Effects of dietary conjugated linoleic acid (CLA) as single or mixed isomers on the expression of genes encoding for proteins involved in cardiac calcium homeostasis in male and female rats

By

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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LIST OF ABBREVIATIONS

AA.....Arachidonic acid

ACBP.....Acyl-CoA binding protein

ANOVA.....Analysis of variance

Ca^{2+}.....Calcium

$[Ca^{2+}]_i$.....Intracellular calcium concentration

CLA.....Conjugated linoleic acid

c.....*cis*-

*c*9,*t*11 CLA.....*cis*-9, *trans*-11 conjugated linoleic acid

CSQ.....Calsequestrin

d.....Day

ddW.....Double distilled water

DMSO.....Dimethyl sulfoxide

DP.....Diastolic pressure

d*P*/dt.....Left ventricular pressure versus time; a change in pressure with respect to time

+d*P*/dt_{MAX}.....Maximum peak positive pressure along the pressure vs. time curve

-d*P*/dt_{MAX}.....Maximum peak negative pressure along the pressure vs. time curve

DTT.....Dithiothereitol

FABP.....Fatty acid binding protein

FA-CoA.....Fatty acyl-CoA

FFA.....Free fatty acid

g.....Gram

GAPDH.....Glyceraldehyde-3-phosphate dehydrogenase

h.....Hour

HDL.....High density lipoprotein

IL-6.....Interleukin-6

kDa .....Kilodalton

Kg.....Kilogram

LA.....Linoleic acid

LCPUFA.....Long chain polyunsaturated fatty acid

L-Type Ca^{2+}.....L-Type calcium channel

LV.....Left ventricle

LVEDP.....Left ventricular end diastolic pressure

LVSP.....Left ventricular systolic pressure

MAP.....Mean arterial pressure

mRNA.....Messenger RNA

n-3.....Omega 3 fatty acid

n-6.....Omega 6 fatty acid

n-9.....Omega 9 fatty acid

NCX.....Sodium/calcium exchanger

p.....Pressure

PGE_2.....Prostaglandin E_2

PLB.....Phospholamban

PMSF.....Phenylmethylsulfonyl fluoride

PPAR.....Peroxisome proliferator-activated receptor

PPRE.....Peroxisome proliferator-activated receptor response element

PUFA.....Polyunsaturated fatty acid

PVDF.....Polyvinylidine diflouride

RA.....Rumenic acid

RT-PCR.....Reverse-transcriptase polymerase chain reaction

RV.....Right ventricle

RXR.....Retinoid X receptor

RyR.....Ryanodine receptor

SDS-PAGE.....Sodium dodecyl sulfate-polyacrilamide gel electrophoresis

SERCA2a.....Sarco(endo)plasmic reticulum calcium ATPase pump 2a isoform

SL.....Sarcolemma

SP.....Systolic pressure

SR.....Sarco(endo)plasmic reticulum

t.....Time

t.....*Trans*

TBS.....Tris-buffered saline

TNF-α.....Tumor necrosis factor-alpha

VA.....Vaccenic acid

VLDL.....Very low density lipoprotein

w/w.....Weight for weight

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ABSTRACT

The aim of the present study was to investigate whether dietary CLA affects the expression of genes encoding for proteins involved in cardiac calcium homeostasis. In particular, we were interested in the effects of CLAs either as single isomers (*cis*-9,*trans*-11 or *trans*-10,*cis*-12) or mixtures of these isomers in fatty acid or triglyceride form on the cardiac gene SERCA2a. Numerous studies have demonstrated that SERCA2a, the key protein involved in cardiac relaxation, decreases progressively as heart disease progresses toward end-stage heart failure, and current research has been focused on methods of increasing functional SERCA2a in failing hearts as a method of improving cardiac function. Left ventricular tissue mRNA was extracted from male and female rat hearts, and reverse-transcriptase polymerase chain reaction was conducted. PCR products were analyzed by gel electrophoresis and the intensity of each band was photographed and quantified as a ratio of target gene over GAPDH. Western blot analyses were performed for SERCA2a and phospholamban protein content, and band intensities were quantified. Calcium uptake and release activities were also determined using isolated SR vesicles. Our results showed that: 1) CLA affected the expression of genes encoding for the proteins responsible for cardiac calcium homeostasis; 2) These changes were isomer-dependent, and also appeared to be dependent upon the form of the mixed isomers (i.e. fatty acid versus triglyceride) and; 3) The observed effects differed between genders. The changes measured for

SERCA2a and phospholamban gene expression did not correlate with protein levels as assessed by Western blot, suggesting post-transcriptional or translational effects of CLA. Results of calcium uptake and release assays revealed that Ca^{2+} release decreased for all groups of males, and Ca^{2+} uptake and release decreased or remained unchanged for female groups. These findings provide evidence that dietary CLA has an effect on the expression of cardiac genes, and that these effects are gender-dependent. Given the decrease in SR calcium release activities, it appears that the main effect of CLA may be antiarrhythmic in nature, rather than increasing the functional level of SERCA2a. Further research is necessary to fully elucidate the effects of CLA on transcriptional and post-transcriptional changes to proteins involved in cardiac calcium homeostasis, as well as to better understand the effect of gender on cardiac response to dietary CLA.

I. INTRODUCTION

Since Ringer's observations in 1883, it has been proven that calcium (Ca^{2+}) is essential for the cardiac excitation-contraction cycle and subsequent propulsion of blood around the circulatory system (Maier and Bers, 2002). It has also been shown that the proteins of the cardiac myocyte sarcolemmal membrane (SL) and sarcoplasmic reticulum (SR), including the sarco(endo)plasmic reticulum ATPase pump (SERCA2a), and the ryanodine receptor (RyR) play a key role in the homeostatic regulation of cardiomyocyte calcium (Dhalla *et al.*, 2000).

Ca^{2+}-overload and oxidative stress are the two major mechanisms that have been implicated in cardiac dysfunction in different pathological conditions (Dhalla *et al.*, 2000a; Dhalla *et al.*, 2000b). Ca^{2+}-overload can be attributed to a number of factors, including the degradation of cardiac cellular proteins such as SERCA2a (Yoshida *et al.*, 1990) and RyR (Rardon *et al.*,1990).

Dietary fat is an essential macronutrient of the diets of all animals, and it provides a source of energy and hydrophobic components for biomolecules (Jump *et al.*, 1996). Beyond its importance as a macronutrient, dietary fat has recently gained prominence for its role in regulating gene expression. Research has demonstrated that polyunsaturated fatty acids (PUFAs) can affect gene expression through various mechanisms, including changes in membrane composition (Clarke and Jump, 1994; Jump and Botolin, 2005), eicosanoid production (Sampath and Ntambi, 2005), and changes to intracellular calcium

levels (Xiao *et al.*, 1997; Honen *et al.*, 2003). Additionally, PUFAs and their various metabolites can act at the nuclear level, working in conjunction with specific nuclear receptors and transcription factors to affect the transcription of a number of genes (Jump and Clarke 1999; Pegorier *et al.*, 2004).

The term conjugated linoleic acid and its acronym CLA refer generally to mixtures of positional and geometric conjugated dienoic isomers of linoleic acid (Griinari *et al.*, 2000; Pariza *et al.*,2000). In recent years, this family of polyunsaturated fatty acids has become a popular health supplement, and scientific interest in conjugated linoleic acid (CLA) has rapidly increased. However, many questions remain to be answered in order to fully elucidate the physiological effects that have been observed in animals fed CLA-enriched diets. To our knowledge, no studies have been performed that have looked at the major CLA isomers or mixtures of these isomers in triglyceride (TG) or fatty acid form (FA) and their effects on the genes that encode for proteins regulating calcium homeostasis in the hearts of male and female rats. An initial study performed in our laboratory demonstrated that dietary CLA, either as single isomers or as mixtures of isomers had significant effects on cardiac hemodynamic performance in male and female rats (Tappia *et al.*, 2006). The present research project therefore sought to determine the underlying mechanisms involved in the hemodynamic changes observed in CLA-fed animals, specifically the expression of genes involved in cardiac calcium handling.

II. LITERATURE REVIEW

A. Conjugated linoleic acid (CLA)

1. Introduction

Dietary lipids have received more attention from health professionals and the public than any other nutrient in the food supply. In the minds of many people, fat has negative health implications; nonetheless, certain types of lipids are an essential component of human health. It is well known that dietary fatty acids can control or modulate a wide variety of different cellular processes (deJonge *et al.*, 1996). CLA is one example of these types of biologically powerful fatty acids.

Literature concerning CLA first emerged in the 1950s and 1960s (Belury, 2002; Reiser, 1950; Scott *et al.*, 1959). However, CLA did not become a serious research area until the 1980s, when Michael Pariza and colleagues coined the acronym CLA and reported that conjugated linoleic acid, isolated from grilled beef or produced by base-catalyzed isomerization of linoleic acid (LA), was an effective inhibitor of benzo[a]pyrene-induced mouse epidermal neoplasia (Pariza *et al.*, 1979; Pariza *et al.*, 1983; Pariza and Hargraves, 1985; Ha *et al.*, 1987; Ha *et al.*, 1990). These researchers subsequently showed that application of both crude extracts and synthetically prepared CLA inhibited chemically-induced epidermal papilomas and decreased tumor incidence (Ha *et al.*, 1990; Ha *et al.*, 1987). This pioneering

research opened the door to further research on the biological functions and possible health benefits of CLA from a number of diverse perspectives, including chemoprevention, immune function enhancement, anti-atherogenic functions, and modulation of body composition (Lee *et al.*, 1994; Miller *et al.*, 1994; Park *et al.*, 1997; Pariza *et al.*, 2001).

2. Description

The term CLA refers generally to mixtures of positional and geometric conjugated dienoic isomers of linoleic acid (Griinari *et al.*, 2000; Pariza *et al*, 2000). Unlike LA, where the double bonds are separated by more than one single bond, making the double bonds isolated or non-conjugated, the double bonds in CLA are conjugated and occur in several positions in the carbon chain, including: 7,9; 8,10; 9,11; 10,12; or 11,13. Furthermore, each double bond can exist in either the *cis* or *trans* form (Belury, 2002).

The major isomer of CLA found naturally in food is *cis*-9, *trans*-11 (*c*9,*t*11 CLA), and this is the predominant isomer in the human diet, consumed together with smaller amounts of *t*7,*c*9 CLA, *c*11,*t*13 CLA and *t*10,*c*12 CLA (Lawson *et al.*, 2001). While dietary sources of CLA isomers are predominantly in the triacyglycerol form, CLA supplements are primarily in the fatty acid form (Nagao and Yanagita, 2005). The main sources of CLA in the human diet are meat and dairy products derived from ruminants. In these products, greater than 90% of CLA exists as the *c*9,*t*11 isomer (McGuire *et al.*,

1999). Recent studies have suggested that the biological effects of CLA are isomer specific (Pariza *et al.*, 2001), and indicate that the *c*9,*t*11 and *t*10,*c*12 isomers of CLA appear to have important potential biological activities (Pariza *et al.*, 2001).

3. Rumenal biohydrogenation of CLA

The rumen of animals such as cows, sheep, goats and deer may be likened to a large, anaerobic "fermentation vat" which contains microbes capable of biohydrogenating ingested PUFAs from forage or other feed sources (i.e. added grain or fish oils) (Wahle *et al.*, 2004). The first step in the biohydrogenation of dietary LA involves the rumenal bacteria *Butyrvibrio fibrisolvens*, and results in the formation of the *c*9,*t*11 isomer, due to the transposition of the delta-12 double bond. As mentioned, this is the most abundant natural isomer present in ruminant tissue fats (over 90% of total CLA) and has been termed rumenic acid (RA) (McGuire *et al.*, 1999; Parodi, 2003). After formation in the rumen, RA may be directly absorbed or further metabolized (biohydrogenated) by rumen microorganisms (Pariza *et al.*, 2001). Further hydrogenation of RA results in the production of *t*11-18:1 vaccenic acid (VA), which is the major *trans*-monounsaturated fatty acid present in the fats of ruminant food products, including milk, yogurt, cheese, butter and meats. It should be noted that the biohydrogenation process is invariably incomplete, otherwise only saturated fatty acids would flow from the rumen,

rather than the mixture of RA, VA, and saturated products that are in fact produced (Wahle *et al.*, 2004). It must also be noted that it is not possible for the relatively high proportion of RA found in cows' milk (2-52 mg/g fat) to be derived solely from the relatively small amount of RA that exits the rumen for distribution to tissues (Wahle *et al.*, 2004). In fact, it has been determined that approximately 70% of RA found in milk fat is derived from VA by the activity of delta-9 desaturase in the mammary tissue of ruminant animals (Griinari and Baumann, 1999).

