA expression in liver of male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

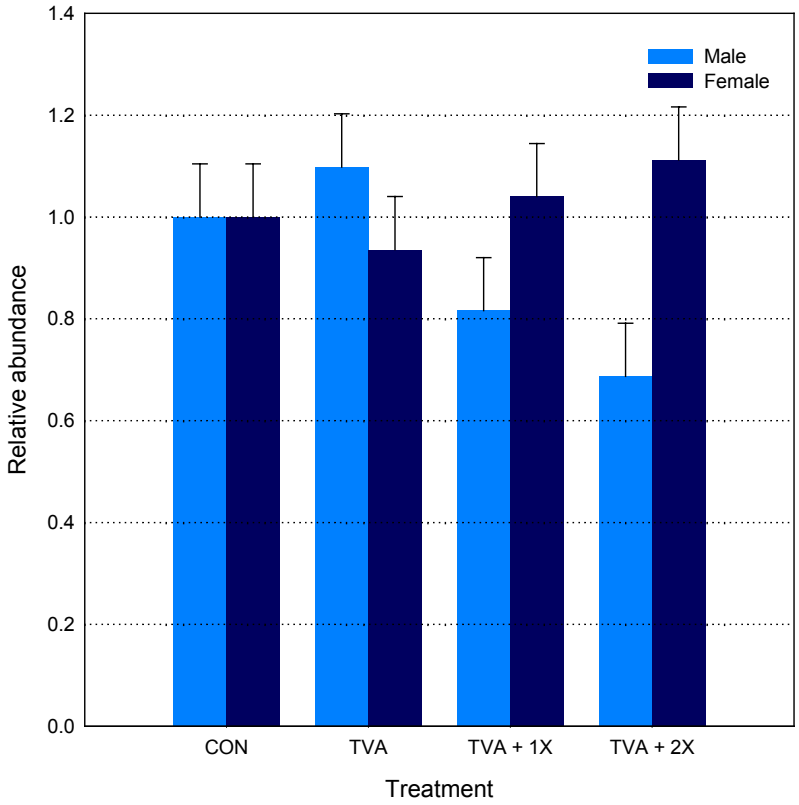

Figure 4-2. SCD mRNA expression in adipose of male and female mice fed diets containing TVA and 0, 0.15 (1X), or 0.30% (2X) *trans*10,*cis*12-conjugated linoleic acid (CLA)

CHAPTER 5

Alterations in CPT-1 mRNA and fatty acid profile in response to *trans*10,*cis*12-conjugated linoleic acid (CLA) in hepatic cell lines

Abstract

*Trans*10,*cis*12-CLA increases liver weights and hepatic lipids in mice. The purpose of this study was to determine the effects of CLA isomers or CPT-1 inhibitors on CPT-1 mRNA, fatty acid profile, and cholesterol synthesis in AML-12 and HepG2 cells. Cells (AML-12 or HepG2) were incubated with control media or media containing 15 μM etomoxir (ETM), 30 μM ETM, 15 μM hemipalmitoylcarnitinium (HPC), 30 μM HPC, 100 μM *cis*9,*trans*11-CLA, or 100 μM *trans*10,*cis*12-CLA for 24 h. Half of the cells were harvested at 24 h, while the remainder were incubated for an additional 24 h in the control media. *Trans*10,*cis*12-CLA was incorporated to a greater extent than *cis*9,*trans*11-CLA in both cell lines. Cells incubated *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA had reduced percentages of 22:6n-3 and 24:0 compared with control, suggesting an increase in peroxisomal oxidation of very long chain fatty acids. Ratios of 16:0 to 16:1 and 18:0 to 18:1 increased in both cell lines in response to *trans*10,*cis*12-CLA, suggesting inhibition of Δ^9-desaturation. Ratio of 18:2n-6 to 20:4n-6 in AML-12 cells increased in response to *cis*9,*trans*11-CLA (0.56 units) or *trans*10,*cis*12-CLA (0.59 units) compared to control (0.27 units). Similarly, ratio of 18:2n-6 to 20:4n-6 in HepG2 cells increased from 0.31 units (control) to 0.54 units (*cis*9,*trans*11-CLA) or 0.58 units (*trans*10,*cis*12-CLA), indicating inhibition of Δ^5 and Δ^6-desaturation. Cells incubated with media containing CPT-1 inhibitors (ETM, HPC) or *trans*10,*cis*12-CLA had higher levels of mRNA for CPT-1 in both cell lines compared with the control, suggesting *trans*10,*cis*12-CLA increased fatty acid oxidation in hepatic cell lines. *Trans*10,*cis*12-CLA increased free cholesterol content of AML-12 and HepG2 cells 4-fold and 5-fold, respectively. Following treatment withdrawal, percentages of *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA remained elevated in cells initially treated with *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA, suggesting a potential for carry-over effects of the CLA isomers. However, mRNA levels for CPT-1 were not different at 48 h, suggesting that the effect

of CPT-1 inhibitors and CLA isomers was reversible. Overall, results demonstrate a potential role for *trans*10,*cis*12-CLA in the modulation of hepatic lipid metaboism.

Key words: conjugated linoleic acid, CPT-1, desaturation, cells

Introduction

Conjugated linoleic acid (CLA) is a unique fatty acid with potential effects on body fat metabolism. Diets containing mixtures of several CLA isomers reduced body fat in mice (1), rats (2), pigs (3), and obese humans (4). However, dietary CLA mixtures also caused an increased fatty acid accumulation in the liver manifested by an increase in liver weights (5, 6, 7, 8). The *trans*10,*cis*12-CLA was identified as the isomer responsible for the liver steatosis (9, Chapter 5). Possible explanations for trigyceride accumulation in the liver of animals fed a CLA mixture or pure *trans*10,*cis*12-CLA include increased uptake of fatty acids, increased lipogenesis, or decreased fatty acid oxidation of fatty acids.

One of the key enzymes regulating the β-oxidation of fatty acids is carnitine palmitoyl transferase-1 (CPT-1). Inhibition of CPT-1 results in arrest of fatty acid oxidation and subsequent lipid accumulation within the liver (10). Because fatty acids are the preferred substrates in conditions such as diabetes, a decrease in their oxidation results in the uptake and utilization of glucose for energy (11). Thus, CPT-1 inhibitors such as etomoxir (ETM) and hemipalmitoylcarnitinium (HPC), have a potential pharmacological role in the treatment of diabetes and the reduction of ketogenesis.

Etomoxir [ethyl-2-(4-chlorophenoxyl) oxirane-2-carboxylate] is a member of a family of chemical compounds that inhibit mitochondrial oxidation of long-chain fatty acids, ketogenesis, and gluconeogenesis (12). After conversion to its CoA ester, ETM binds irreversibly to the active catalytic site of CPT-1 and inhibits the transport of long chain fatty acids into the mitochondria (11). Inhibition of fatty acid oxidation results in a shift in substrate utilization from fatty acids to glucose as a source of energy (12). Thus, ETM is a potentially effective drug in the treatment of non-insulin dependent diabetes.

Another potential CPT-1 inhibitor is HPC, which belongs to a class of carnitine acyl transferase inhibitors known as hemiacylcarnitiniums (13). Like ETM, HPC competitively binds to the catalytic site of CPT-1, thus preventing the entry of fatty acids

into the mitochondrion for oxidation. However, while ETM binds irreversibly to CPT-1, the binding of HPC to the catalytic site of CPT-1 is reversible (13).

This study was designed to explore the effects of purified *trans*10,*cis*12-CLA and *cis*9,*trans*11-CLA on CPT-1 mRNA expression in normal mouse hepatocytes (AML-12) and human hepatoma cells (HepG2) after a 24-h incubation. Data obtained from these cell lines possibly could provide insight into *in vivo* effects of the treatments in mice and humans. Although the HepG2 cell line is derived from human hepatoma, it retains many normal hepatic functions, including cholesterol synthesis and lipoprotein secretion (14). Because ETM and HPC are potent inhibitors of CPT-1, they also were used as treatments in this study for comparison with *trans*10,*cis*12-CLA. Additionally, the effects of the CLA isomers and CPT-1 inhibitors on fatty acid profile and free cholesterol synthesis in both cell lines was tested. Alteration in CPT-1 mRNA expression, fatty acid profile, and the amount of free cholesterol in cells following a 24-h withdrawal of treatments also was examined.

Materials and Methods

Human hepatoma (HepG2) cells, mouse hepatocytes (AML-12), and Vitacell® Minimum Essential Eagle Medium were purchased from American Type Cell Culture (Manassas, VA). *Trans*10,*cis*12-CLA (>95% purity) and *cis*9,*trans*11-CLA (>90% purity) were obtained from Natural Lipids (Hordebygda, Norway). Fetal bovine serum (FBS), 10X Trypsin-EDTA, 100X Antibiotic-Antimycotic solution (containing 10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin B), and Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 (containing 2.5 mM L-glutamine, 1.2 g/L sodium bicarbonate, 15 mM HEPES, and 0.5 mM sodium pyruvate), were purchased from Atlanta Biologicals (Norcross, GA). Bovine serum albumin (BSA), insulin, transferrin, selenium, HEPES, and dexamethasone were obtained from Sigma (St. Louis, MO). Etomoxir (ETM) was purchased from Dr. H. P. O. Wolf (GMBH, Germany). Hemipalmitoylcarnitinium (HPC) was donated by Dr. Richard Gandour (Department of Chemistry, Virginia Tech.). Amplex Red® was purchased from Molecular Probes Inc. (Eugene, OR). Horseradish peroxidase, cholesterol oxidase, free cholesterol standard, cholic acid, and Triton® X-100 were purchased from Sigma (St. Louis, MO). Human and mouse CPT-1 cDNA probes were donated by Dr. Nigel Price (Dept. of Cellular Biochemistry, Hannah Research Institute, Scotland, UK).

Isomers of CLA (*cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA) were bound to BSA according to the method outlined by Spector (15). The molar ratio of fatty acids to BSA was kept at 2:1. Briefly, the desired amount of fatty acid was weighed out in a 10 ml screw-capped tube filled with nitrogen and dissolved in 1 ml hexane. After adding 40 μl of 1 M KOH, the hexane layer was evaporated thoroughly under nitrogen. The residue was dissolved in 3 ml double distilled water and heated at 50 ^{o}C in a water bath under nitrogen until the solution became clear. The warm fatty acid solution was then added slowly to 0.5 mM BSA solution in 2X PBS. The pH was adjusted to 7.2 and the final volume was made up to 50 ml with double distilled water. The fatty acid-albumin solution was sterilized by filtering through a 0.45 μm filter (Nalgene, NY) and stored at -

80 ^{o}C. The fatty acid complex was diluted in the growth media to obtain the desired concentrations of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA.