A wide spectrum of minor geometrical and positional isomers of CLA is also produced during rumenal biohydrogenation of LA, and range from *t*6,*t*8-18:2 to *t*13,*t*15-18:2, with a number of *cis-trans*, *trans-cis*, *trans-trans*, and *cis-cis* positional isomers between the two extreme positions on the acyl chain (McGuire *et al.*, 1999; Parodi, 2003). To date, little is known about any beneficial or detrimental metabolic effects of the minor isomers found in natural CLA mixtures.

4. Commercial production of CLA

CLA is chemically synthesized by alkali isomerization from LA or oils rich in this fatty acid, such as sunflower or safflower oils, which can be in the form of TGs, FAs or fatty acid esters (Reany *et al.*, 1999; Saebo, 2001). While earlier commercial preparation methods focused primarily on maximizing the yield of CLA, current production procedures aim at a product containing the

two main CLA isomers, *c*9,*t*11 and *t*10,*c*12 CLA (Reany *et al.*, 1999). A few pure CLA isomers (*c*9,*t*11, *c*9,*c*11, *t*9,*t*11 and *t*10,*c*12) are also now available (Kramer *et al.*, 2004). Nonetheless, commercially produced CLA still contains some level of minor isomers. Furthermore, the conditions used to conjugate LA have little or no effect on either monounsaturated or saturated fatty acids that may also be present in the initial oil; however, any PUFAs present may also become conjugated, thereby producing potentially undesirable products (Reany *et al.*, 1999).

5. Dietary sources of CLA

The major dietary sources of CLA are animal products, with meat derived from ruminants containing more CLA than the meat derived from non-ruminants (MacDonald, 2000). In humans, it is estimated that dairy products contribute approximately 60% of total dietary CLA, followed by beef at 32% (Shantha and Decker, 1993). Of the total CLA present in dairy products, 73% to 93% is the biologically active *c*9,*t*11 isomer, while 57% to 85% of the total CLA found in beef is the *c*9,*t*11 isomer (Shantha and Decker, 1993; Shantha *et al*, 1999; Thorsdottir *et al.*, 2004).

Among dairy products, cheese is the major source, contributing roughly 30% and 33% of total CLA intake in men and women, respectively (Chin et al., 1992). Among meat sources of CLA, veal has the least CLA at 2.7 mg CLA/g fat (including intramuscular fat), while lamb has the highest amount of CLA at 5.6 mg CLA/g fat. The content of CLA in fish and fish-derived food

products is negligible in relation to the CLA content of dairy products, containing 0.1 to 0.9 mg/g fat in most common marine foods (Shantha and Decker, 1993; McGuire *et al.,* 1999). While fatty acids with conjugated double bonds do occur in many seed oils, CLA is not found in any of the vegetable oils commonly consumed by humans. However, small amounts of CLA are produced during the heating, bleaching and deodorisation associated with refining processes, and the CLA concentration in vegetable oil can range from 0.6 to 0.9 mg/g fat (Wahle *et al.*, 2004).

CLA content in milk varies with the type of feed given to cows and also varies with season, with spring grasses producing higher ruminant levels of CLA (Ha *et al.*, 1987). Processing conditions also influence CLA content of dairy foods (Scimeca 1998; Shantha *et al.*, 1999; Thorsdottir *et al.*, 2004); for instance, aged cheeses have lower amounts of CLA than cheeses with a shorter ripening time (Chin *et al.*, 1992). In processed cheeses, higher processing temperatures, the addition of sodium caseinate, use of hydrogen donors (butylated hydroxytoluene, propyl gallate or ascorbic acid), as well as the addition of whey powder, non-fat dry milk or iron all serve to increase CLA content (Shantha *et al.*, 1999). On the other hand, storage or processing of dairy products such as low-fat and regular-fat yogurt, low-fat and regular-fat ice cream, sour cream or cheeses at low temperatures do not produce any change in CLA content (Pariza *et al.*, 1979). According to Ha *et al* (1987), grilling beef can increase CLA content by about four-fold. Conversely,

Shantha *et al* (1999) reported that cooking did not increase the CLA content of beef; they suggested instead that CLA is stable and therefore not destroyed by cooking or storage. Table 1 (non-exhaustive list) shows the CLA content of various foods.

Table 1: Conjugated linoleic acid content of various foods[1]

Dairy products	mg/g fat	Meats/Fish	mg/g fat
Homogenized milk	5.5	Fresh ground beef	4.3
2% milk	4.1	Veal	2.7
Butter fat	6.1	Lamb	5.8
Condensed milk	7.0	Pork	0.6
Cultured butter milk	5.4	Chicken	0.9
Butter	4.7	Fresh ground turkey	2.6
Sour cream	4.6	Salmon	0.3
Ice cream	3.6	Egg yolk	0.6
Low-fat yogurt	4.4	**Vegetable Oil**	
Medium Cheddar	4.1	Safflower oil	0.7
American processed	5.0		

[1]Adapted from Chin *et al* (1992)

6. CLA intakes in humans

Methodologies used to estimate daily intake of CLA in human populations range from large dietary surveys to more detailed dietary assessments of small population sub-groups (Fritsche *et al.*, 1999). Various quantification tools employed in these assessments include the use of disappearance data, dietary recalls, food frequency questionnaires, weighed food records and biochemical analysis of food duplicates. However, all of these techniques have inherent limitations. According to a study done on college-aged students in the United States (Ritzenthaler *et al.*, 2001), total CLA intake estimated using 3d food duplicates was found to be 212 ± 14 and 151 ± 14 mg/d for men and women, respectively, while intake of RA was estimated to be 193 ± 13 mg/d for men and 140 ± 14 mg/d for women. In another study, typical human consumption of CLA estimated from 3d written dietary records was reported as 139 mg/d in young men and women (Jiang *et al.*, 1999).

CLA intakes in other countries appear to be comparable to that of the U.S. population. As estimated by 7d weighed dietary records and 24h recalls (Salminen *et al.*, 1998), mean CLA intake in older Swedish men was 160 mg/d. According to a national consumption survey of the German population (Ritzenthaler *et al.*, 2001), German men consumed approximately 430 mg RA/d and women consumed 350 mg RA/d. It is interesting to note that these estimated intakes are approximately twice those for the U.S. population. Given that the average German consumes 10% more energy from fats than does the

average American, the higher level of CLA intake in the German population becomes plausible (Ritzenthaler *et al.*, 2001).

Average *c*9,*t*11 linoleic acid intake estimated by 7d diet records in a small group of young Canadians (Ens *et al.*, 2001) was determined to be 94.9 ± 40.6 mg/d, but ranged between 15-174 mg/d. Intake of the *c*9,*t*11 isomer of CLA, when expressed as mg CLA per unit of energy consumed significantly correlated with the intake of saturated fat ($r=0.62$, $P<0.002$), but not with intake of total fat ($r=0.39$, $P<0.08$).

According to animal studies, consumption of a diet containing as little as 0.1 g CLA/100 g dry weight is sufficient to significantly reduce tumorigenesis, delay atherosclerosis and increase HDL-cholesterol concentrations (Rudel, 1999). If this were extrapolated to humans, RA intake would have to be 620 mg/day for men and 441 mg/day for women in order to attain cancer protective properties and exert an anti-atherosclerotic effect. Nonetheless, existing literature suggests that current as well as chronic total intakes of CLA and RA in men and women do not exceed 500 mg/day. It is important to note, however, that the average estimated intake of CLA probably does not reflect the total CLA available to an individual because of endogenous conversion of VA in dairy products to CLA via the delta-9 desaturase enzyme (Santora *et al.*, 2000). In fact, it has been estimated that close to 20% of ingested VA is converted to CLA in humans (Turpeinin *et al.*, 2002). This would increase the amount of CLA available to people consuming an average

North American diet (with CLA intake estimated at 400-600 mg/d) to between 600 and 800 mg/d (Santora *et al.*, 2000).

Due to lack of and inconsistency in experimental human data, as well as the wide variety of effects reported in animal models, it would be prudent to await further CLA research before increasing CLA intake to therapeutic levels in healthy humans.

7. Safety of CLA isomers

Safety of a substance, whether a food ingredient or a dietary supplement, is of utmost importance, regardless of whether it has been found to be physiologically effective. The safety of CLA has been evaluated in a number of animal toxicologic studies (Pariza, 2004). One such study involved a 36-week feeding trial of Fischer 344 rats (Scimeca, 1998), in which the rats were fed either a control diet or a diet supplemented with 1.5% (w/w) CLA mixture. The amount of CLA consumed by the rats in this study was 80-fold greater than the estimated 50th percentile of daily CLA intake for teenaged American boys, and 50-fold greater than the estimated 90th percentile of daily CLA intake for teenaged American boys. No adverse effects were observed in terms of food disappearance, body weights, cage-side examinations, or hematologic and histopathologic analyses done on 15 major organs. In a 90d oral rat toxicity study, O'Hagan and Menzel (2003) performed a variety of *in vitro* genotoxicity studies typical for assessment of food ingredient safety using

a commercial CLA preparation (*c*9,*t*11:*t*10,*c*12, 1:1). They concluded that the "*no observed adverse effect levels*" of CLA for male and female rats were 2,433 and 2,728 mg/kg body weight/d, respectively.

A number of human studies have been conducted with high quality CLA preparations, consisting almost entirely (>90%) of the 2 most biologically active isomers, *c*9,*t*11 and *t*10,*c*12, in approximately equal amounts (~ 45% of each). In numerous human studies, when ~ 90% pure CLA was administered at 3-6 g/d, no adverse effects were observed (Kamphius *et al.*, 2003; Kamphius *et al.*, 2003; Kelley and Erickson, 2003; Larsen *et al.*, 2003).

Nonetheless, there still remain concerns regarding the safety of CLA isomers given the induction of fatty liver, insulin resistance, and lipodystrophy observed in mice fed with CLA-supplemented diets (Plaa *et al.*, 1986; Riserus *et al.*, 2002; Larsen *et al.*, 2003). In human trials, enhanced C-reactive protein, lipid peroxidation, unfavourable changes in serum lipids in obese individuals, and reduced milk fat during lactation have emerged as safety concerns (Riserus *et al.*, 2002).

Hamsters and female rats fed diets supplemented with 15% (w/w) CLA also exhibit enlarged livers, but this enlargement has been reported to be liver hypertrophy as opposed to fat accumulation (O'Hagan and Menzel, 2003). It should be noted that toxicologists consider neither fatty liver nor liver hypertrophy to be toxic effects (Larsen *et al.*, 2003). Moreover, the liver hypertrophy observed in female rats fed a diet supplemented with 15% (w/w)

CLA was completely reversible when the animals were switched to a CLA free diet (O'Hagan and Menzel, 2003).

Concerns about elevations in oxidative stress and unfavourable changes in blood lipids also exist. One study (Risérus *et al.*, 2002) investigated the effects of CLA supplementation (3.4 g CLA/d) in men with metabolic syndrome. This study compared a typical high quality CLA preparation consisting of equal amounts of *c*9,*t*11 and *t*10,*c*12 CLA (35.4% and 35.9%, respectively), with a supplement that was enriched for *t*10,*c*12 CLA (76.5%) but contained very little *c*9,*t*11 CLA (2.9%). Increased lipid peroxidation (based on measurement of urinary 8-iso-prostaglandin F(2α) and 15-keto-dihydro-prostaglandin F(2α) as markers of *in vivo* oxidative stress and inflammation, respectively), enhanced C-reactive protein in serum, and elevated very low density lipoprotein (VLDL) coupled with reduced high density lipoprotein (HDL) were significant relative to placebo for the patients taking *t*10,*c*12 CLA supplements. These parameters were reduced in patients taking the typically commercially available CLA (mixed isomer) supplement relative to placebo. Hence it was concluded from this study that *t*10,*c*12 CLA could enhance inflammation and the risk of cardiovascular disease.

However, other studies have demonstrated that CLA reduces inflammation. For instance, pigs supplemented with 2% (w/w) mixed isomer CLA (43.3% *c*9,*t*11 and 40.91% *t*10,*c*12 CLA) and subsequently exposed to pro-inflammatory lipopolysaccharide exhibited decreased levels of the

cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Changhua *et al*, 2005). Similar findings have been reported in earlier studies. Akohashi *et al* (2002) reported deceased serum TNF-α levels in mice fed a diet supplemented with 1% (w/w) mixed isomer CLA. Turek *et al* (1998) supplemented the diet of male Sprague-Dawley rats with 1% (w/w) mixed isomer CLA. These researchers found decreased levels of prostaglandin E_2 (PGE_2) as well as significantly lower levels of the pro-inflammatory cytokines TNF, IL-1 and IL-6 compared to control.

It is also documented that *t*10,*c*12 CLA reduces milk fat. This has been concluded from studies in cows and lactating women (Masters *et al.*, 2002; Park *et al.*, 1999; Petersen *et al.*, 2003), and may mean that less energy is available for the nursing infant. However, in a study with rats, the pups nursing dams fed CLA-supplemented diet actually grew to a larger size (Chin *et al.*, 1994).

In reviewing the published CLA literature, it is apparent that a number of valid safety concerns remain in regard to CLA intake. Clearly, more extensive research is needed so that the benefits of CLA intake can be more knowledgably weighed against the risks.

8. Absorption, metabolism and tissue incorporation of CLA

The metabolism and tissue incorporation of CLA has been characterized in rats, and it is believed that not only is this process similar to the metabolism

of other long-chain fatty acids, it is also similar in other species, including humans (Banni *et al.*, 2004). One possibility for CLA's distinct actions in biological systems (i.e. *c*9,*t*11 and *t*10,*c*12 isomers) may be that it is active at a metabolic level. This may be possible since CLA's conjugated diene structure gives its isomers and their metabolites a distinct pattern of incorporation into the lipid fraction. Several mechanisms of action have been proposed for the two main CLA isomers, although there is not as yet consensus on these mechanisms, since the *c*9,*t*11 and *t*10,*c*12 isomers have different biological activities depending on the experimental system. One possibility for the distinct actions of CLA isomers at the level of absorption/metabolism may be explained by the fact that CLA appears to undergo similar transformations as LA and other PUFA (i.e. omega 3 [n-3], omega 6 [n-6] and omega 9 [n-9] PUFA), but with subtle isomer differences (Banni, 2002). For instance, metabolism of dietary CLA appears to be less efficient, as evidenced by the accumulation of CLA itself, rather than its derivatives, in various tissues (Banni *et al.*, 1995; Sebedio *et al.*, 1997). Nonetheless, one mechanism by which CLA exerts different biological actions may be related, at least in part, to its metabolic fate and subsequent perturbation of fatty acid metabolism (Banni *et al.*, 2001).

In rats, dietary CLA is transported from the intestine in chylomicrons, similar to other long chain dietary fatty acids (Sugano *et al.*, 1997); however, the release of CLA from chylomicrons for tissue incorporation has been

reported to be lower than that of its parent fatty acid, LA (Sugano *et al.*, 1997). In fact, the absorption rates of individual CLA isomers differ, with more *trans-trans* than *cis-trans* and *trans-cis* isomers detected in chylomicrons (Sugano *et al.*, 1997). The distribution of these isomers in the three stereospecific positions of TG molecules also differs, with *trans-cis* and *cis-trans* isomers incorporated equally into all three positions (i.e. sn-1, sn-2 and sn-3) of the TG molecule, and *trans-trans* isomers incorporated preferentially into the sn-1 and sn-3 positions (Sugano *et al.*, 1997).

Further, the composition of CLA isomers differs among tissues; for instance, one study showed that *trans-trans* isomers accumulated significantly in adipose tissues, but not at all in brain tissues of rats fed CLA (Fa *et al.*, 2005). The distribution of CLA in various lipid fractions also differs, where all CLA isomers appear in triglyceride (neutral lipid) but only certain isomers appear in specific tissue phospholipids (i.e. only the *c*9,*t*11 CLA isomer is found in liver phospholipids) (Sugano *et al.*, 1997; Kramer *et al.*, 1998). In humans, CLA has been identified in a number of tissues, including blood, bile, adipose tissue, mammary tissue and in human breast milk (Cawood *et al.*, 1983; Iverson *et al.*, 1984; Harrison *et al.*, 1985; Yurawescz *et al.*, 1998).

Following incorporation into chylomicrons, CLA (as with other PUFAs), enters the circulation, and consequently undergoes cellular uptake similar to other long-chain polyunsaturated fatty acids (LCPUFA). Triglycerides are hydrolyzed by lipoprotein lipase, enabling free fatty acids to

enter cells through membrane-associated fatty acid transporters (Hui and Bernlohr, 1997). At least five plasma membrane proteins have been identified as potential fatty acid transporters, including a) fatty acid binding protein (FABP); b) fatty acid translocase; c) caveolin; d) 56-kDa kidney fatty acid binding protein; and e) fatty acid transport protein (Jump and Clarke, 1999). More specifically, evidence now exists for a saturable transporter for LCPUFA in several tissues, including the heart (Hirsch *et al.*, 1998; Kalant and Cianflone, 2004). Several proteins have been identified as candidates for mediating fatty acid uptake in cardiac tissue, including CD36 fatty acid transporter, caveolin, and fatty acid transport proteins (Kalant and Cianflone, 2004).

Once in the cell, most fatty acids (and presumably CLA) are noncovalently bound to proteins (i.e. FABP) (Gossett *et al.*, 1996). A requisite step for fatty acid entry into several metabolic pathways is the formation of fatty acyl-CoA thioesters (FA-CoA), which are catalyzed by FA-CoA synthetases. Six such synthetases have so far been identified (Coleman *et al.*, 2000). Several FA-CoA synthetase subtypes display fatty acid chain length specificity (Jump and Clarke, 1999), and the products of these enzymes are sequestered through binding to acyl-CoA binding proteins (Gosset *et al.*, 1996). These fatty acid CoA binding proteins exist within both the cytosol and the nucleus (Borderwick *et al.*, 1989; DuBois *et al.*, 1998), suggesting that fatty acids or FA-CoA may be in the nucleus and serve as ligands capable of

regulating the activity of specific transcription factors (Jump and Clarke, 1999). The total intracellular FA-CoA level is highly variable and depends on the cell in question (i.e. in the liver, FA-CoA levels range from 11,000-15,200 nmol/g wet weight tissue, and in the heart, levels range from 10-70 nmol/g wet weight tissue) (Brugengraber *et al.*, 1978; DuBois *et al.*, 1998). Note that, although the intracellular concentration of fatty acid and FA-CoA that is not bound to protein is low (i.e. <10 μM), it is the fatty acid or FA-CoA in this fraction that likely serves as regulatory ligands for specific transcription factors (DuBois *et al.*, 1998).

Fatty acids (i.e. as FA-CoAs) may alternately be channeled to various other fates. They can be incorporated into complex lipids such as TG and phospholipids, or they may undergo metabolic conversion by elongation, desaturation and oxidation (Brugengraber *et al.*, 1978; McGarry and Foster, 1980; Neat *et al.*, 1980; Nilsson *et al.*, 1986; Flatmark *et al.*, 1988; Fitzpatrick and Murphy, 1989; Karara *et al.*, 1989; Oliw, 1994; Goetzl *et al.*, 1995; Jump *et al.*, 1996; Luthria *et al.*, 1996; Makita *et al.*, 1996; DuBois *et al.*, 1998). One major route for fatty acid metabolism (including CLA) is through oxidation in mitochondria and peroxisomes (McGarry and Foster, 1980; Reddy and Mannaerts, 1994). As will be discussed in a later section, these metabolites in turn can have profound effects on gene regulation and cell-signaling pathways.

As mentioned, CLA and CLA metabolites differ from their parent LA by distinct patterns of incorporation into tissues (Banni *et al.*, 2001). For instance, whereas LA and its metabolites are mainly incorporated into phospholipids, CLA isomers are mainly incorporated into neutral lipids (Banni *et al.*, 2001). It is known that an increasing number of *cis* bonds favour incorporation of PUFA into phospholipids rather than neutral lipids, and it is likely that the presence of a conjugated diene structure drives the preferential incorporation of CLA into neutral lipids (Banni *et al.*, 2004). In mice fed dietary CLA, isomers were incorporated more readily into neutral lipids than into phospholipids, although the neutral lipid CLA disappeared more rapidly than phospholipid CLA when supplementation was discontinued (Ha *et al.*, 1990). In another study, researchers fed mice differing levels of CLA for 6 weeks (Belury and Kempa-Steczko, 1997). They reported that dietary CLA was incorporated into liver lipids by displacement of LA and arachidonic acid (AA). Further, the effect of dietary CLA on the level of these n-6 PUFA was more marked in neutral lipids than it was in phospholipids. These researchers concluded that CLA affects metabolic conversion of fatty acids in the liver, and eventually results in modification of the n-6 composition of phospholipids.

An *in vitro* study compared the incorporation of CLA with that of LA and AA into HEL-30 murine keratinocyte cells (Liu and Belury 1998). The researchers reported that approximately 50% of LA and AA and only 30% of CLA were incorporated into the phospholipid phosphatidylcholine of these

cells, with more CLA incorporated into phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine, indicating that CLA was incorporated into phospholipids in a significantly different manner than either LA or AA. Given that phospholipids play a key role in cell membrane structure and function, it is possible that CLA exerts at least some of its biological effects through its effect on the n-6 fatty acid composition of phospholipids. Belury and Kempo-Steczko (1997) postulated that CLA may be desaturated to an 18:3 product, similar to the conversion of its parent fatty acid to γ-linolenic acid. In this way, CLA may compete with LA as a substrate for delta-6 desaturase and thereby inhibit the desaturation of other n-6 fatty acids.

The presence of CLA metabolites in tissues of rats fed partially hydrogenated vegetable oil was first reported by Banni and associates over a decade ago (Banni *et al.*, 1995). More recent studies have demonstrated that conjugated metabolites of CLA (i.e. conjugated dienes 16:2, 18:3, 20:2, 20:3 and 20:4) are present in tissues of animals fed CLA, as well as in cultured cells treated with CLA. The CLA metabolites reportedly originate from the elongation, desaturation and peroxisomal or mitochondrial β-oxidation of CLA (Sebedio *et al.*, 2001; Banni *et al.*, 2004; Park *et al.*, 2005). Although much research remains to be done to fully elucidate the potential biological role of these CLA metabolites *in vivo*, it is possible that the various effects observed to

date with CLA may be mediated not only by CLA itself, but also by its conjugated metabolites.

9. Biological effects of CLA

a. General considerations

The first and most obvious role of fat and fatty acids is as an essential nutrient, as well as an ideal storage form of energy (Sampath and Ntambi, 2005). At the cellular level, fatty acids form an essential part of the cell membrane phospholipid bilayer, and serve as precursors to signaling molecules such as steroids and prostaglandins (Pariza *et al.*, 2000; Sampath and Ntambi, 2005). Apart from these well-established roles for fatty acids, however, it has become increasingly clear that PUFAs, including CLA, can specifically and rapidly effect changes in cellular metabolism, differentiation and growth through alterations in gene expression patterns (Sampath and Ntambi, 2005).

CLA has often been treated as a compound whose beneficial effects are not directly linked to its fatty acid nature. Indeed, early research regarding CLA's mechanisms of action hypothesized antioxidant activity to be the basis of its reported beneficial health effects, although this has since been refuted (van den Berg *et al.*, 1995; Carta *et al.*, 2002). Subsequently, the discovery that CLA can be elongated and desaturated as a regular fatty acid elicited the theory that the activity of CLA may be related to its properties as an unsaturated fatty acid (Banni *et al.*, 1995).