Growth medium for HepG2 cells contained 89% Vitacell® Minimum Essential Eagle Medium, 10% FBS and 1% antibiotic-antimycotic solution. Medium for AML-12 cells was prepared by adding 10% FBS, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone to Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 medium. Cells were maintained in their respective growth media at 37^{o}C under standard atmospheric conditions (95% air and 5% CO_2). Cells were seeded into 24-well collagen treated plates at a density of 0.5 x 10^5 cells per well with 0.5-ml growth medium. Cells were allowed to grow for 24 h, then growth media was removed and 0.5 ml of growth media (**CON**), or growth media containing 15 μM etomoxir (**ETM**), 30 μM ETM, 15 μM hemipalmitoylcarnitinium (**HPC**), 30 μM HPC, 100 μM *cis*9,*trans*11-CLA (**9,11-CLA**) or 100 μM *trans*10,*cis*12-CLA (**10,12-CLA**) were added to the wells. Half of the cells were harvested to determine fatty acids, CPT-1 mRNA expression, and cholesterol concentration at 24 h. Treatment media was replaced with the control media at 24 h in the remaining cells. After an additional 24-h of incubation, the cells were harvested (48-h group). The 48-h group was used for testing the reversibility of ETM and the effect of withdrawal of CLA isomers on fatty acid profiles.

A 1X solution of trypsin-EDTA was prepared by dissolving 1 ml of 10X trypsin-EDTA in 9 ml Dulbecco's phosphate buffer saline (DPBS). Media was removed and stored at -80 ^{o}C for fatty acid analysis. Then 100 μl of 1X trypsin-EDTA was added to each well and the plates were incubated at 37 ^{o}C for 10 min. DPBS (250 μl) was added to each well, cells were transferred to 1.5 ml microfuge tubes, and cells were stored at -80 ^{o}C for fatty acid analysis and cholesterol assay.

Fatty acids from cells were extracted according to the Folch procedure (16) with modifications (17), and the resulting fatty acids were transesterified according to the method of Park and Goins (18) as described in Chapter 2. Methyl esters of fatty acids

were separated on an Agilent 6890N gas chromatograph equipped with 7683 autosampler and a flame ionization detector (Agilent Technologies, Palo Alto, CA). Agilent ChemData software was used for data acquisition, integration and quantification. Methyl esters of fatty acids obtained from cells were run splitless using hydrogen as the carrier gas. Temperature conditions were similar to those described for bone fatty acids in Chapter 2. Desaturation ratios were calculated as described in Chapter 2.

Cholesterol was extracted from the cells based on the procedure outlined by Heider and Boyett (19) with modifications. Briefly, tubes containing cells were centrifuged at 5000 x *g* for 10 min in a Micromax microfuge (IEC, Needham Heights, MA). The resulting pellet was homogenized in 500 μl of isopropanol. Tubes were centrifuged at 3000 x *g* for 15 min. Supernatant was decanted into acid-washed 12 x 15 mm glass tubes and evaporated to dryness under nitrogen in an N-Evap (Model # 112, Organomation Inc., South Berlin, MA) at 45 ^{o}C to obtain the cholesterol fraction.

Free cholesterol was determined using the Amplex® Red based fluorometric procedure (20). Briefly, the cholesterol obtained after evaporation under nitrogen, was dissolved in 900 μl of 1X reaction buffer (5 ml 0.5 M potassium phosphate, pH 7.4, 0.05 M NaCl, 5 mM cholic acid, 0.1% Triton® X-100). Fifty microliters of sample, standard, or a control, were pipetted into separate wells of a 96-well microplate and an equal volume of Amplex Red working solution (containing 2 U/ml horseradish peroxidase and 2 U/ml cholesterol oxidase in Amplex Red reagent) was added. Plates were incubated at 37 ^{o}C for 30 min. Fluorescence was measured in a microplate reader (Bio-Tek Instruments Inc., Winooski, VT) using excitation and emission wavelengths of 530 and 590 nm respectively.

For RNA extraction, 100 μl of Tri Reagent® (MRC, Cincinnati, OH) was added to each well after removal of the overlying media. Plates were incubated at room temperature for 5 min. Cells were harvested into 1.5 ml microfuge tubes and RNA and DNA were extracted according to the procedure outlined in Chapter 3. Gel electrophoresis of RNA, membrane transfer, and a DIG hybridization and detection

procedure using a CPT-1 cDNA probe were carried out as described previously (Chapter 3). Intensity of the mRNA bands was measured using the GelWorks® 1D intermediate software, version 4.01 (UVP Inc., Upland, CA) and were normalized to β-actin bands.

Statistical Analysis

Data were analyzed as a completely randomized design using the MIXED procedure of SAS according to the following model:

$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$

where Y_{ij} is the observation for the j^{th} well supplemented with the i^{th} treatment.

μ = grand mean,

α_i = effect of treatment i [i = 1 (control; **CON**), 2 (**15 μM ETM**), 3 (**30 μM ETM**), 4 (**15 μM HPC**), 5 (**30 μM HPC**), 6 (100 μM *cis*9,*trans*11-CLA; **9,11-CLA**) or 7 (100 μM *trans*10,*cis*12-CLA; **10,12-CLA**)]

ε_{ij} = error effect associated with Y_{ij} (j = 1, .,3 wells).

All data are reported as least square means ± SEM. Non-orthogonal contrasts were performed to compare the control (**CON**) with each treatment. Contrasts were tested at $P < 0.05$ using the Dunnett's procedure.

Because the variance of observations within each treatment differed extensively from one treatment to another, the ratio of the highest to lowest variance was used to detect whether a log transformation would reduce the disparity. Log transformation caused a several-fold reduction in error variance. Data are therefore reported as least square means ± SEM of raw and log transformed values, whereas tests of significances were made on transformed data only.

Results

Table 5-1 shows the fatty acid profile of AML-12 cells, 24 h after the application of treatments. Both *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA were incorporated into the cells and comprised 24 and 34% of the total fatty acids respectively. Percentages of 18:0, *cis*11-C18:1, 22:6n-3, and 24:0 decreased, while 18:2n-6 increased due to the CLA isomers compared with the control. Cells treated with the *trans*10,*cis*12-CLA also had lower percentages of 16:0, *cis*9-16:1, and *cis*9-C18:1 compared with the control. Etomoxir and HPC did not alter the fatty acid profile of AML-12 cells.

Changes in fatty acid profile of AML-12 cells at 48 h (48-h groups), following withdrawal of treatments, are shown in **Table 5-2**. Percentages of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA were 10 and 16% respectively in cells that were initially treated with the *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA. Percentages of *cis*9-18:1, *cis*11-18:1, 20:4n-6, 22:5n-3, 22:6n-3, and 24:0 were lower, while 18:2n-6 remained elevated upon withdrawal of the CLA isomers from the media compared with the control. However, percentage of 16:0 was increased following withdrawal of *trans*10,*cis*12-CLA from the media. Cells initially treated with ETM also had lower 24:0 compared with the control.

Table 5-3 shows the fatty acid composition of AML-12 media at 24 h. The *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA comprised approximately 11% and 11.5% of the total fatty acids respectively, in media containing *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA. Like AML-12 cells, percentages of 22:5n-3 and 22:6n-3 were lower in media containing *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA compared with the control.

Compared with the control, percentage of *cis*9,*trans*11-CLA in media remained higher even after withdrawal of *cis*9,*trans*11-CLA from the media (48-h group) (**Table 5-4**). Similarly, percentage of *trans*10,*cis*12-CLA in media tended to be higher even after removal of *trans*10,*cis*12-CLA from the media.

Like AML-12 cells, the *trans*10,*cis*12-CLA and *cis*9,trans11-CLA were incorporated into HepG2 cells and comprised approximately 18 and 28% of the total fatty acids respectively (**Table 5-5**). Both CLA isomers decreased percentages of 14:0, 16:0, *cis*9-16:1, *cis*11-18:1, 20:4n-6, 22:6n-3, 24:0, and increased 18:2n-6 and 20:2n-6, compared with the control. Cells treated with *trans*10,*cis*12-CLA had an increased percentage of 18:3n-3. Supplementing HepG2 cells with ETM or HPC decreased percentages of 22:6n-3, while ETM also lowered 24:0 compared with the control.

Following treatment withdrawal (48-h groups), percentages of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA were 9.5 and 15% respectively in cells initially treated with *cis*9,*trans*11-CLA and the *trans*10,*cis*12-CLA (**Table 5-6**). Withdrawal of *trans*10,*cis*12-CLA resulted in increased percentages of 16:0 and 18:0. Percentages of cis9-16:1, cis9-18:1, cis11-18:1, 20:4n-6, 22:6n-3, and 24:0 remained lower, while 18:2n-6 and 20:2n-6 remained elevated in cells initially treated with the CLA isomers. Withdrawal of treatments did not alter fatty acid profile in the ETM and HPC groups in HepG2 cells.

Percentages of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA were higher in media containing *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA compared with the control (**Table 5-7**). Percentages of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA in media continued to remain higher even after withdrawal of *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA from the media (48-h group) (**Table 5-8**).

Fatty acid ratios of 16:0 to *cis*9-16:1 and 18:2n-6 to 20:4n-6 increased in AML-12 cells at 24 h in response *trans*10,*cis*12-CLA (**Table 5-9**). Ratio of 18:2n-6 to 20:4n-6 was also elevated in the *cis*9,*trans*11-CLA treatment group compared with the control. Ratios followed a similar pattern 24h after withdrawal of *trans*10,*cis*12-CLA and *cis*9,*trans*11-CLA from the media (**Table 5-9**). Cells treated with ETM had no effect on fatty acid ratios at 24 h or upon withdrawal of treatments. Although, HPC did not alter fatty acid ratios at 24 h, ratio of 18:2n-6 to 20:4n-6 were reduced following treatment withdrawal in the 15 μM HPC group.