It is also becoming increasingly evident that the different CLA isomers, for instance, *c*9,*t*11 CLA and *t*10,*c*12 CLA, might have different actions and effects in biological systems, and also react differently to biological and physiological conditions and stimuli (Pariza *et al.*, 2000; Khosla and Fungwe, 2001). Note that, although our study focuses on cardiac effects of CLA, the evidence cited below comes from studies of various disease states; as mentioned, virtually nothing is presently known regarding CLA's effects in terms of cardiac function. It is conceivable, however, that the differences in isomer actions and effects observed in current studies may also be applicable to the heart. In animal studies, CLA is usually given as a 50:50 mixture of the two main CLA isomers, namely *c*9,*t*11 and *t*10,*c*12 CLA, although in natural food sources the *c*9,*t*11 isomer predominates over the *t*10,*c*12 isomer by a ratio of 30-70:1 (Parodi, 1997). However, the effects of the individual isomers both *in vitro* and *in vivo* can vary dramatically (Miller *et al.*, 1994; Park *et al.*, 1997; Parodi, 1997; McGuire *et al.*,1999; Parodi, 2003). For instance, research has shown that different CLA isomers exert opposite effects on gene expression (Majumder *et al.*, 2002; Palombo *et al.*, 2002; Ochoa *et al.*, 2004).

Just as current studies have indicated that different CLA isomers may have different actions under the same biological conditions, current literature also suggests that the effects observed may also be influenced by the animal species used (Azain *et al.*, 2000; Keim, 2003), and differences in effects have

also been observed in humans (Atkinson, 1999; Blankson *et al.*, 2000; Smedman and Vessby, 2001; Thom *et al.*, 2001).

Clearly, in order to understand the beneficial/detrimental effects specific to each individual isomer, it is important to investigate how these isomers differ in their actions in a biological system. Further, it has become evident that the effects of CLA may be species-dependent, and this must also be taken into consideration when interpreting the results of animal research trials and extrapolating these results to humans.

Although CLA isomers have been reported to exhibit antioxidant properties (Yu 2001; Kim *et al.*, 2004), to have anticarcinogenic effects via induction of apoptosis and cytotoxic activity (Chujo *et al.*, 2003; Yamasaki *et al.*, 2003; Song *et al.*, 2004), to modulate fatty acid composition (Li and Watkins, 1998; Noone *et al.*, 2002), enhance immune function (Song *et al.*, 2005), alleviate hyperinsulinemia (Nagao *et al.*, 2003), and to reduce body fat in animals (Terpstra, 2004) and possibly humans (Terpstra, 2004), as well as affect the expression of cytokines and growth factors (Cho *et al.*, 2003, Kim *et al.*, 2003; Luongo *et al.*, 2003), there is as yet very limited information available on the impact of CLA isomers or mixtures on specific cardiac cellular mechanisms. This detailed baseline information is crucial for the assessment of any future therapeutic applications as well as for establishment of potential risks and benefits of CLA isomers on heart function during different pathophysiological conditions.

Since the present research project examines specifically the effect(s) of dietary CLA on cardiac gene expression, the innumerable other physiological/biological effects of CLA will not be further explored. Instead, the following sections (b. through e.) discuss the possible role of CLA in cardiovascular function.

b. CLA and atherosclerosis

Cardiovascular disease is regarded as a complex disease with strong lipid metabolic and inflammatory components that are targeted on the vascular endothelium (Wahle and Rotondo, 1999). Although there are no epidemiological studies demonstrating the effect of CLA on the prevention of atherosclerosis, animal studies have suggested that CLA supplementation decreases the development of early atherosclerotic lesions (Kritchevsky *et al.*, 2000; Lee *et al.*, 1994). Studies published to date have used a mixture of CLA isomers, and thus there is as yet no clear consensus on the possible effects of specific CLA isomers on atherosclerosis (Kritchevsky *et al.*, 2000; Kritchevsky *et al.*, 2002). Table 2 gives a non-exhaustive summary of animal studies that have examined the effects of CLA on atherosclerosis. The next section discusses direct cardiac effects of CLA.

Table 2: Summary of studies examining the effects of CLA on atherosclerosis [1]

Animal Model	Diet	Reported Effect	Reference
Rabbit	High fat (14% w/w), 0.1% cholesterol, 0.5 g CLA/day	Decreased aortic atherosclerosis	Lee *et al.*, 1994
Rabbit	High fat (14% w/w), 0.1% cholesterol + isomeric mixture of 40% *c*9,*t*11 CLA and 40% *t*10,*c*12 CLA	Decreased aortic atherosclerosis	Kritchevsky, 2000
Rabbit	0.2% cholesterol + *c*9,*t*11 (90% pure); 0.2% cholesterol + *t*10,*c*12 CLA (90% pure); 0.2% cholesterol + isomeric mixture of isomers; or, same CLA with cholesterol-free diet	Decreased severity of existing atherosclerotic lesions; inhibited development of atherogenesis	Kritchevsky *et al.*, 2004
Hamster	Chow-based hypercholesterolemic diet supplemented with 20% coconut oil, 2% safflower oil, 0.12% cholesterol alone or + 1% (w/w) CLA as free fatty acid (FFA) or + 1% (w/w) LA as FFA	CLA diet group developed significantly less early aortic atherosclerosis compared to control or LA diets	Wilson *et al*, 2000
Hamster	Chow-based hypercholesterolemic diet with 10% coconut oil and 0.1% cholesterol alone or + 0.5% (w/w) *c*9,*t*11 CLA or + 0.5% (w/w) *t*10,*c*12 CLA, or + 0.5% (w/w) LA	*c*9,*t*11 CLA tended to decrease atherosclerotic lesions; *t*10,*c*12 tended to increase atherosclerotic lesions	Wilson *et al.*, 2006
Hamster	High-fat, high-cholesterol diet with 1% (w/w) c9,t11 CLA; or 1% (w/w) t10,c12 CLA; or 1% (w/w) LA	Compared to LA group both single isomer groups showed non-significant decrease in fatty aortic streaks	Mitchell *et al.*, 2005
Mouse	Atherogenic diet + 5 g CLA/kg; or + 2.5 g/kg CLA + 2.5 g/kg LA; or 5 g/kg LA	CLA increased the development of aortic fatty streaks	Munday *et al.*, 1999
Diabetic ApoE deficient Mouse	Semi-purified diet supplemented with 0.9% (w/w) *c*9,*t*11 CLA	*c*9,*t*11 CLA failed to reduce severity of atherosclerotic lesions	Nestel *et al.*, 2006
ApoE knockout Mouse	Western-type diet enriched with LA (control); with 1.0% (w/w) *c*9,*t*11 CLA; or with 1.0% (w/w) *t*10,*c*12 CLA	*t*10,*c*12 CLA promoted atherosclerosis; *c*9,*t*11 did not	Arbones-Mainar *et al.*, 2006

[1] Non-exhaustive list

c. Direct cardiac effects of CLA

As mentioned, some studies have shown that CLA can exert cardiovascular benefits through its hypolipidemic and antiatherosclerotic effects (Rudel, 1999; Khosla and Fungwe, 2001; Toomey *et al.*, 2003). A blood pressure lowering effect of *t*10,*c*12 in obese rats (Inoue *et al.*, 2004) and in spontaneously hypertensive rats (Nagao *et al.*, 2003) has also been reported. In addition, CLA has been reported to depress the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in neonatal cardiomyocytes (Xiao *et al.*, 1997).

Intensive research has shown that diets rich in n-3 PUFAs such as eicosapentanoic acid and docosahexanoic acid are associated with decreased incidence and severity of coronary heart disease (Vanden Heuvel, 2004). It has also become evident from animal studies that CLA has effects on risk factors for coronary heart disease that resemble those of n-3 PUFAs (Vanden Heuvel, 2004). It has been postulated that cognate receptors must exist that preferentially respond to a particular structure of fatty acid, and that these specific "lipid sensors" affect gene expression in a tissue-specific, sex-specific, and developmentally-specific manner, thereby affecting the development of coronary heart disease by altering enzymes and proteins involved in the transport or metabolism of cholesterol and fatty acids (Vanden Heuvel, 2004).

One possible family of proteins that may act as lipid sensors are the nuclear receptors, a "superfamily" of receptors that act as intracellular transcription factors that directly regulate gene expression in response to

lipophilic molecules (Wahli and Martinez, 1991; McDonnell *et al.*, 1993; Weatherman *et al.*, 1999; Honkakoski and Negishi, 2000; Kahn and Vanden Heuvel, 2003). A number of nuclear receptors have evolved to respond to dietary lipids, including the peroxisome proliferator activated-receptor (PPAR), and the retinoid X receptor (RXR), both of which are active in cardiac tissue (Clarke and Jump, 1996; Kahn and Vanden Heuvel, 2003). The PPARs are ligand-activated transcription factors involved in gene expression in a tissue-sex- and species-dependent manner (Hihi *et al.*, 2002; Kahn and Vanden Heuvel, 2003). More specifically, PPARs regulate the expression of genes involved in fatty acid uptake and oxidation, lipid metabolism, and inflammation (Kersten *et al.*, 2000). Three different isoforms of PPAR have been identified, namely PPARα, PPARβ, and PPARγ, and all three are encoded by different genes (Vanden Heuvel, 2004). PPARα is expressed mainly in tissues that have a high level of catabolism, such as liver, kidney, skeletal muscle and heart tissue (Braissant *et al.*, 1996). PPARβ is ubiquitously expressed, whereas PPARγ expression is limited primarily to white and brown adpose tissue, with limited amounts found in skeletal and heart tissue (Braissant *et al.*, 1996). It has been demonstrated that PPARβ is the predominant PPAR isoform in cardiac cells, and is the primary PPAR responsible for regulation of cardiac lipid metabolism (Gilde *et al.*, 2003). Furthermore, fatty acids have been shown to act as endogenous ligands for PPARβ in the context of cardiac muscle (Gilde *et al.*, 2003). It has also been

shown that all three PPAR subtypes, when activated, are able to inhibit cardiac hypertrophy (Yamamoto *et al.*, 2001; Asakawa *et al.*, 2002; Planavila *et al.*, 2005).

All three PPAR isoforms function by forming heterodimers with RXR and binding to a prescribed DNA sequence, known as the PPAR response element (PPRE) (Desvergne and Wahli, 1999). The PPRE comprises direct repeat sequences separated by a single nucleotide spacer (AGGTCA_AGGTCA), and the binding of the PPAR/RXR heterodimer to the PPRE affects transcription of the target gene (Ijpenberg *et al.*, 1997). In general, all of the n-3 and n-6 fatty acids activate the three PPAR isoforms (Sampath and Ntambi, 2004); however, their affinities for the receptor vary, which suggests a role for site-specific availability and metabolism of particular fatty acids, and differences in their affinity for specific PPAR subtypes (Sampath and Ntambi, 2004).

With regard to CLA, the *c*9,*t*11 CLA isomer is a potent PPARα ligand (Vanden Heuvel, 2004). PPARα affects PPAR-responsive enzymes, including acyl-CoA oxidase (Moya-Camarena *et al.*, 1999), and controls lipid transport proteins such as FABP and acyl-CoA binding protein (ACBP), both active in cardiac cells, as well as affecting genes involved in fatty acid transport (Escher and Wahli, 2000; Kirsten *et al.*, 2000). Additionally, CLA isomers, particularly *c*9,*t*11 and *t*10,*c*12, are ligands for PPARγ, which is found in vascular cells (Willson and Wahli, 1997; Claudel *et al.*, 2001). It has been

shown that activated PPARγ has an inhibitory effect on vascular cell adhesion molecules and intercellular adhesion molecules (Pasceri *et al.*, 2000), inhibits the migration of vascular smooth muscle cells (Marx *et al.*, 1998), and also inhibits monocyte/macrophage homing to atherosclerotic plaques (Pasceri *et al.*, 2000). As a ligand for PPARγ, CLA may play a role in preventing the development of risk factors for heart disease. CLA isomers are also activators of PPARβ, which, as mentioned, plays a role in cardiac lipid metabolism (Moya-Camarena *et al.*, 1999; Gilde *et al.*, 2003).

Retinoid X receptors also exist in the three subtypes α, β, and γ; RXRα and β are ubiquitously distributed, while RXRγ is expressed in specific organs, including the heart (Vanden Heuvel, 2004). The *c*9,*t*11 CLA isomer has been shown to be the most potent activator of RXRα, and research has demonstrated that activated RXR is capable of reducing atherosclerosis in apolipoprotein E knockout mice (Claudel *et al.*, 2001).

d. CLA effects on cardiac hemodynamics

Hemodynamics refers to the forces that affect the circulation of blood throughout the body (Hebra *et al.*, 2006). By extension then, cardiac hemodynamics refers to the forces within the heart that affect blood circulation. This is, of course, a simplistic definition, and cardiac hemodynamic function requires a more thorough explanation.

Generally, in the absence of valvular disease, left ventricular (LV) pressures obtained during cardiac catheterization are rarely used for more than measurement of peak systolic and end-diastolic pressures (Eucker *et al.*, 2001). In pathophysiological studies, however, assessment of systolic and diastolic function may also be performed on the basis of the hemodynamic waveform. Usually, hemodynamic assessment involves the measurement of selected points on the LV pressure (P) versus time (t) contour, and/or its derivative, dP/dt, where dP/dt denotes the time derivative of pressure (i.e. a change in pressure with respect to time) (Euker SA *et al*, 2001). Such selected measurements often include maximum LV systolic pressure (SP), LV end-diastolic pressure (LVEDP), and peak positive dP/dt ($+dP/dt_{max}$), and peak negative dP/dt ($-dP/dt_{min}$), as well as mean arterial pressure (MAP), which is the average pressure within an artery over a complete cycle of one heartbeat (Recordati, 1999). The physiological and clinical significance of these parameters is well-established, and they are widely used in both clinical and research settings (Zile and Brutsaert, 2002). It should be noted that several of these hemodynamic measurements are related; for instance, SP and $+dP/dt_{max}$ are measures of LV inotropic performance, where $+dP/dt_{max}$ signifies the point along the LV pressure versus time curve where maximum pressure (inotropic/systolic) is achieved, and $-dP/dt_{min}$ signifies the point along the same curve where minimum lusitropic pressure (relaxation) is achieved. Diastolic

pressure (DP) is also associated with the lusitropic LV effect, as it is the pressure measured in the LV during cardiac relaxation (diastole).

Our previous study on CLA has provided some interesting data regarding the effects of CLA supplementation on the cardiac hemodynamic function of rats (Tappia *et al.*, 2006). In this study, Sprague Dawley rats (30 male and 30 female) were randomized at 3 weeks of age to receive 1 of 5 diets between 4 and 20 weeks of age. The diets included a modified AIN-93G control diet and four treatment diets, consisting of the AIN-93G diet with either single isomer (i.e. *c*9,*t*11 or *t*10,*c*12 CLA) in fatty acid form or mixed isomer CLA (i.e. 50/50 mixture *c*9,*t*11 and *t*10,*c*12 isomers) in fatty acid or triglyceride form. The CLA isomers were obtained from Lipid Nutrition, a division of Loders Crokklan (Channahon, IL, USA). Details of diet formulations are listed in Table 4 in the Materials and Methods Section.

In our previous study, following dietary treatment, LV function was assessed in the CLA fed rats by the *in vivo* catheterization technique (Cheema *et al.*, 2005). Measurements for heart rate, SP, DP, MAP, left ventricular systolic pressure (LVSP), LVEDP, $+dP/dt_{max}$, and $-dP/dt_{min}$ were recorded. Hemodynamic data were computed and displayed using AcqKnowledge Software version 3.7.1 (MP System "Quick Start", Biopac System Inc., Goleta, CA, USA). Microcal Origin version 7.5 (Origin Lab Corp., Northampton, MA, USA) was used for statistical analysis of the data. The differences between all dietary groups and gender were evaluated by two-way analysis of variance

(ANOVA) followed by Tukey post-hoc multiple comparison tests. A probability of 95% or more ($P<0.05$) was considered significant.

As expected, there was a difference in body weight between male and female rats, but no significant differences in body weight due to dietary treatment were observed within gender groups. No differences were seen in heart weight, or in the weight of the right and left ventricle or the atria, indicating that no hypertrophic response had occurred as a result of 16 weeks of dietary CLA treatment. The hemodynamic results of this study are summarized in Table 3.

Table 3: Summary of changes in hemodynamic measurements of male and female rats supplemented with CLA isomers or mixtures of isomers for 16 weeks

Hemodynamic Measurement [1,2]	*c9,t11* Diet		*t10,c12* Diet		Fatty Acid Mix		Triglyceride Mix	
	Male	Female	Male	Female	Male	Female	Male	Female
HR	- 9%	+ 7%	- 6%	NC	- 9%	NC	-7%	NC
SP	- 15%	- 10%	+24%	NC	- 15%	- 12%	NC	NC
DP	NC	NC	NC	+ 16%	NC	- 19%	NC	NC
MAP	NC	- 9%	- 19%	NC	- 19%	- 18%	- 14%	NC
LVSP	- 14%	NC	+ 9%	NC	- 17%	- 11%	- 15%	- 11%
LVEDP	NC	NC	NC	NC	NC	NC	NC	+ 51%
+dP/dt$_{max}$	- 16%	- 14%	NC	- 15%	- 28%	- 8%	- 12%	- 23%
-dP/dt$_{max}$	NC	NC	+35%	NC	NC	NC	NC	- 30%

[1] Abbreviations: HR = heart rate; SP = systolic pressure; DP = diastolic pressure; MAP = mean arterial pressure; LVSP = left ventricular systolic pressure; LVEDP = left ventricular end diastolic pressure; +dP/dt$_{max}$ = peak positive LV pressure; -dP/dt$_{max}$ = peak negative LV pressure.

[2] Percentages (+/-) indicate change in hemodynamic measurement compared to same-gender control. NC = No change (<5%). (Adapted from Tappia *et al.*, 2006)

Since these data have been described in detail previously (Tappia *et al*, 2006), for the purposes of this discussion, only a few highlights will be mentioned. As can be seen in Table 3, there are some notable hemodynamic changes. In all groups of male rats, heart rate decreased. This is a desirable change if contractility parameters (i.e. SP, LVSP, and/or $+dP/dt_{max}$) also increase, as the combination of decreased heart rate and increased contractility denotes a positive inotropic effect, most likely due to increased Ca^{2+} available to the cardiac contractile machinery (Cotton *et al.*, 2001). This was observed in male rats fed the *t*10,*c*12 CLA isomer diet. In contrast, the *c*9,*t*11, FA, and TG mixtures decreased heart rate as well as all inotropic parameters in male rats, demonstrating decreased contractility, possibly due to decreased availability of Ca^{2+}. In female rats, the *c*9,*t*11 CLA diet produced an increase in heart rate and a decrease in contractile parameters, which may show that contractile ability was decreased, causing the heart to work harder in order to maintain cardiac output. While heart rate remained unchanged in the female rats fed the CLA fatty acid mix, all inotropic parameters decreased, and it is possible that heart rate would have to increase at some point to compensate for decreasing cardiac contractility due to decreased cardiac Ca^{2+} availability. Of final note are the effects of the CLA triglyceride mix in female rats, where systolic parameters decreased (i.e. LVSP, $+dP/dt_{max}$), while diastolic parameters increased (i.e. LVEDP, $-dP/dt_{max}$). In this group, it is possible that as contractile performance decreased, the heart pumped less blood out to the

arteries with each contraction, leaving blood in the LV chamber (therefore increasing LVEDP). It is conceivable that heart rate would soon increase in an attempt to maintain cardiac output. Clearly, this earlier study suggests that there are differences in digestion, absorption and transport of CLA isomers, as the free fatty acid and triglycerol forms had different effects on the cardiac responses observed through hemodynamic measurement.

It should be noted that a number of previous studies have shown that PUFA, including CLA, have effects on the contraction of cardiac myocytes, indicating an effect on the excitability (automaticity) of myocytes (Billman *et al.*, 1994; Kang and Leaf, 1994; Kang *et al.*, 1995; McLennan and Dallimore, 1995; McLennan 2001). It has been shown that PUFA can significantly reduce the rate of contraction of neonatal cardiomyocytes without changing the amplitude of contraction (Kang and Leaf, 1994). While this effect is strongest for the n-3 PUFA eicosahexapentanoic acid, it was also observed, although to a smaller extent, with CLA (Kang and Leaf, 1994).

It is also interesting to note that while several studies have shown incorporation of CLA into myocardial membrane phospholipids of experimental animals fed different CLA isomers (Kramer *et al.*, 1998; Alaisner *et al.*, 2002; Yang *et al.*, 2003), other researchers have concluded that neither incorporation of PUFA into the membrane phospholipid nor covalent linking of CLA to membrane components is required for their action (Kang and Leaf, 1994). These researchers also concluded that the free fatty acid is the form

responsible for the changes observed in heart rate and rate of contraction (+dP/dt) (Kang and Leaf, 1994). It has been postulated that a decrease in the excitability of the cells may result from: 1) elevation of the threshold to a more positive membrane potential of the threshold; 2) an increase in the resting potential (i.e. a more negative resting potential); or 3) a decrease in the rate of depolarization of resting potential (Berne, 1993). It has been further postulated that changes in the activity of Ca^{2+}, Na^{+} or K^{+} channels may be responsible for the above effects (Berne, 1993).

It is clear that further work must be undertaken to elucidate the mechanisms of action of CLA isomers on heart function as well as to determine possible gender differences in response to CLA supplementation. Such an investigation should measure the effect of CLA isomer incorporation into the cardiomyocyte SR membrane on the expression of genes that regulate calcium handling in cardiomyocytes, and thus identify possible effects on the cardiac calcium excitation pathways, and ultimately, cardiac contractile performance. In this regard, it is conceivable that the incorporation of CLA isomers as well as their metabolites into the SR membrane could be accompanied by changes in the expression of the genes responsible for calcium uptake and release from cardiomyocytes, thereby producing the hemodynamic changes observed in our previous study.

e. Fatty acid regulation of mammalian gene expression: a possible explanation for the effect of CLA on cardiac hemodynamic function

One of the first reports regarding the role of polyunsaturated fatty acids in the regulation of mammalian gene expression came in the mid-1960s, when the upregulation of fatty acid synthesizing enzymes in the liver and epididymal fat pads of mice fed high-carbohydrate diets was described (Allman and Gibson, 1965). In this study, enzyme activities of fatty acid synthetase, malic enzyme and glucose-6-dehydrogenase were decreased, suggesting rapid, direct, and specific regulation of enzymes of lipid metabolism by a particular fatty acid. These findings were subsequently confirmed a decade later (Wahle and Radcliffe, 1977; Jeffcoat and James, 1978). It was not until 1982, however, that a possible mechanism of action was suggested, when Schwartz and Abraham (1982) showed that corn oil supplementation of a high carbohydrate diet reduced the rate of synthesis of fatty acid synthetase protein in mouse liver. It has since been shown that several fatty acids (including CLA), as well as their metabolites, are capable of specifically effecting changes in gene expression patterns in liver, adipose and other tissues (Sampath and Ntambi, 2005).

It was the eventual discovery of nuclear receptors capable of binding fatty acids that established a direct role for fatty acids in gene regulation (Gottlicher *et al.*, 1992). Although the nuclear actions of PUFAs were first

studied in hepatic cells, their actions have since been confirmed in cultured 3T3-L1 adipocytes (Tebbey *et al.*, 1994; Sessler *et al.*, 1996; Mater *et al.*, 1998), as well as various tissues, including small intestine (Niot *et al.*, 1997), pancreas (Brun and Spiegelman, 1997), immune cells (Tebbey and Butke, 1993) and neonatal mouse brain (DeWille and Farmer, 1993). It is conceivable, therefore, that CLA, as a polyunsaturated fatty acid, might also have a direct role in control of gene regulation and gene expression in the heart.