Both *trans*10,*cis*12-CLA and *cis*9,*trans*11-CLA reduced ratios of 16:0 to *cis*9-16:1 and 18:2n-6 to 20:4n-6 in HepG2 cells at 24 h (**Table 5-10**). In addition, *trans*10,*cis*12-CLA caused a reduction in the ratio of 18:0 to *cis*9-18:1 compared with the control. Withdrawal of treatments did not alter the fatty acid ratios in HepG2 cells (**Table 5-10**).

Amount of free cholesterol in AML-12 cells was not altered upon application or withdrawal of treatments (**Table 5-11**). However, free cholesterol amounts were increased in HepG2 cells due to *trans*10,*cis*12-CLA treatment and remained elevated upon withdrawal of *trans*10,*cis*12-CLA from the media (**Table 5-11**).

Expression of CPT-1 mRNA was elevated in response to 30 μM ETM, 15 μM HPC, and *trans*10,*cis*12-CLA, compared with the control (**Figure 5-1**). However, upon withdrawal of treatments from the media (48-h groups), CPT-1 mRNA levels were not different from the control (**Figure 5-2**). Similarly, CPT-1 expression was increased in HepG2 cells in response to 30 μM ETM, 15 μM HPC, 30 μM HPC, and *trans*10,*cis*12-CLA, compared with the control (**Figure 5-3**). Following withdrawal of treatments from the media (48-h group), levels were similar to the control (**Figure 5-4**). The *cis*9,*trans*11-CLA did not alter CPT-1 mRNA expression in AML-12 and HepG2 cells.

Discussion

Lipid content of hepatocytes is regulated by several enzymes that catalyze fatty acid uptake, synthesis, secretion, and oxidation. When hepatic fatty acid uptake and lipogenesis exceeds the capacity of the liver to oxidize and secrete lipids, hepatic steatosis occurs. Thus, impairment of fatty acid oxidation by CPT-1 inhibitors such as ETM results in elevated liver lipid content (21). Similarly, dietary *trans*10,*cis*12-CLA also increases hepatic lipid accumulation and liver weights mice (9, Chapter 3). However, the effect of pure *cis*9,*trans*11 and *trans*10,*cis*12 isomers on CPT-1 mRNA expression and fatty acid profile in hepatocytes has not been reported. This study therefore focussed on the effect of addition and withdrawal of *trans*10,*cis*12-CLA, *cis*9,*trans*11-CLA, and CPT-1 inhibitors on CPT-1 mRNA expression, fatty acid profile, and cholesterol synthesis in AML-12 and HepG2 cells.

HepG2 cells incubated with 1 mmol/L of *trans*10,*cis*12-CLA or *cis*9,*trans*11-CLA for 5 h had a higher percentage of *trans*10,*cis*12-CLA than *cis*9,*trans*11-CLA (22), suggesting that the *trans*10,*cis*12-CLA was incorporated to a greater extent than the *cis*9,*trans*11-CLA in this cell line. Similarly, in the current study, although AML-12 and HepG2 cells were treated with similar concentrations of *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA, percentage of *trans*10,*cis*12-CLA was greater in both cell lines compared with the *cis*9,*trans*11-CLA. Possibly, the *cis*9,*trans*11-CLA is converted to its conjugated metabolites such as 18:3 and 20:3 conjugated fatty acids (23), thereby decreasing the proportion of *cis*9,*trans*11-CLA within cells.

Increased incorporation of *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA in cells treated with the *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA reduced the percentages of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) of both cell lines compared with the control. However, desaturation ratios of SFA to MUFA were higher in both cell lines treated with the *trans*10,*cis*12-CLA compared with the control, suggesting an inhibition of Δ^9-desaturase in response to *trans*10,*cis*12-CLA treatment. These findings are consistent with *in vitro* data obtained from HepG2 cells (24) and *in vivo* data (25,

26), which indicate that *trans*10,*cis*12-CLA is able to decrease Δ^9-desaturase activity and mRNA expression.

The Δ^5 and Δ^6-desaturation of 18:2n-6 is a key step in the synthesis of arachidonic acid (20:4n-6), which is further converted to eicosanoids such as PGE_2 (27). Eicosanoids are involved in several health processes modulated by CLA, such as immune-modulation, cancer, and cardiovascular diseases. In the current study, cells treated with *trans*10,*cis*12-CLA and *cis*9,*trans*11-CLA had increased percentages of 18:2n-6 compared with the control. Percentage of 20:4n-6 was reduced in response to CLA isomers in HepG2 cells, but was not affected in AML-12 cells. However, ratios of 18:2n-6 to 20:4n-6 increased in both cell lines in response to *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA, suggesting inhibition of Δ^5 and Δ^6-desaturase. Bretillon et al. (25) demonstrated that both the *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA inhibited Δ^6-desaturase activity in rat liver microsomes. HepG2 cells treated with *trans*10,*cis*12-CLA had elevated ratios of linoleic to arachidonic acid suggesting inhibition of Δ^5 and Δ^6-desaturation (24). Results from the current study also support our previous finding that purified *trans*10,*cis*12-CLA increased ratio of 18:2n-6 to 20:4n-6 in mouse liver (Chapter 3).

Percentages of 22:6n-3 and 24:0 were reduced in both AML-12 and HepG2 cells treated with the cis9,trans11-CLA or trans10,cis12-CLA. Possibly, the CLA isomers increase peroxisomal fatty acid oxidation of the very long chain fatty acids (23), leading to a reduction in their percentage in cells. Reductions in percentage of 22:6n-3 was observed in HepG2 cells treated with CPT-1 inhibitors. Also, 24:0 was reduced in HepG2 cells compared with control in response to ETM. Reasons for this decrease however, are not clearly understood.

Treatment of HepG2 cells with a CLA mixture increased acetate incorporation into cholesterol (28). In the current study, amount of free cholesterol in AML-12 cells was numerically higher in response to *trans*10,*cis*12-CLA compared with the control. Free cholesterol was significantly greater in HepG2 cells treated with the *trans*10,*cis*12-

CLA compared with the control, suggesting enhanced cholesterol synthesis by *trans*10,*cis*12-CLA. These results are in agreement with a previous report that fatty acids have a stimulatory effect cholesterol synthesis (29) and enhance activity of HMG-CoA reductase (30), the rate-limiting step in cholesterol synthesis (31). Also, HepG2 cells appear to be more responsive to *trans*10,*cis*12-CLA than AML-12 cells, with respect to cholesterol synthesis.

Injection of ETM caused an increase in mRNA levels for CPT-1 in the liver of rats (11). Because ETM or HPC bind competitively to the catalytic site of CPT-1, they inhibit oxidation of fatty acids by preventing their entry into the mitochondria. The accumulated fatty acids act as ligands for peroxisome-proliferator activated receptor- alpha (PPAR-α) (11). The PPARs are ligand inducible transcription factors that control the expression of several genes involved in lipid metabolism, including CPT-1 (32). Thus inhibition of β-oxidation by CPT-1 inhibitors, would indirectly increase the expression of CPT-1 mRNA.

A dietary CLA mixture containing equal proportions of *cis*9,*trans*11-CLA and *trans*10, *cis*12-CLA increased hepatic mRNA for CPT-1 in mice coupled with elevated CPT-1 activity (8). Because CLA is a potent ligand for PPAR-α, it induces hepatic fatty acid oxidation (33), possibly through increased expression of CPT-1 mRNA.

In the current study, mRNA for CPT-1 was elevated in response to ETM and HPC in both cell lines compared with the control. Increased CPT-1 mRNA suggests inhibition of fatty acid oxidation in response to the CPT-1 inhibitors, in agreement with the findings of Hegardt et al. (11). *Trans*10,*cis*12-CLA also increased mRNA for CPT-1 in both cell lines compared to the control suggesting enhanced oxidation of fatty acids. Previous studies reported that *trans*10,*cis*12-CLA is associated with liver steatosis in mice (9, Chapter 3). The concomitant increase in both hepatic fatty acid oxidation and lipid accumulation can be speculated from data obtained from several studies. It has been documented that dietary *trans*10,*cis*12-CLA decreased adipose tissue mass in mice (34, Chapter 3) by increasing lipolysis (34). Enhanced lipolysis results in an increased pool of circulating free fatty acids that could be taken up by the liver for

triglyceride synthesis. Also, *trans*10,*cis*12-CLA increases lipogenesis in liver of mice (9). Although, *trans*10,*cis*12-CLA increases fatty acid oxidation, the net uptake of fatty acids and synthesis by the liver probably exceeds its capacity to oxidize lipids, thereby resulting in hepatic steatosis.

Approximately, 40 to 50% of the *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA was retained in both AML-12 and HepG2 cell lines 24 h after removal of the treatment media (48-h group) suggesting a potential for carry-over effects of the CLA isomers. The residual effects were evident in the fatty acid profiles and ratios in AML-12 and HepG2 cells at 48 h, which closely resembled the 24-h group. Also, percentages of *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA in media remained higher even after removal of *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA from the media, suggesting secretion of these fatty acids by the cells into the media. Likewise, cholesterol synthesis remained elevated in HepG2 cells treated initially with the *trans*10,*cis*12-CLA, suggesting a potential carry-over effect.

Etomoxir has been reported to bind irreversibly CPT-1 (12), while the binding of HPC to CPT-1 is reversible (13). In the current study, levels of mRNA for CPT-1 in cells initially treated with CPT-1 inhibitors or CLA isomers were not different from the control following withdrawal of the treatment media. Results therefore suggest that the binding of ETM to CPT-1 probably was reversed upon removal of ETM from the media. However, because the effect of ETM on CPT-1 mRNA is indirect, as discussed earlier, reversibility of ETM cannot be concluded from the available data.

Conclusions

This is the first study to demonstrate the effect of purified CLA isomers and CPT-1 inhibitors on expression levels of mRNA for CPT-1, fatty acid profile, and cholesterol synthesis in both HepG2 and AML-12 cell lines. Moreover, the effects following withdrawal of CLA isomers from the media in hepatic cell lines have not been reported earlier. Both *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA decreased Δ^5 and Δ^6 desaturation in HepG2 and AML-12 cell lines. Due to the role of Δ^5 and Δ^6 desaturases in eicosanoid synthesis, the *trans*10,*cis*12-CLA could indirectly modulate the immune-system and cancer development. In addition, *trans*10,*cis*12-CLA also inhibited Δ^9 desaturation.

*Trans*10,*cis*12-CLA increased oxidation of fatty acids in hepatic cell lines , as indicated by increased expression of mRNA for CPT-1. Additionally, cholesterol synthesis was elevated in hepatic cells treated with *trans*10,*cis*12-CLA. Increased fatty acid oxidation could potentially result in accumulation of ketone bodies and decreased utilization of glucose as a substrate for energy. Intake of this CLA isomer might therefore be contraindicated in individuals suffering from Type-2 diabetes. Further research is necessary to elucidate the molecular aspects underlying alterations in hepatic lipid metabolism caused by *trans*10,*cis*12-CLA.

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Table 5-1. Fatty acid composition of AML-12 cells at 24 h

Fatty acid	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
				% total fatty acids				
14:0	5.92	4.28	4.25	4.51	4.00	3.28	3.21	1.33
16:0	22.51	22.63	22.72	23.19	21.69	19.76*	18.52#	0.86
*cis*9-16:1	4.66	5.41	4.18	3.56	3.96	2.23*	1.31#	0.66
*trans*9-16:1	0.16	0.19	0.24#	0.23	0.18	0.14	0.09	0.02
18:0	18.10	16.97	17.90	18.23	17.42	13.78#	12.49#	0.94
*cis*9-18:1	20.64	21.35	20.35	20.15	22.14	13.41*	8.49#	1.97
*cis*11-18:1	5.14	5.69	5.50	5.42	5.99	2.32#	1.80#	0.59
*trans*9-18:1	0.42	0.45	0.42	0.43	0.41	0.24#	0.30	0.04
18:2n-6	1.69	1.78	1.91	2.15	1.81	3.64#	4.07#	0.18
*cis*9,*trans*11-18:2	0.07	0.07	0.14	n.d.	n.d.	23.60#	0.85	0.74
*trans*10,*cis*12-18:2	0.04	n.d.	n.d.	n.d.	n.d.	0.55	33.98#	0.71
18:3n-3	0.08	0.09	n.d.	0.06	0.05	0.16	0.23	0.04
20:0	1.27	1.65	1.03	1.02	0.99	0.91	0.79	0.31
20:4n-6	4.66	4.40	4.55	4.90	4.46	2.89	2.87	0.58
20:5n-3	0.42	0.43	0.48	0.49	0.42	0.18#	0.27	0.04
22:5n-3	1.89	2.13	2.13	2.18	1.92	1.26	1.15	0.23
22:6n-3	2.79	2.80	3.01	2.88	2.67	1.56#	1.52#	0.26
24:0	0.99	0.86	0.78	1.10	1.10	0.44#	0.24#	0.09
24:1	0.17	0.31	0.28	0.25	0.60	2.22#	0.76	0.30

n.d., not detectable; * Treatment vs Control, $P < 0.10$; # Treatment vs Control, $P < 0.05$

Table 5-2. Fatty acid composition of AML-12 cells at 48 h

Fatty acid	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
				% total fatty acids				
14:0	3.13	3.73	3.42	3.58	3.33	3.16	3.73	0.20
16:0	20.87	22.36	21.28	21.78	21.65	21.72	24.93#	0.62
*cis*9-16:1	4.12	4.23	4.68	3.76	3.47	4.41	2.25*	0.49
*trans*9-16:1	0.16	0.19	0.20	0.18	0.18	0.14	0.13	0.02
18:0	17.58	17.21	17.19	17.87	17.81	16.84	17.12	0.42
*cis*9-18:1	23.14	22.44	21.96	22.04	21.81	16.66#	10.11#	0.59
*cis*11-18:1	5.46	5.50	5.46	5.41	5.42	3.20#	2.17#	0.20
*trans*9-18:1	0.36	0.29	0.41	0.33	0.29	0.29	0.46	0.05
18:2n-6	2.19	2.26	2.11	2.42	2.26	3.59#	4.14#	0.14
*cis*9,*trans*11-18:2	0.03	n.d.	n.d.	n.d.	n.d.	10.45#	0.78	0.22
*trans*10,*cis*12-18:2	n.d.	n.d.	n.d.	n.d.	n.d.	0.33	16.31#	0.16
18:3n-3	0.07	0.14	0.08	0.16*	0.13	0.08	0.09	0.03
20:0	1.19	0.91	1.22	1.25	1.17	1.47	0.90	0.23
20:4n-6	5.64	5.02*	5.00#	5.13	5.18	4.35#	4.07#	0.17
20:5n-3	0.29	0.28	0.32	0.25	0.29	0.31	0.13	0.07
22:5n-3	2.36	2.17	2.24	1.99#	2.15	1.73#	1.90#	0.09
22:6n-3	3.61	3.24	3.36	3.30	3.38	2.57#	2.54#	0.12
24:0	1.36	1.16*	1.10#	1.32	1.47	0.80#	0.78#	0.05
24:1	0.85	0.95	0.76	0.73	0.88	1.11	0.80	0.10

n.d., not detectable; * Treatment vs Control, $P < 0.10$; # Treatment vs Control, $P < 0.05$

Table 5-3. Fatty acid composition of AML-12 media at 24 h

Fatty acid	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
				% total fatty acids				
14:0	2.79	3.45#	3.54#	3.49#	3.44#	2.74	2.72	0.11
16:0	25.37	26.83	26.65	26.96	28.61	21.31#	21.24#	0.92
*cis*9-16:1	4.73	3.50	4.07	3.78	3.68	2.90	3.37	0.53
*trans*9-16:1	0.17	0.14	0.18	0.18	0.20	0.16	0.19	0.01
18:0	14.82	17.29	16.79	16.99	11.22	14.22	14.29	2.16
*cis*9-18:1	16.51	15.93	15.11	15.50	17.22	15.72	12.93	0.73
*cis*11-18:1	5.11	4.79	4.73	4.64	4.83	3.54#	3.29#	0.16
*trans*9-18:1	0.23	0.22	0.26	0.24	0.32	0.13	0.21	0.06
18:2n-6	4.77	3.21	2.73	2.97	4.86	5.49	5.68	0.96
*cis*9,*trans*11-18:2	0.24	0.26	0.35	0.42	0.25	11.14#	0.70	0.86
*trans*10,*cis*12-18:2	n.d.	0.09	0.09	0.44	0.26	0.71	11.61#	0.41
18:3n-3	0.52	0.45	0.45	0.51	0.53	0.47	0.51	0.08
20:0	0.84	0.63	0.77	0.75	0.72	0.53	1.53	0.36
20:4n-6	5.80	5.21	5.15	5.14	5.68	3.85#	3.73#	0.27
22:5n-3	2.25	1.81	1.84	1.77	1.94	1.38#	1.53#	0.16
22:6n-3	3.59	3.21	3.09	3.36	3.16	2.58#	2.38#	0.20
24:0	2.00	1.96	1.88	1.82	2.02	1.34#	1.51#	0.09
24:1	1.06	1.90	1.91	1.90	1.75	2.07#	1.62	0.24

n.d., not detectable; # Treatment vs Control, $P < 0.05$

Table 5-4. Fatty acid composition of AML-12 media at 48 h

Fatty acid	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
				% total fatty acids				
14:0	3.30	2.96	3.28	3.35	2.98	3.25	2.92	0.18
16:0	27.64	25.14	27.44	27.62	27.56	26.90	26.52	0.96
*cis*9-16:1	3.84	3.37	5.15	3.52	4.06	3.55	2.97	0.53
*trans*9-16:1	0.16	0.17	0.18	0.16	0.17	0.18	0.19	0.02
18:0	16.35	15.42	16.75	16.20	15.50	15.40	18.47	1.77
*cis*9-18:1	16.83	16.35	12.06	16.61	16.89	16.04	14.36	1.48
*cis*11-18:1	5.36	4.70	4.65	5.32	5.33	4.87	4.27	0.42
*trans*9-18:1	0.43	0.37	0.23	0.46	0.17	0.30	0.24	0.09
18:2n-6	4.13	4.19	4.28	4.36	3.99	4.45	4.74	0.33
*cis*9,*trans*11-18:2	0.35	0.30	0.42	0.23	0.40	1.62$^{\#}$	0.48	0.09
*trans*10,*cis*12-18:2	0.25	0.25	0.15	0.06	0.15	0.30	0.75*	0.12
18:3n-3	0.33	0.76	0.48	0.53	0.47	0.54	0.50	0.12
20:0	0.45	0.56	1.02$^{\#}$	0.41	0.51	0.57	0.45	0.12
20:4n-6	5.30	4.68	5.31	5.15	5.39	5.02	4.57	0.30
22:5n-3	2.11	1.90	2.22	2.21	2.00	2.02	1.83	0.12
22:6n-3	2.73	2.80	2.91	2.98	3.35	3.01	2.69	0.23
24:0	1.95	1.76	1.88	1.67	1.91	1.86	1.62	0.14
24:1	1.96	2.00	2.14	2.12	2.26	1.98	1.92	0.32

n.d., not detectable; * Treatment vs Control, $P < 0.10$; # Treatment vs Control, $P < 0.05$