B. Sarcoplasmic reticulum and cardiac oxidative stress: new thoughts for treating heart disease

1. The cardiac contractile cycle

It has been established that Ca^{2+} plays a major role in the process of excitation-contraction coupling in cardiomyocytes (Bassani *et al.*, 1994). In the normal heart, the sarcoplasmic reticulum works in concert with the sarcolemma (SL) to maintain a 10,000-fold gradient of Ca^{2+} across the cardiomyocyte plasma membrane (MacLennan and Wong, 1971; Jorgensen *et al.*, 1982). At the beginning of the cardiac contractile cycle, depolarization of the SL allows the entry of a small amount of Ca^{2+} from the extracellular space into the cytosol via voltage-dependent L-type Ca^{2+}-channels (Bodi *et al.*, 2005). This small influx of Ca^{2+} in turn triggers the release of a large amount

of Ca^{2+} from the SR via the calcium release channels (calcium-induced calcium release), including the RyR (Fabiato, 1983), which results in an increase in the $[Ca^{2+}]_i$ and thereby induces cardiac contraction. The amount of Ca^{2+} released during this cycle depends on the amount of Ca^{2+} stored in the SR as well as the fraction of the total Ca^{2+} reserve that is available for release (Bassani *et al.*, 1994; Bers, 1998). In the SR lumen, Ca^{2+} is bound to a negatively charged storage protein, calsequestrin (Csq), which stores Ca^{2+} for release during the next wave of depolarization (Dhalla and Temsah, 2001). During diastole, the $[Ca^{2+}]_i$ is restored to its resting level of 10^{-7} M. This occurs mainly due to the action of the SERCA2a pump, which actively pumps Ca^{2+} back into the lumen of the SR (MacLennan and Wong, 1971; Dhalla *et al.*, 1982). SERCA2a, which is located in the SR, transports two moles of Ca^{2+} at the expense of one mole of ATP. A relatively small amount of Ca^{2+} is extruded by the SL Na^+/ Ca^{2+}-exchange, as well as an SL Ca^{2+}-pump ATPase mechanism (Dhalla *et al.*, 1982; Bers *et al.*, 1998).

It should be noted that the abovementioned $[Ca^{2+}]_i$ restoration mechanisms are species-dependent (Bassani *et al.*, 1994; Bers *et al.*, 1998). For instance, it has been shown that SR SERCA2a contributes approximately 92% of cytosolic Ca^{2+}-reuptake in the rat heart, and about 70-75% of this function in guinea pig, cat, rabbit and human hearts. Conversely, SL Na^+/ Ca^{2+}-exchange is responsible for 25-30% of Ca^{2+} restoration in all species, but only about 7% in rats (Bassani *et al.*, 1994). While a great deal of research and

attention has focused on SR/SL as sites for cardiac drug development, very little attention has been paid to exploring SR sites, particularly the regulatory mechanisms that control Ca^{2+} movements in the SR membrane, as possible targets for improving therapy for heart disease. Figure 1 depicts the movement of calcium within the cardiomyocyte.

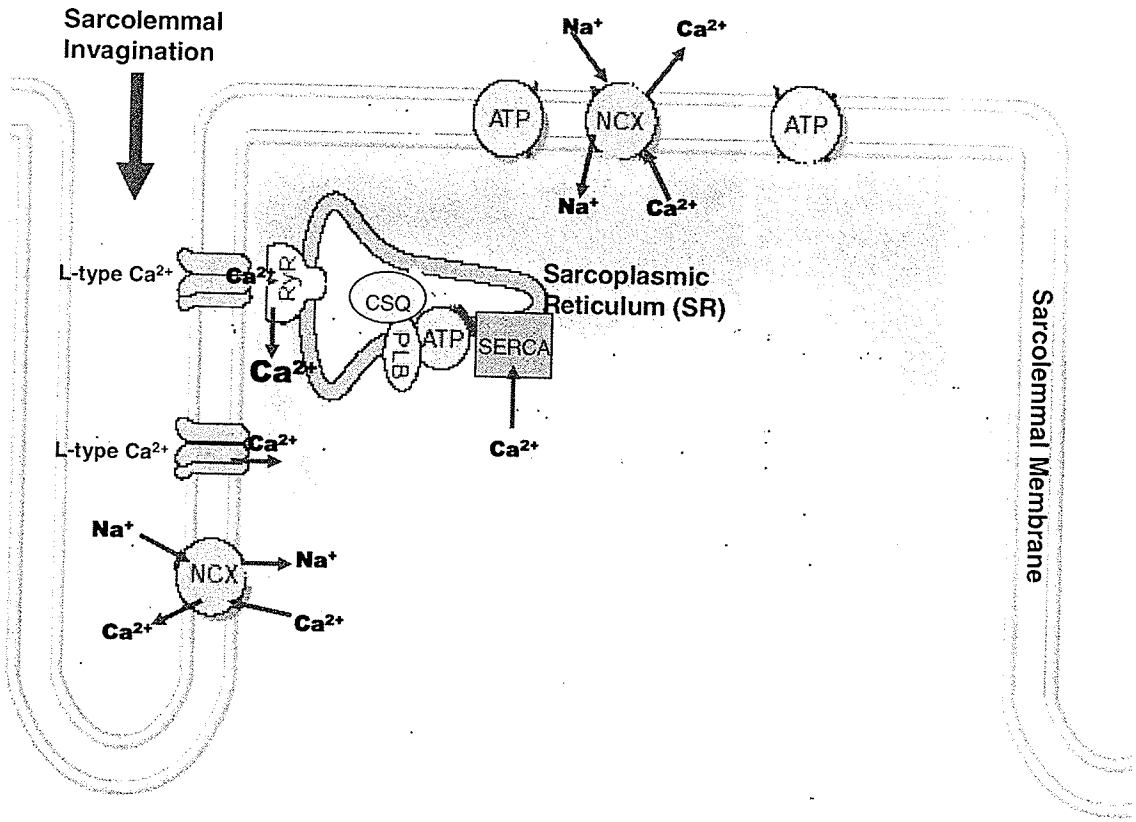


Figure 1: Calcium movement within the cardiomyocyte

The L-type Calcium Channels (L-type Ca^{2+}), located along the sarcolemmal membrane, allow the entry of a small amount of calcium ions into the cardiomyocyte cytosol. This small amount of calcium triggers the release of a much larger amount of calcium (calcium-induced calcium release) from the sarcoplasmic reticulum (SR) via the ryanodine receptors (RyR). This increase in intracellular calcium concentration causes contraction of the cardiomyocyte (ventricular systole). Following contraction, the sarcoendoplasmic reticulum ATPase pump (SERCA) actively removes the excess calcium from the cytosol into the lumen of the SR, where it is held by the SR storage protein calsequestrin (CSQ). In order for SERCA to function maximally, phospholamban (PLB), a negative regulator of SERCA, must be phosphorylated. A small amount of calcium is also extruded from the cell via the sodium-calcium exchanger (NCX). When intracellular calcium concentration $[Ca^{2+}]_i$ returns to 10^{-7} M, the mycocyte relaxes (ventricular diastole). Cartoon adapted from www.edoc.hu-berlin.de/.../HTML/chapter1.html.

2. Pathophysiology of heart disease: Ca^{2+}-overload and oxidative stress

Ca^{2+}-overload and oxidative stress are the two major mechanisms that have been implicated in cardiac dysfunction in different pathological conditions (Dhalla *et al.*, 2000a; Dhalla *et al.*, 2000b). Ca^{2+}-overload was first described by Fleckenstein (1971) as an increase in intracellular Ca^{2+} levels such that cellular toxicity resulted. This toxic increase can be attributed to a number of factors, including the degradation of cellular proteins such as SERCA2a (Yoshida *et al.*, 1990) and the RyR (Rardon *et al.*, 1990).

The effects of oxidative stress were first identified by Gerschman *et al* (1954). There are several underlying reasons for the occurrence of oxidative stress, including impairment of the antioxidant defense system, excess generation of free radicals, or a combination of both. As the result of oxidative stress, the SR membrane lipid bilayer arrangement is disrupted, which alters its functional properties (Grinna, 1977).

There is an interactive relationship between Ca^{2+}-overload and oxidative stress, although it is unknown whether these pathogenic phenomena are causally related or complementary mechanisms of cellular injury (Dhalla *et al.*, 2000a; Schumacher *et al.*, 1998). SR dysfunction due to excessive generation of free radicals and leading to Ca^{2+}-overload in cardiomyocytes is represented by a marked decrease in Ca^{2+}-uptake and Ca^{2+}-release activities (Netticadan *et al.*, 1999; Temsah *et al.*, 1999). This decrease in SR activities has been

partially attributed to depressed levels of RyR, SERCA2a and phospholamban (PLB) protein content (Temsah *et al.,* 1999). It has been observed that the expression levels of genes that encode SR proteins are depressed after episodes of oxidative stress, such as that seen in ischemia-reperfused hearts, and it is postulated that these alterations may lead to long-term heart dysfunction (Gwathmey *et al.,* 1987). It should also be noted that several researchers have reported elevated diastolic force, reduced systolic force and slowed relaxation in cardiac muscle strips and cardiomyocytes isolated from patients with end stage heart failure (Gwathmey and Hajjar, 1990; Morgan *et al.,* 1990; Zarain-Hertzberg *et al.,* 1996). In both animals and humans, abnormalities in Ca^{2+} homeostasis appear to be associated with decreased SR Ca^{2+}-uptake and Ca^{2+}-ATPase activities (Gwathmey and Morgan, 1993; Schmidt *et al.,* 1998; Netticadan *et al.,* 2000). In addition to SR dysfunction, a decrease in the relative ratio of PLB:SERCA2a has also been observed in failing hearts (Hajjar *et al.,* 1997; Meyer *et al.,* 1999; Bartling *et al.,* 1999). Note that *in vivo* studies suggest that the "functional stoichiometry" of PLB:SERCA2a is less than 1:1 in native cardiac sarcoplasmic reticulum membranes (Kadambi *et al.,* 1996). The ratio of SR SERCA2a:SL Na^{+}/Ca^{2+}-exchange was also reduced in left ventricular specimens from patients with end-stage heart failure compared to patients with normal heart function (Bartling *et al.,* 1999).

3. Is it in the genes?

Patients with heart failure are characterized by multiple cardiac defects, but alterations in Ca^{2+} handling have been shown to be the primary cause of systolic and diastolic dysfunction (Gwathmey *et al.,* 1987; Gwathmey and Hajjar, 1990). These abnormalities are tightly linked to downregulation of SERCA2a expression and activity (Zarain-Hertzberg, *et al.,* 1996). Although not all studies have produced consistent results, most investigators have shown that expression of genes concerned with Ca^{2+} transport by the SR, such as SERCA2a (Mercadier *et al.,* 1990; Arai *et al.,* 1994; Mewe *et al.,* 1994; Schwinger *et al.,* 1995; Mattiello *et al.,* 1998; Kubo *et al.,* 2001), PLB (Linck *et al.,* 1996; Marx *et al.,* 2000), and the RyR (Go *et al.,* 1995) are all reduced in the end-stage failing human heart. Patients with dilated cardiomyopathy have lower levels of SERCA2a and PLB messenger RNA (mRNA) than normal patients (Yasumura *et al.,* 2003). In contrast, NCX mRNA in end-stage failing hearts has been reported to be increased (Struder *et al.,* 1994; Flesch *et al.,* 1996). Modifications in the expression of genes that encode SR proteins have been suggested as potential strategies to normalize cardiac dysfunction as seen in the hypertrophic heart. Overexpression of SERCA2a in isolated myocytes as well as intact animals has been shown to enhance cardiac contraction and relaxation (He *et al.,* 1997).

Pharmacological interventions are considered to be the traditional form of treatment therapy for heart disease. Based on expanding knowledge of the

functional importance of proteins and their interaction with other functional players or signaling pathways, as well as the significance of Ca^{2+}-related therapies, it is possible that new therapies for heart failure that aim to improve SR function may include upregulation of SERCA2a, RyR and PLB. It may be possible to effect this upregulation, at least in part (i.e. as an adjunct to gene therapy), through nutrition interventions with physiologically active nutrients such as CLA.