Table 5-5. Fatty acid composition of HepG2 cells at 24 h

Fatty acid	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
				% total fatty acids				
14:0	3.39	3.97*	3.94*	3.24	3.01	2.77#	2.65#	0.15
16:0	25.05	25.99	25.64	25.67	25.16	22.29#	21.17#	0.42
*cis*9-16:1	6.98	6.88	7.76	6.93	6.90	4.55#	2.71#	0.36
*trans*9-16:1	0.10	0.10	0.07	0.10	0.07	0.06	0.06	0.03
18:0	10.84	11.35	10.08	10.63	9.70	8.33	9.15	0.76
*cis*9-18:1	19.54	19.23	20.22	20.42	21.16	17.06*	11.21#	0.62
*cis*11-18:1	12.34	12.02	12.88	12.98	14.04	8.13#	6.36#	0.67
*trans*9-18:1	0.23	0.31	0.21	0.27	0.26	0.18	0.16	0.02
18:2n-6	2.14	1.78	1.70	1.96	1.92	3.44#	3.72#	0.13
*cis*9,*trans*11-18:2	n.d.	n.d.	n.d.	n.d.	0.07	17.67#	0.81	0.27
*trans*10,*cis*12-18:2	n.d.	n.d.	n.d.	n.d.	n.d.	0.59	27.67#	0.61
18:3n-3	0.05	0.03	0.04	n.d.	0.07	0.10	0.18#	0.03
20:0	0.57	0.48	0.41	0.47	0.42	0.32#	0.32#	0.05
20:2n6	n.d.	n.d.	n.d.	n.d.	n.d.	0.58*	1.22#	0.14
20:4n-6	4.67	4.35	3.99#	4.34	4.27	2.98#	2.64#	0.12
20:5n-3	0.17	0.11	0.09	0.04	n.d.	0.26	0.07	0.06
22:5n-3	0.60	0.60	0.61	0.49	0.42*	0.67	0.53	0.04
22:6n-3	4.51	4.19#	3.96#	4.07#	4.01#	2.63#	2.33#	0.08
24:0	1.27	0.88#	0.78#	1.20	1.10*	0.61#	0.58#	0.04
24:1	0.79	0.82	0.73	0.83	0.74	1.12	0.58	0.10

n.d., not detectable; * Treatment vs Control, $P < 0.10$; # Treatment vs Control, $P < 0.05$

Table 5-6. Fatty acid composition of HepG2 cells at 48 h

Fatty acid	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
				% total fatty acids				
14:0	3.35	3.72*	3.94#	2.92#	2.88#	3.22	3.64	0.09
16:0	24.62	24.43	24.84	25.24	25.22	24.18	27.28#	0.32
*cis*9-16:1	8.47	9.17	9.26*	8.64	8.26	6.92#	3.29#	0.20
*trans*9-16:1	0.05	0.06	0.05	0.05	0.04	0.04	0.04	0.005
18:0	8.52	8.23	8.95	7.87	8.16	7.53*	11.21#	0.24
*cis*9-18:1	21.35	21.17	20.62	22.39	22.13	19.59#	12.40#	0.34
*cis*11-18:1	13.76	13.44	12.78	15.04*	15.20#	10.10#	6.69#	0.29
*trans*9-18:1	0.14	0.22	0.22	0.20	0.18	0.19	0.26#	0.3
18:2n-6	1.91	1.77	1.68#	1.68#	1.67#	2.85#	3.15#	0.05
*cis*9,*trans*11-18:2	0.16	0.19	0.04	0.10	0.05	9.44#	0.52	0.12
*trans*10,*cis*12-18:2	n.d.	n.d.	n.d.	n.d.	n.d.	0.26	14.94#	0.32
18:3n-3	n.d.	0.01	0.02	0.01	0.02	0.04	0.16#	0.01
20:0	0.42	0.46	0.56	0.37	0.37	0.32	0.36	0.05
20:2n6	n.d.	n.d.	n.d.	n.d.	n.d.	0.66#	2.22#	0.04
20:4n-6	4.21	4.00	3.84#	3.75#	3.93*	3.35#	3.02#	0.07
20:5n-3	0.08	0.07	0.08	0.06	0.04	0.22*	0.14	0.03
22:5n-3	0.49	0.51	0.53	0.38	0.31#	0.64*	0.61	0.04
22:6n-3	4.46	3.39#	3.87#	3.81#	3.88#	2.98#	2.43#	0.10
24:0	1.15	0.80#	0.79#	1.02*	1.03	0.68#	0.63#	0.03
24:1	0.57	0.68	0.75	0.52	0.53	0.73	0.56	0.08

n.d., not detectable; * Treatment vs Control, $P < 0.10$; # Treatment vs Control, $P < 0.05$

Table 5-7. Fatty acid composition of HepG2 media at 24 h

Fatty acid	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
				% total fatty acids				
14:0	3.71	4.83	4.59	3.70	3.57	8.11*	3.92	1.10
16:0	31.00	32.40	31.37	30.89	31.10	34.97	30.68	1.72
*cis*9-16:1	3.34	3.53	3.42	3.38	3.56	3.03	2.92	0.18
*trans*9-16:1	0.16	0.18	0.17	0.15	0.15	0.26	0.17	0.03
18:0	13.82	13.22	12.73	17.31	13.30	11.71	13.57	1.58
*cis*9-18:1	15.57	16.54	15.51	15.11	16.08	17.00	15.34	0.65
*cis*11-18:1	5.15	4.97	5.07	5.14	5.65	3.16#	4.25	0.38
*trans*9-18:1	0.41	0.46	0.54	0.40	0.30	0.20	0.28	0.09
18:2n-6	4.11	3.93	4.11	3.72	3.93	3.26	4.35	0.35
*cis*9,*trans*11-18:2	0.26	0.38	0.31	0.21	0.22	2.72#	0.55	0.34
*trans*10,*cis*12-18:2	0.39	0.38	0.29	0.16	0.40	0.38	4.42#	0.09
18:3n-3	0.25	0.33	0.25	0.28	0.30	0.26	0.20	0.05
20:0	0.88	0.95	0.91	0.89	0.93	0.72	0.97	0.11
20:4n-6	4.79	4.53	4.78	4.67	4.89	2.97#	4.25	0.40
20:5n-3	0.63	0.62	0.61	0.53	0.66	0.44	0.80	0.08
22:5n-3	1.97	1.62	1.99	1.93	1.82	1.12#	1.54	0.15
22:6n-3	3.10	2.64	3.12	2.99	3.35	1.99#	2.62	0.26
24:0	1.78	1.56	1.67	1.60	1.88	1.08#	1.49	0.16
24:1	1.24	1.22	1.53	1.32	1.78	1.18	1.29	0.20

n.d., not detectable; * Treatment vs Control, $P < 0.10$; # Treatment vs Control, $P < 0.05$

Table 5-8. Fatty acid composition of HepG2 media at 48 h

Fatty acid	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
				% total fatty acids				
14:0	4.00	3.83	3.84	4.14	3.24	3.59	3.51	0.41
16:0	30.80	30.97	30.65	30.39	29.64	30.31	29.98	0.86
*cis*9-16:1	3.84	4.36	3.94	4.04	4.45	4.28	3.25	0.38
*trans*9-16:1	0.11	0.12	0.11	0.16	0.12	0.11	0.12	0.02
18:0	12.34	12.29	12.36	11.69	12.06	12.93	13.32	0.34
*cis*9-18:1	15.53	15.39	15.67	18.71	17.00	15.06	14.84	0.88
*cis*11-18:1	5.49	5.53	5.59	5.86	6.37	4.98	4.94	0.33
*trans*9-18:1	0.34	0.46	0.24	0.24	0.34	0.36	0.28	0.11
18:2n-6	3.95	3.66	3.96	3.84	4.02	4.05	4.40	0.14
*cis*9,*trans*11-18:2	0.28	0.32	0.23	0.33	0.26	1.94$^{\#}$	0.32	0.07
*trans*10,*cis*12-18:2	0.44	0.36	0.39	0.28	0.36	0.49	2.93$^{\#}$	0.09
18:3n-3	0.37	0.32	0.38	0.27	0.27	0.21$^{\#}$	0.24*	0.03
20:0	0.93	1.27	1.04	0.87	0.92	1.21	0.69	0.18
20:4n-6	4.88	4.97	5.16	4.74	4.35	4.94	5.34	0.32
20:5n-3	0.77	0.78	0.77	0.61	0.75	0.81	0.74	0.07
22:5n-3	1.56	1.68	1.78	1.69	1.83	1.60	1.97	0.15
22:6n-3	3.41	3.21	3.51	3.40	3.70	3.20	3.35	0.26
24:0	1.73	1.72	1.81	1.71	1.98	1.80	1.89	0.14
24:1	2.02	1.74	1.90	1.46	1.74	1.89	1.29	0.27

n.d., not detectable; * Treatment vs Control, $P < 0.10$; # Treatment vs Control, $P < 0.05$

Table 5-9. Fatty acid ratios of AML-12 cells at 24 and 48 h

Ratio	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
AML-12 (24 h)								
16:0 to *cis*9-16:1	0.83	0.81	0.84	0.87	0.85	0.90	0.93#	0.02
18:0 to *cis*9-18:1	0.47	0.45	0.47	0.47	0.44	0.51	0.59*	0.03
18:2n-6 to 20:4n-6	0.27	0.29	0.30	0.31	0.29	0.56#	0.59#	0.02
AML-12 (48 h)								
16:0 to *cis*9-16:1	0.83	0.84	0.82	0.85	0.86	0.83	0.92#	0.02
18:0 to *cis*9-18:1	0.43	0.43	0.44	0.45	0.45	0.50#	0.63#	0.01
18:2n-6 to 20:4n-6	0.28	0.31	0.30	0.32#	0.30	0.45#	0.50#	0.01

* Treatment vs Control, $P < 0.10$; # Treatment vs Control, $P < 0.05$

Table 5-10. Fatty acid ratios of HepG2 cells at 24 and 48 h

Ratio	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
HepG2 (24 h)								
16:0 to *cis*9-16:1	0.78	0.79	0.77	0.79	0.78	0.83[#]	0.89[#]	0.01
18:0 to *cis*9-18:1	0.36	0.37	0.33	0.34	0.31	0.33	0.45[#]	0.02
18:2n-6 to 20:4n-6	0.31	0.29	0.30	0.31	0.31	0.54[#]	0.58[#]	0.01
HepG2 (48 h)								
16:0 to *cis*9-16:1	0.74	0.73	0.73	0.74	0.75	0.78[#]	0.89[#]	0.005
18:0 to *cis*9-18:1	0.28	0.28	0.30	0.26	0.27	0.28	0.47[#]	0.01
18:2n-6 to 20:4n-6	0.31	0.31	0.30	0.31	0.30	0.46[#]	0.51[#]	0.006

[#] Treatment vs Control, $P < 0.05$

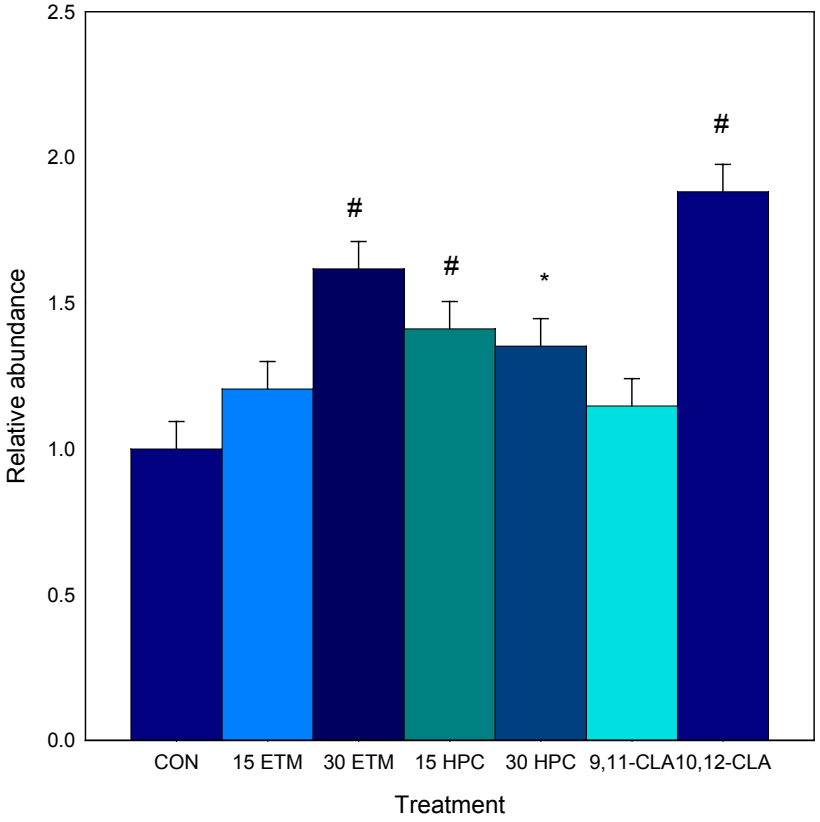

Figure 5-1. CPT-1 mRNA in AML-12 cells at 24 h

* Treatment vs Control, P < 0.10
# Treatment vs Control, P < 0.05

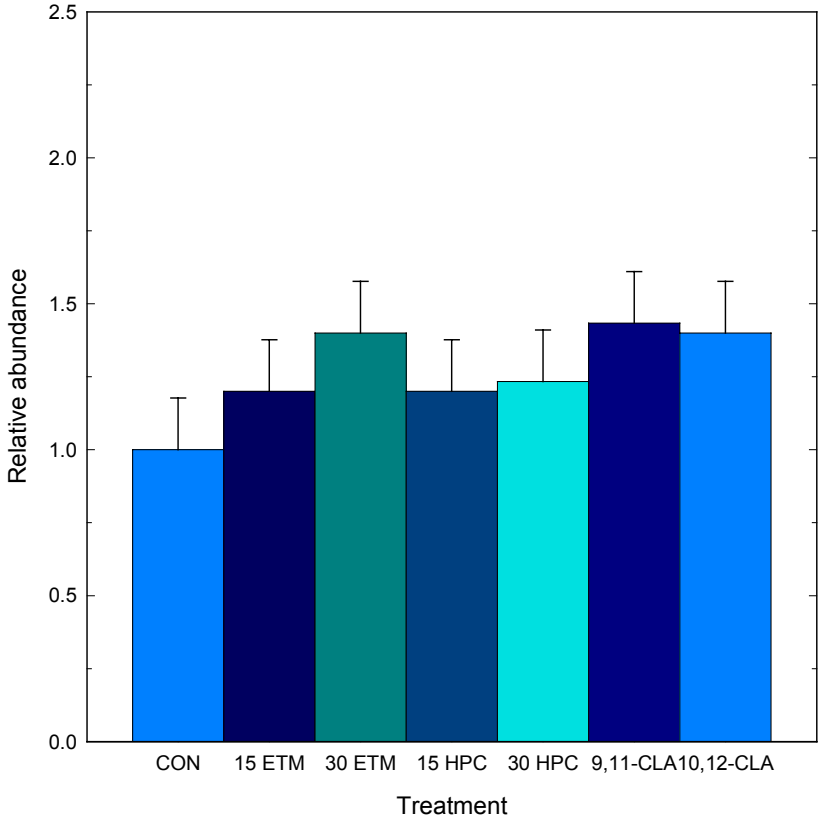

Figure 5-2. CPT-1 mRNA in AML-12 cells at 48 h

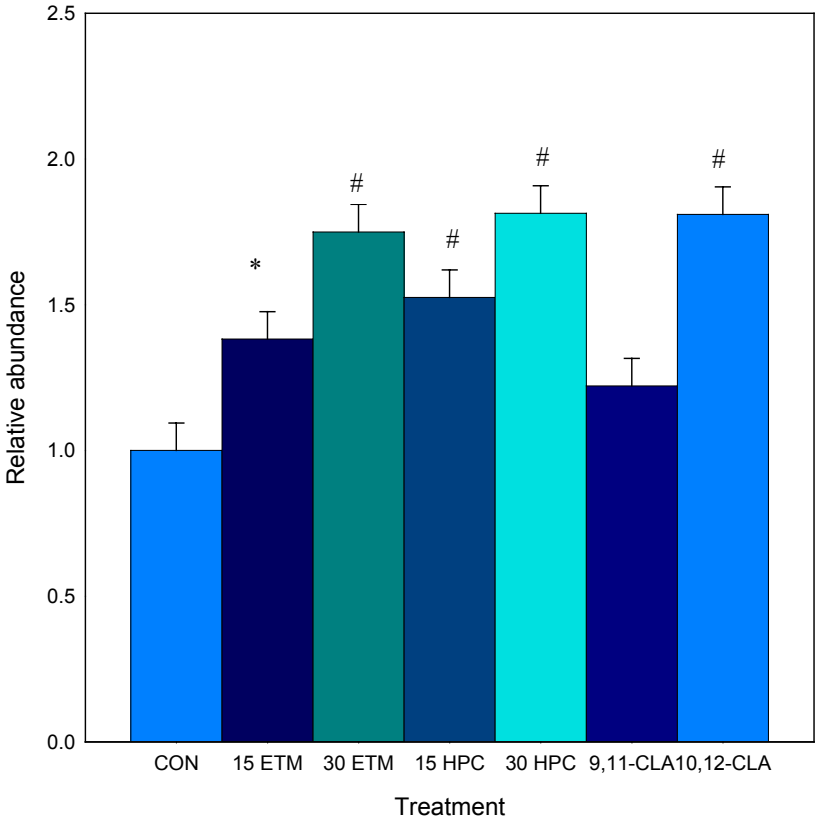

Figure 5-3. CPT-1 mRNA in HepG2 cells at 24 h

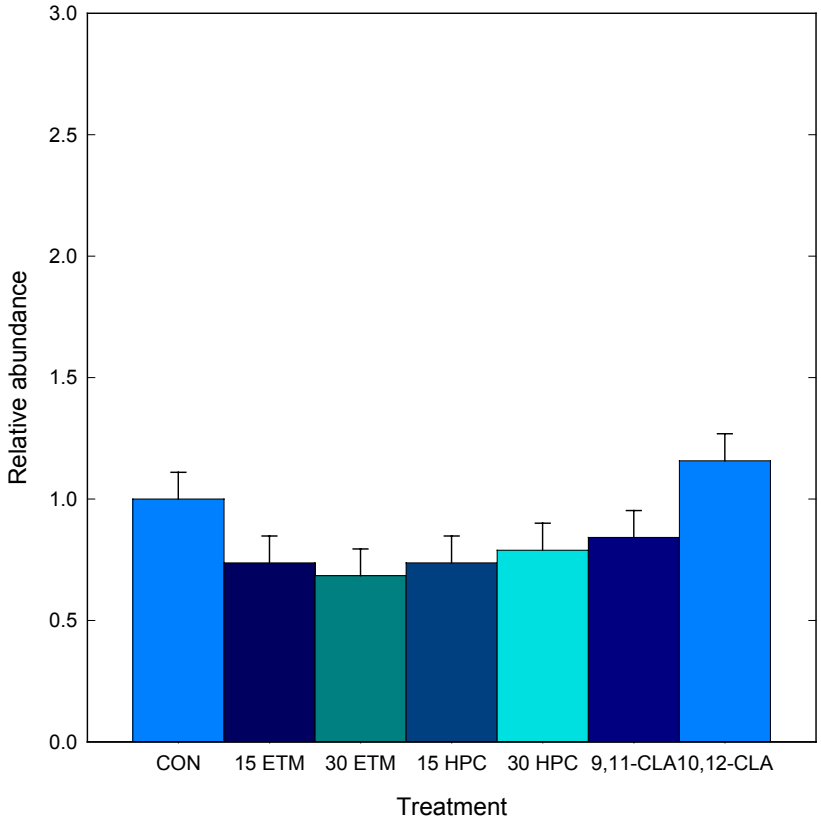

Figure 5-4. CPT-1 mRNA in HepG2 cells at 48 h

* Treatment vs Control, P < 0.10
# Treatment vs Control, P < 0.05

Table 5-11. Free cholesterol (μg per well) in AML-12 and HepG2 cells

	CON	15 μM ETM	30 μM ETM	15 μM HPC	30 μM HPC	9,11-CLA	10,12-CLA	SEM
AML-12 cells								
Free Cholesterol (24 h)								
• Raw data	11.58	7.69	25.81	17.58	11.07	13.77	46.81	11.08
• Log transformed	2.19	1.96	3.06	2.45	2.22	2.53	3.40	0.52
Free Cholesterol (48 h)								
• Raw data	9.20	3.88	13.36	6.93	5.94	10.30	13.14	3.06
• Log transformed	2.05	1.10	2.45	1.72	1.66	2.31	2.45	0.40
HepG2 cells								
Free Cholesterol (24 h)								
• Raw data	19.84	9.27	9.53	21.08	10.40	9.03	92.83	11.67
• Log transformed	2.88	2.12	2.11	2.64	2.34	2.20	4.46$^{\#}$	0.36
Free Cholesterol (48 h)								
• Raw data	8.28	9.84	10.56	15.60	11.95	24.17	104.36	12.46
• Log transformed	2.11	2.27	2.32	2.73	2.34	2.95	4.52$^{\#}$	0.30

* Treatment vs Control, $P < 0.05$; $^{\#}$ Treatment vs Control, $P < 0.01$. Raw data were not tested.