However, while new findings in gene therapy are on the horizon, certain precautions must be taken into consideration. First, genes must be overexpressed in a controlled fashion such that the interference with the machinery that transcribes and transports mRNA to its final destination does not occur. Also, it is important to ensure that the overexpressed protein is not only translated, but also incorporated into the membrane and that it is functional (He *et al.,* 1997). Further, the overexpression of a protein should not interfere with the expression of other proteins. Protein overexpression (as in the case of SERCA2a) should not create a dilemma as far as energy demand is concerned. Negative outcomes associated with pharmacological therapies may be ameliorated through accessory nutritional therapies such as supplementation with CLA, which may decrease toxic side effects while assisting in producing the desired effect on cardiac SR gene expression and protein function.

C. Production of functional protein from a gene sequence: transcription and translation

One of the most important activities of a cell is the production of proteins that perform major functions within the cell (i.e. structural, enzymatic, hormonal, etc.). The following section will give a very brief description of the process of protein production within a cell.

Within every eukaryotic cell reside complex polymeric molecules known as DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). In eukaryotic cells, DNA resides exclusively within the nucleus, whereas different types of RNA are found within both the nucleus and cytoplasm. The double stranded structure of DNA is the result of the formation of hydrogen bonds between the nitrogenous bases on one strand of DNA with the nitrogenous bases on the other strand of DNA (Wikipedia, 2006). There are four such bases found in DNA, namely thymine, adenine, cytosine and guanine, commonly abbreviated A, C, T and G. In RNA, thymine is replaced by the nitrogenous base uracil (U). These bases exhibit complementarity, meaning that each nitrogenous base can only form a hydrogen bond with one other nitrogenous base, namely A with T, C with G (A-T, C-G) (Wang *et al.*, 1999). Note that in RNA, uracil pairs only with adenine (A-U). Complementarity allows DNA to replicate itself, and also allows it to be rewritten in the form of RNA (Pearson Prentice Hall, 2006).

A protein is a chainlike molecule built of subunits of smaller molecules known as amino acids; during protein synthesis, the separate amino acids are reassembled into new chains (Wikipedia, 2006). The code for ordering the amino acids of a protein is written as a sequence of bases in the DNA. This sequence of DNA is known as a gene or gene sequence (http://home.homecast.net).

Cells use the two-step process of transcription and translation to read each gene and produce the string of amino acids that makes up a protein. The term "transcription" literally means to "write across" or convert information, where the transcription process converts the double-stranded DNA code into a single strand RNA format (King, 2006). This single strand chain of nucleotides, or messenger RNA (mRNA) leaves the cell nucleus, carrying an accurate reproduction of the information coded in the original DNA (Wang *et al.*, 1999). The formation of mRNA encompasses the process of transcription.

The molecules of mRNA leave the cell nucleus, entering the cytoplasm, where they are attracted to structures known as ribosomes. Within the cytoplasm is a second type of RNA molecule called transfer RNA (tRNA). mRNA lines up at the ribosome, tRNA in turn attaches to mRNA and subsequently donates amino acids to the mRNA chain (King, 2006). This assembly of amino acids, in a sequence of amino acids specified by the order of nucleotides in the mRNA (which was initially determined by the sequence

of amino acids in the original DNA and governed by complementarity) into a protein is called translation (Wikipedia, 2006).

D. Preliminary findings

In order to assess the viability of the current proposed study, we performed some initial assessments of LV tissue obtained from the original CLA study. RNA was isolated from male LV tissue from each of the treatment and control groups, and expression of a number of genes responsible for calcium handling within cardiomyocytes was measured using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). The results of the PCR amplification were analyzed by gel electrophoresis and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada) and calculating the intensity as a ratio of a target gene over the housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase, GAPDH). These preliminary trials indicated that there were significant changes in the expression of the SERCA2a gene in the LV tissue of male rats supplemented with the single isomer *t*10,*c*12 CLA and single isomer *t*9,*c*11 CLA, and an additive increase in SERCA2a gene expression in the LV of male rats fed the fatty acid CLA mixture. We concluded that these results warranted a more in-depth investigation of the effects of CLA supplementation on the expression of the genes encoding cardiomyocyte SR protein in growing rats. We focused

our investigation on the genes encoding the principal SL and SR calcium-handling proteins, as previous research has demonstrated that calcium handling is key to maintenance of cardiac contractility and relaxation and normal heart function (del Monte *et al.*, 2004) and we believed that the hemodynamic changes observed in our previous study could have been due to changes in the expression of the genes encoding for key calcium handling cardiac proteins. We also felt that it was essential to investigate possible gender differences in the effect of CLA supplementation on cardiac function.

III. RATIONALE OF THE STUDY

In recent years, CLA has become a popular health supplement, and scientific interest in this linoleic acid derivative has rapidly increased. However, many questions remain to be answered in order to fully elucidate the physiological effects that have been observed in animals fed CLA-enriched diets. To our knowledge, no studies have been performed that have examined the major CLA isomers (i.e. *c*9,*t*11 and *t*10*c*12) or mixtures of these isomers in triglyceride or fatty acid form, and their effects on the genes that encode the proteins regulating calcium homeostasis in the hearts of male and female rats. Current research has also identified specific cardiac genes that exhibit reduced expression during progression to heart failure. This research will therefore seek to determine the underlying mechanisms involved in the hemodynamic

changes observed in CLA-fed animals, specifically the expression of genes involved in cardiac calcium handling. Information may be yielded that supports the possibility that CLA may have use as a novel agent for the treatment of heart failure.

IV. OBJECTIVES AND HYPOTHESES

Objective 1: To examine the effect of dietary CLA as single isomers *c*9,*t*11 or *t*10,*c*12 in fatty acid form, or as mixed isomers in fatty acid or triglyceride form on the expression of the cardiac genes responsible for calcium handling in left ventricular tissue from male and female rats.

To accomplish this objective, 30 male and 30 female rats were assigned to one of 5 dietary groups (n=6 in each group) and received dietary treatment from age 4 weeks until age 20 weeks. At 20 weeks of age, animals were sacrificed and RNA was isolated from LV tissue from each of the CLA treatment (*c*9,*t*11, *t*10,*c*12, fatty acid mix, triglyceride mix) and control-fed groups of male and female rats. RT-PCR was performed using primers specific for calcium-handling genes, including L-Type Ca^{2+} channel, SERCA2a, PLB, Csq, NCX and RyR. The PCR products were analyzed by electrophoresis in 2% agarose gels. The intensity of each band was photographed and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada) calculating the intensity as a ratio of a target gene over the housekeeping gene, GAPDH.

Hypothesis 1: CLA supplementation with *c*9,*t*11 in fatty acid form will significantly increase the mRNA levels of some of these genes, specifically SERCA2a.

This first hypothesis was based on existing literature that has shown the *c*9,*t*11 CLA isomer to be the most biologically active form of CLA (Pariza *et al.*, 2001). Other literature describes the *c*9,*t*11 CLA isomer to be the most potent ligand for PPARα, a member of the PPAR superfamily of nuclear receptors that have been shown to affect gene expression, and which is present in cardiac tissue (Vanden Heuvel, 2004). At a basic level, it has been shown that several fatty acids, including CLA, are capable of specifically effecting changes in gene expression patterns in liver, adipose and other tissues (Brun and Spigelman, 1997; Niot *et al.*, 1997; Mater *et al.*, 1998; Sampath and Ntambi, 2005). Although there is no literature to date that has shown that CLA can effect changes in cardiac gene expression it is conceivable that CLA, as a PUFA, might also have a direct role in control of gene expression in the heart. Finally, we hypothesized that the *c*9,*t*11 single isomer in fatty acid form would increase the expression of certain of the genes under study, particularly SERCA2a. This was based on our preliminary findings where SERCA2a mRNA levels were in fact elevated in the group of male rats supplemented with this isomer.

Objective 2: To examine the effect of dietary CLA as single isomers *c*9,*t*11 or *t*10,*c*12 in fatty acid form, or as mixed isomers in fatty acid or

triglyceride form on protein levels of LV SERCA2a and phospholamban in male and female rats.

To accomplish this objective, LV tissue was used from the 30 male and 30 female rats that had been assigned to one of 5 dietary groups (n=6 in each group) and had received dietary treatment from age 4 weeks until age 20 weeks. Protein isolates from male and female rats, aged 20 weeks, were analyzed by Western blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SERCA2a and PLB (unphosphorylated dimer) were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Boehringer Mannheim, Laval, PQ), and band intensities of the Western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS 800).

Hypothesis 2: Dietary *c*9,*t*11 CLA in fatty acid form will increase SERCA2a protein levels in male rats while decreasing PLB protein levels.

This hypothesis was based on several of our initial findings. First, preliminary tests for mRNA levels showed higher SERCA2a mRNA levels in male rats fed *c*9,*t*11 CLA, and thus we assumed that greater mRNA levels could be translated into higher SERCA2a protein levels. Further, our previous hemodynamic study showed that in male rats, heart rate decreased while cardiac output remained normal (as evidenced by unchanged LVEDP). This may have been due to upregulation of cardiac SERCA2a pump activity

combined with decreased PLB inhibition of pump activity (hence decreased PLB protein levels).

Objective 3: To examine the effect of dietary CLA as single isomers *c*9,*t*11 or *t*10,*c*12 in fatty acid form, or as mixed isomers in fatty acid or triglyceride form on calcium uptake and release by sarcoplasmic reticulum (SR) membrane from male and female rats.

To accomplish this objective, LV tissue was used from the 30 male and 30 female rats that had been assigned to one of 5 dietary groups (n=6 in each group) and had received dietary treatment from age 4 weeks until age 20 weeks. LV SR vesicles were isolated from male and female rat LV tissue and protein content assessed by the Lowry method (Sapan *et al.*, 1999). Calcium uptake and release in the isolated SR was assessed via a ^{45}Ca assay modified from the original method of Hawkins *et al.* (1994).

Hypothesis 3: Dietary *c*9,*t*11 CLA and mixed isomer CLA in fatty acid form will decrease both sarcoplasmic reticulum Ca^{2+} intake and sarcoplasmic reticulum Ca^{2+} release in male and female rats.

This hypothesis was based on extensive research demonstrating that PUFAs (in fatty acid form), including CLA, have antiarrhythmic effects following ischemic events (Negretti *et al.*, 2001; Swan *et al.*, 2003). First, PUFAs inhibit the SR Ca^{2+} release mechanism by decreasing the relative time that the RyR remain in the open state, P_o (Swan *et al.*, 2003). PUFA have also been shown to decrease $[Ca^{2+}]_i$, meaning that less cytosolic Ca^{2+} is available

for the SERCA2a pump to bring into the SR, as well as increasing the amount of time it takes for this pump to bring in sufficient Ca^{2+} for the next wave of ventricular polarization (Negretti *et al.*, 2001). In this way, it was predicted that *c*9,*t*11 CLA in the fatty acid form would also decrease Ca^{2+} uptake.

V. MATERIALS AND METHODS

A. Methods for sample preparation

1. Background

It should be noted that the animals used in the previous hemodynamic study were in fact part of another principle study that was designed to examine the effects of CLA parathyroid hormone in relation to bone health (Weiler H, unpublished findings) The principle investigator of this study was interested in identifying isomer-specific (i.e. *c*9,*t*11 or *t*10,*c*12) and form-specific (i.e. fatty acid or triglyceride) effects of dietary CLA, and was also interested in possible gender differences in the respone to CLA supplementation. Although the animals and diets were primarily for use in this bone study, it was deemed that the animals chosen and diets formulated would also be appropriate for a preliminary study that explored cardiac hemodynamic function, particularly since the principle design incorporated differences in isomer effects, form effects and gender effects. To our knowledge, this would be the first study to look at the effects of dietary CLA on cardiac hemodynamic performance.

The following sections describe the animals and diets used for the principle bone study, our supplementary hemodynamic study, and this thesis project.

2. Feeding of animals

Sprague-Dawley rats (30 male and 30 female) were randomized at 3 weeks of age to receive 1 of 5 diets between 4 and 20 weeks of age. Sprague-Dawley rats were chosen for two reasons: first, this animal breed is a common model for bone-related studies; second, Sprague-Dawley rats are aseptically bred and housed, and are therefore generally healthy. The diets were formulated to provide equal quantities of total fat on a weight-for-weight basis. A modified AIN-93 G diet for growth was used throughout the study. The diets were:

1. Control: The AIN-93G diet normally contains 7% (w/w) soybean oil (n-6:n-3 ratio approximately 7:1) (Reeves *et al*, 1997). Since CLA was added to the treatment diets, additional soybean oil was added so that all diets would contain an equal amount of fat. Therefore, the AIN-93G diet was modified such that it contained 8.35% (w/w) soybean oil.