CHAPTER 6
Epilogue

Research on the potential effects of dietary CLA isomers on health has received considerable attention over the last few years. The *cis*9,*trans*11-CLA isomer apparently is a potent anticarcinogen, but *trans*10,*cis*12-CLA is associated with a reduction in body fat deposition. Recent research suggests *trans*10,*cis*12-CLA however, may have deleterious effects on lipid metabolism that accompany the reduction in body fat.

The initial study reported in this dissertation was the first to evaluate low dietary doses of *trans*10,*cis*12-CLA in growing male and female mice. A marked reduction in adipose weights caused by a decrease in the mRNA for FAS and SCD was noted for mice fed diets containing *trans*10,*cis*12-CLA. However, mice fed *trans*10,*cis*12-CLA had an enlarged liver due to fatty acid accumulation. We speculated that a reduction in oxidation of fatty acids might have led to hepatic fatty acid accumulation. On the contrary, *trans*10,*cis*12-CLA caused an increase in mRNA for CPT-1 in hepatic cell lines, suggesting an increase in fatty acid oxidation. Increased oxidation of fatty acids reduces glucose utilization and may be undesirable, especially in diabetics. Results, therefore, question the usage of *trans*10,*cis*12-CLA in body fat reduction for diabetic and obese individuals.

*Trans*10,*cis*12-CLA reduced the ratio of linoleic to arachidonic acid in hepatic cell lines, but increased this ratio in bone and muscle in mice. Because arachidonic acid is a substrate for the synthesis of pro-inflammatory factors such as prostaglandins and eicosanoids, modulation of arachidonic acid synthesis by *trans*10,*cis*12-CLA could possibly affect the inflammatory process. The metabolic role of *trans*10,*cis*12-CLA in muscle and bone needs to be investigated further with emphasis on the expression and activities of fatty acid desaturases *in vivo* and *in vito*.

APPENDIX

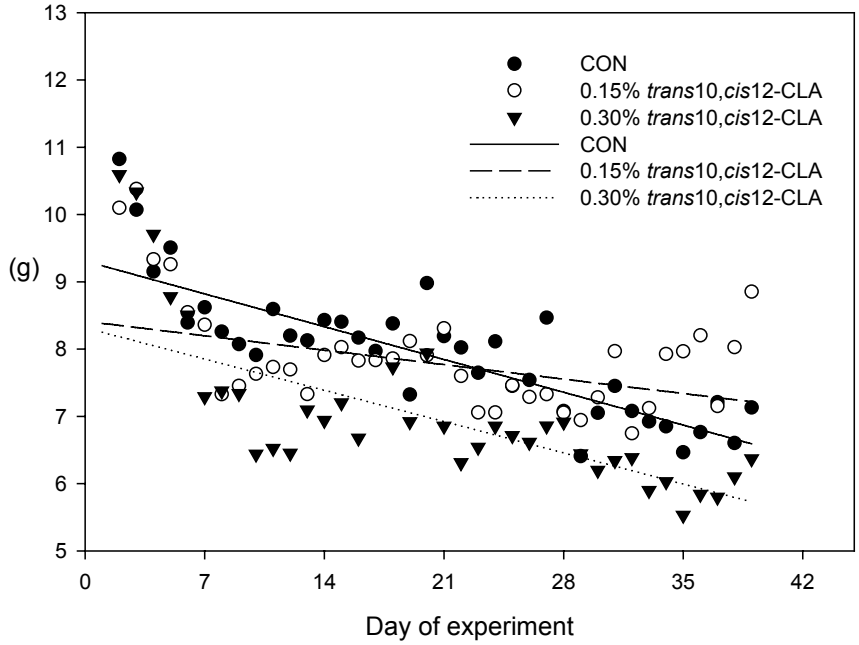

Figure A-1. Daily food intake by female mice fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA. Data from mice sacrificed at wk 6 (n = 7 per treatment group).

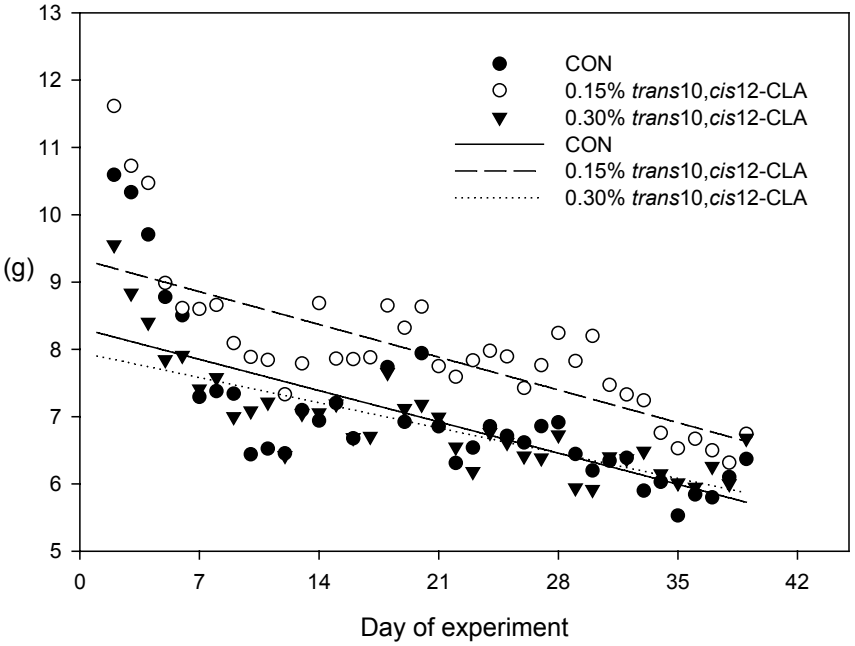

Figure A-2. Daily food intake by male mice fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA. Data from mice sacrificed at wk 6 (n = 7 per treatment group).

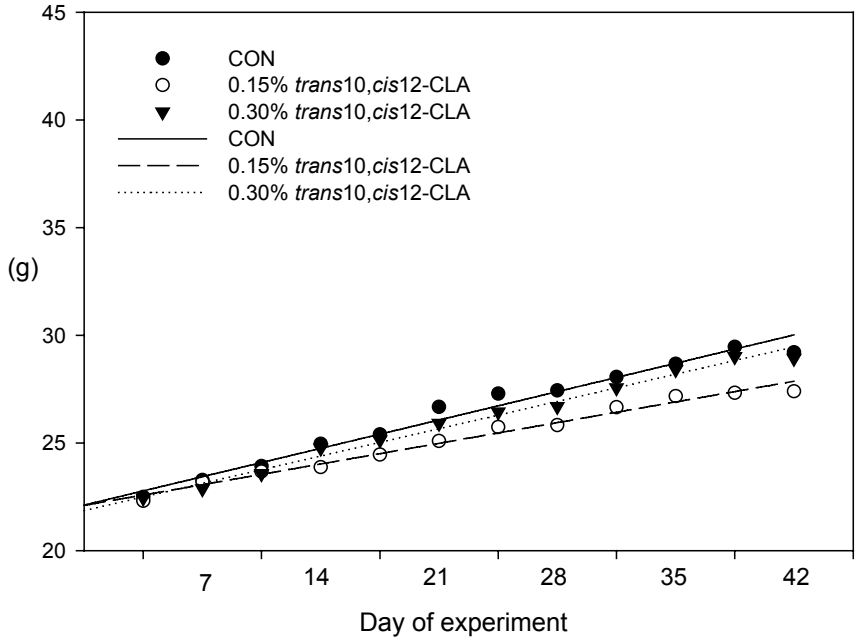

Figure A-3. Body weights of female mice fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA. Data from mice sacrificed at wk 6 (n = 7 per treatment group).

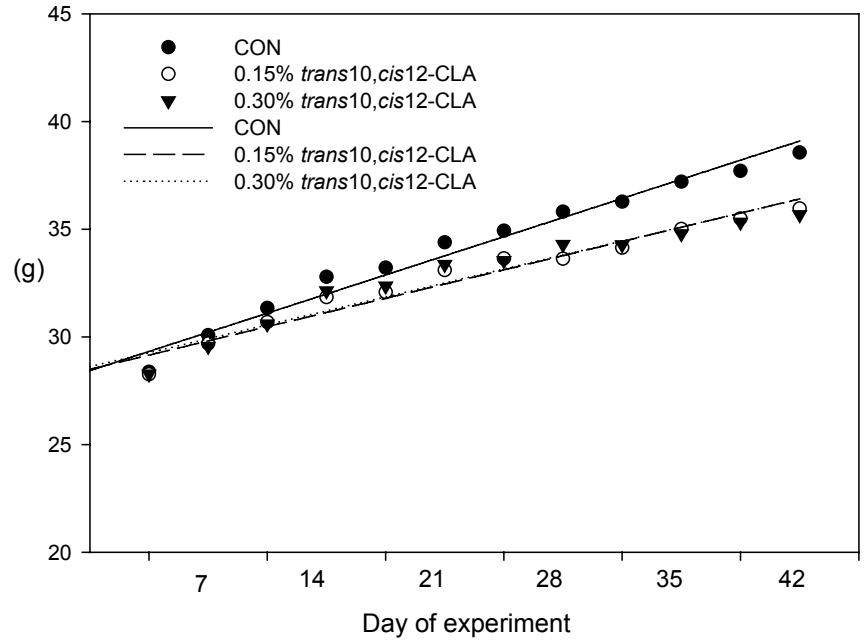

Figure A-4. Body weights of male mice fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA. Data from mice sacrificed at wk 6 (n = 7 per treatment group).

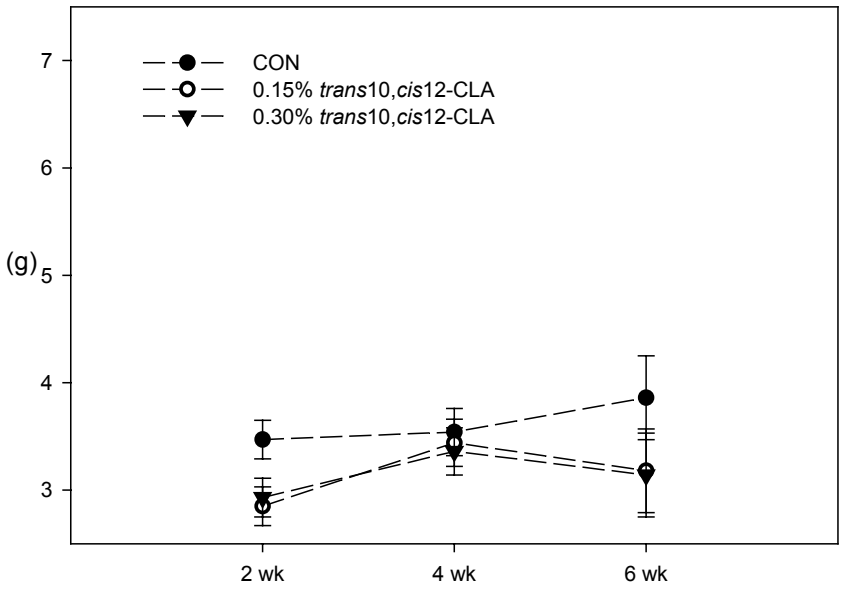

Figure A-5. Carcass weights of female mice fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA.

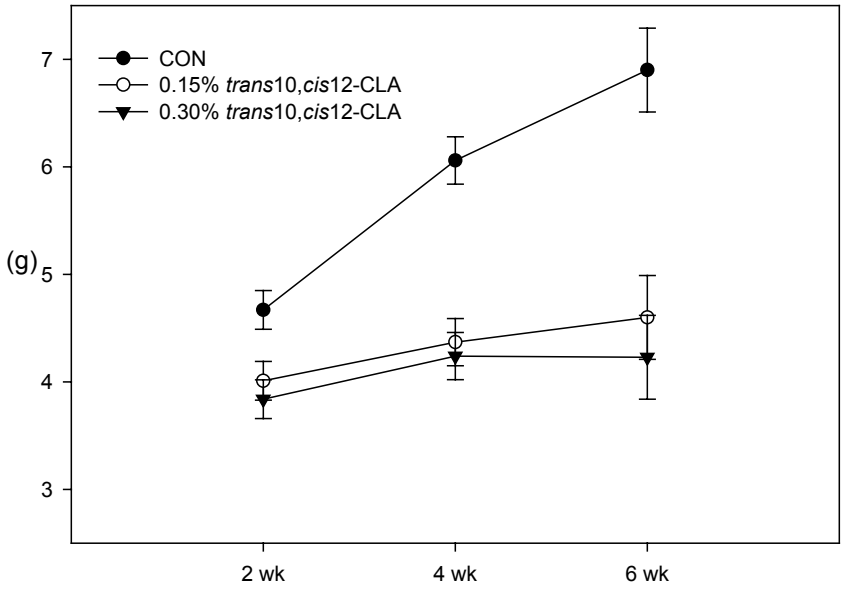

Figure A-6. Carcass weights of male mice fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA.

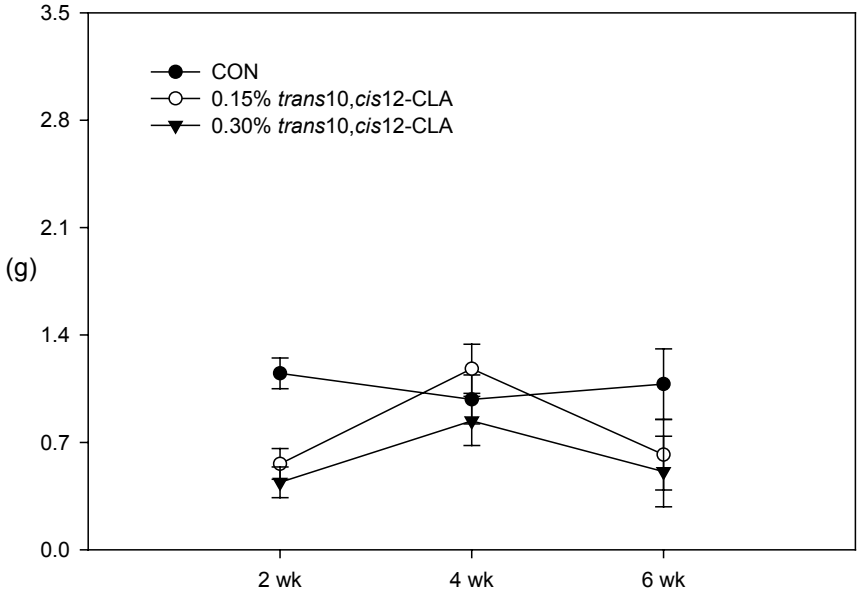

Figure A-7. EE (g) of female mice fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA.

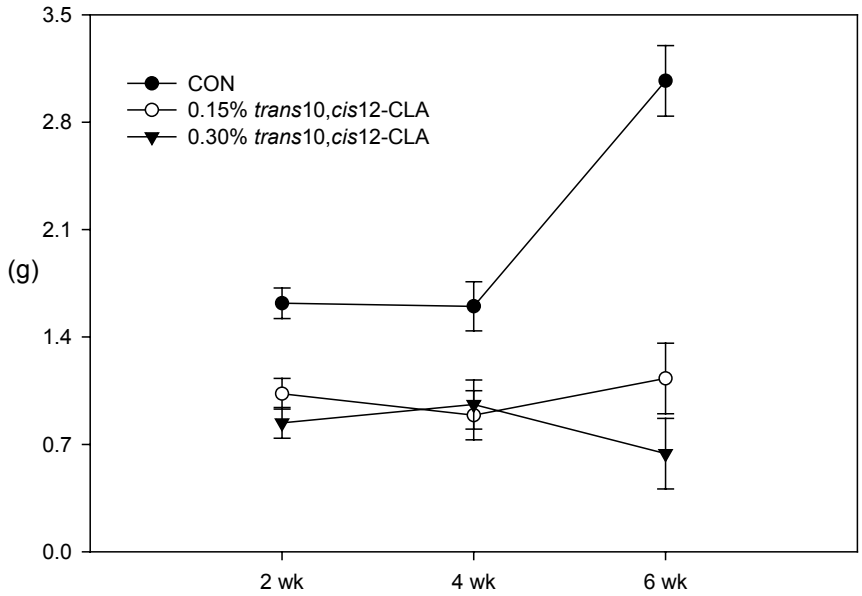

Figure A-8. EE (g) of male mice fed diets containing 0, 0.15, or 0.30% *trans*10,*cis*12-CLA.

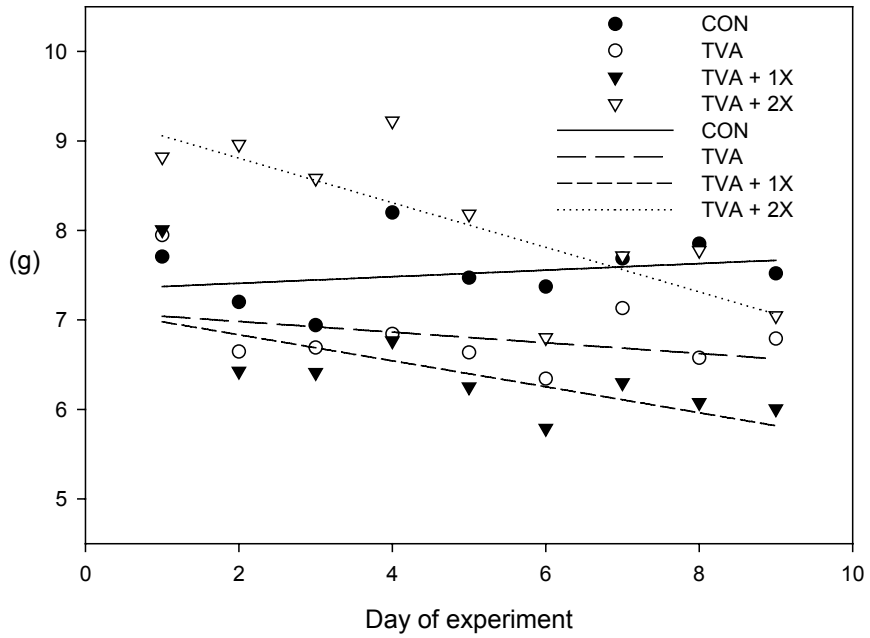

Figure A-9. Daily food intake by female mice fed diets containing TVA, TVA plus 0 (CON), TVA plus 0.15 (1X), or TVA plus 0.30% (2X) *trans*10,*cis*12-CLA.

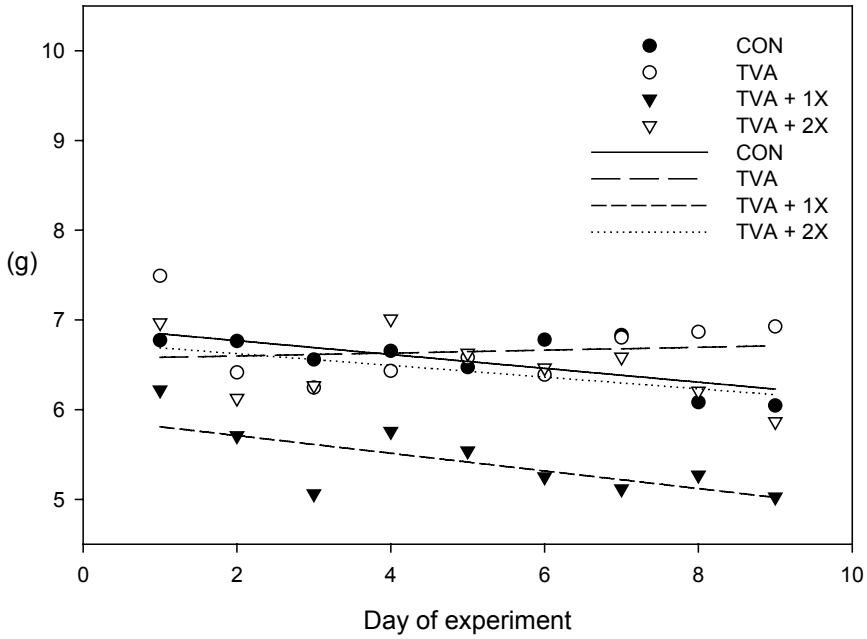

Figure A-10. Daily food intake by male mice fed diets containing TVA, TVA plus 0 (CON), TVA plus 0.15 (1X), or TVA plus 0.30% (2X) *trans*10,*cis*12-CLA.

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