2. AIN-93G diet containing 0.5% (w/w) *c*9,*t*11 CLA as fatty acid and 8.35% (w/w) total fat. Since the single isomer CLA was not 100% pure, additional CLA oil was added to ensure that the diet contained exactly 0.5% *c*9,*t*11 CLA. The isomer content of the CLA oil was 84% *c*9,*t*11 plus 16% *t*10,*c*12 CLA in free fatty acid form.

3. AIN-93G diet containing 0.5% (w/w) *t*10,*c*12 CLA as fatty acid and 8.35% (w/w) total fat. The isomer content of the CLA oil was 84% *t*10,*c*12 plus 16% *c*9,*t*11 CLA in fatty acid form. Since the single isomer CLA was not 100% pure, additional CLA oil was added to ensure that the diet contained exactly 0.5% *t*10,*c*12 CLA.

4. AIN-93G diet containing 0.5% (w/w) *c*9,*t*11 + 0.5% (w/w) *t*10,*c*12 CLA as fatty acid. Since the fatty acid mixed isomer CLA was not 100% pure, adjustment was made such that the diet contained the two isomers at exactly 0.5% (w/w) each. Total fat content of this diet was 8.35% (w/w).

5. AIN-93G diet containing 0.5% (w/w) *c*9,*t*11 + 0.5% (w/w) *t*10,*c*12 CLA in triglyceride form. Since the triglyceride mixed isomer CLA was not 100% pure, adjustment was made such that the diet contained the two isomers at exactly 0.5% (w/w) each. Total fat content of this diet was 8.35% (w/w).

All CLA isomers and mixed isomers were obtained from Lipid Nutrition, a division of Loders Crokklan (Channahon, IL, USA), and were independently analysed for fatty acid composition by the Quality Control Department of Wormerveer (Channahon IL). Table 4 shows diet formulations.

Table 4: Formulations for control and CLA diets [1,2]

Ingredient	Control (AIN-93G)	*c*9,*t*11 Diet	*t*10,*c*12 Diet	Fatty Acid Mix	Triglyceride Mix
Cornstarch[3]	398	398	398	398	398
Casein	200	200	200	200	200
Maltodextrin	132	132	132	132	132
Dextrose	100	100	100	100	100
Soybean oil	83.5	75	76.3	70.1	70.1
***c*9,*t*11 CLA**[4]	-	8.1	-	-	-
***t*10,*c*12 CLA**[4]	-	-	7.2	-	-
CLA fatty acid mix[4]	-	-	-	13.4	-
CLA triglyceride mix[4]	-	-	-	-	13.4
Cellulose	50	50	50	50	50
Mineral mix (AIN-93G-MX)	35	35	35	35	35
Vitamin mix (AIN-93-VX)	10	10	10	10	10
L-cysteine	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
TertButylhydroqinone	0.014	0.014	0.014	0.014	0.014

[1] Total fat in each diet equals 8.35% (w/w). All ingredients listed as grams per kilogram of diet.
[2] All ingredients obtained from Harlan Teklad (Madison, WI, USA) unless otherwise specified.
[3] Obtained from Best Food (Etobicoke, ON, Canada)
[4] Obtained from Lipid Nutrition, a division of Loders Crokklan (Channahon, IL, USA).

At the end of the feeding period, cardiac function was assessed by the *in vivo* catheterization technique as previously described (Tappia *et al.*, 1999). Following hemodynamic assessment, hearts were removed from all animals, separated into LV, right ventrical (RV) and atria, then immediately frozen in liquid nitrogen and stored at -80°C.

B. Methods

1. RNA isolation

Total RNA was isolated from LV tissue using TRIzol reagent (Life Technologies, Burlington, ON). Previously harvested LV tissue was removed from -80°C storage, and kept on ice in a -20°C freezer until ready for use. One sample at a time was removed from frozen storage, cut with scissors and weighed to obtain a 150-200 mg sample. The sample was placed into a mortar, liquid nitrogen added, and the sample crushed to a powder with a pestle. The sample was immediately mixed with 200 µl TRIzol reagent, and using the pestle, stirred to remove tissue from the sides of the mortar. This mixture was poured into 50 ml Corningware tubes, and homogenized with a PowerGen 125 homogenizer (Fisher Scientific) as follows: 20 seconds homogenization followed by 10 seconds rest (repeated 5 times). This process was repeated for all samples, and the remaining LV tissue was returned immediately to -80°C storage. The homogenate in each screwcap tube was divided equally between two microfuge tubes, and 200 µl chloroform added to each tube. The tubes

were then flipped 50 times by hand. Following this the samples were centrifuged at 4°C, 10,000 g for 15 minutes using a Heraeus Centrifuge 28RS (Sepatech). The aqueous phase was removed and transferred to a new microfuge tube. Isopropyl alcohol (500 µl) was added to each tube, inverted by hand 20 times, then allowed to rest at room temperature for 10 minutes. Samples were then centrifuged at 4°C, 10,000 g for 20 minutes to create a pellet. Following centrifugation, the supernatant was poured off, and the pellet was redissolved by adding 1 ml of 75% ethanol and agitated with a pipette. The resuspension was then centrifuged at 4°C, 10,000 g for 10 minutes. The supernatant was poured off, and the inside of the microfuge tubes wiped with a Kimwipe to remove as much remaining liquid as possible without disturbing the RNA pellet. The pellets were dried at room temperature for 20 minutes, then reconstituted by adding 50 µl nuclease-free water and agitating with a pipette (50 times). The resulting solution was heated in an Isotemp 500 Series Laboratory Oven (Fisher Scientific) at 55°C for 10 minutes, then placed on ice.

2. Assessment of RNA concentration

To assess RNA concentration, a zero value was obtained first by using RNAse free water and measuring its concentration spectrophotometrically using a Spectronic 601 Spectrophotometer (Fisher Scientific) at 260λ, then repeating the assessment at 280λ. Following this, 4.4 µl of each RNA solution obtained from RT-PCR was removed by pipette and added to a separate Eppendorff tube containing 996 µl RNAse free water. RNA concentration was

subsequently assessed by spectrophotometry, using the same spectrophotometer, first by measuring at 260λ, then repeating the assessment at 280λ. The concentration was then calculated as the ratio of 260λ:280λ. A concentration of 1.6 μg/μl or higher was considered acceptable. RNA samples were then stored at -80°C until further use.

3. Semi-quantitative RT-PCR

Reverse transcription (RT) was conducted using the Superscript Preamplification System for first strand cDNA synthesis (Life Technology, ON, Canada) as previously described (Meadus WJ, 2003). Temperatures used for PCR were as follows: denaturation at 94°C for 30 seconds, annealing at 62°C for 60 seconds, and extension at 68°C for 120 seconds, with a final extension for 7 min; 25 amplification cycles were used for each individual primer set. For the purpose of normalization of the data, GAPDH primers were used as the housekeeping gene. The PCR products were analyzed by electrophoresis in 2% agarose gels. The intensity of each band was photographed and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada) calculating the intensity as a ratio of a target gene over the housekeeping gene, GAPDH. Primers used for amplification were synthesized as follows:

GAPDH: 5'-TGAAGGTCGGTGTCAACGGAT TTG-3' (forward)

5'-GCATGTCAGATCCACAACGGATAC-3' (reverse)

Genes encoding for Sarcolemmal proteins:

Na^+/Ca^{2+} Exchanger:

5'-TTGGCATCATGGAGGTGAAG-3' (forward)

5'-CCCCACATTCATCATCATCG-3' (reverse)

L-Type Calcium Channel Receptor:

5'-TTCATCTACGCCGTCATTGG-3' (forward)

5'-CGTCCGTACCAAAGCAAACA-3' (reverse)

Genes encoding for Sarcoplasmic Reticulum proteins:

SERCA2a:

5'-GAAACTGTCACTGCCTTTGTGG-3' (forward)

5'-TCGGATCTTGCCAATCTCAGTA-3' (reverse)

Phospholamban:

5'-TTGAAATGCGCTCAGCAAGC-3' (forward)

5'-TGTCAGATCAGCAGCAGACAT-3' (reverse)

Calsequestrin:

5'-TTCCAAGAGGCAGCTGAACA-3' (forward)

5'-TGGGTCAATCCACAAGATGC-3' (reverse)

Ryanodine Receptor:

5'-TTGAAATGCCTCAGCAAGC-3' (forward)

5'-TGCAGATCAGCAGCAGACAT-3' (reverse)

4. Western blot of SERCA2a and PLB proteins

High-molecular-weight markers (Bio-Rad, Hercules, CA, USA) and 20 μg total membrane proteins were separated on 10% SDS-PAGE as previously described (Asemu *et al.,* 2003; Dent *et al.,* 2004). Separated proteins were transferred onto 0.45-μm polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% skim milk and probed with mouse monoclonal primary SERCA2a or PLB (unphosphorylated) antibody (Upstate Biotechnology, NY, USA). Primary SERCA2a or PLB antibodies were diluted in TBS-T (1:2000 according to the manufacturer's instructions). Horseradish peroxidase-labeled anti-mouse IgG (Bio-Rad, CA, USA) was diluted 1:3000 in TBS-T and used as the secondary antibody. SERCA2a and PLB proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Boehringer Mannheim, Laval, PQ). Band intensities of the Western blot were quantified using a CCD camera imaging densitometer (Bio-Rad GS 800). The linearity of the Western blot procedure used for the quantification of SERCA2a and PLB has been previously determined (Asemu *et al.,* 2003). Exposure time used was 5 minutes.

5. SR membrane isolation

The SR membrane was isolated according to the following method. Reagents used included:

1. DTT + PMSF in DMSO: Stock mixture prepared by dissolving 154.2 mg of Dithiothereitol (DTT) and 17.4 mg of phenylmethylsulfonyl fluoride (PMSF) in 10 ml of dimethyl sulfoxide (DMSO).
2. Solutions used included:

i. Solution A: 0.9% saline

ii. Solution B: 10 mM $NaHCO_3$, 5 mM NaN_3, 15 mM Tris-HCL, pH 6.8. 0.5 ml of DTT + PMSF in DMSO was added to 500 mL of solution A and the pH adjusted to 6.8 with drop wise addition of HCl.

iii. Solution C: 0.6 M KCl, 20 mM Tris-HCl, pH 6.8. 0.5 ml of DTT + PMSF in DMSO was added to 500 ml of solution A and the pH adjusted to 6.8 with drop wise addition of HCl.

iv. Solution D: 250 mM sucrose, 10 mM histidine, pH 7.0. 0.25 ml of DTT + PMSF in DMSO was added to 500 ml of prepared solution A and the pH adjusted to 7.0 with drop wise addition of HCl.

Left ventricular tissue was removed from -80°C storage, and placed on ice. LV (whole, no specified weight) was then placed in 10 ml cold Solution A, and chopped with scissors. The aqueous portion was removed by suction, and 10 ml of Solution B was added to the remaining tissue. This was

homogenized at 12,000 rpm with a Polytron homogenizer (Brinkman, Westbury, NY) for 45 seconds (20 seconds homogenization, one minute rest, 25 seconds homogenization). The tubes were balanced using Solution B, then centrifuged at 9,500 g in a Beckman centrifuge at 4°C for 20 minutes. The resultant pellet was discarded, the supernatant balanced with Solution B, followed by centrifugation at 19,000 g for 45 minutes at 4°C. The resulting pellet was suspended by hand in 8 ml of Solution C, the tubes balanced with Solution C and centrifuged at 19,000 g for 45 minutes at 4°C. The resulting pellet was suspended in 8 ml Solution D. All steps were performed in the cold room (0-4°C), and the resulting SR and cytosolic suspensions were stored at -80°C and used later for calcium uptake and release assays. The protein concentration of the SR preparation was determined by the Lowry method (Sapan *et al.*, 1999).

6. Calcium uptake and release

The Ca^{2+} uptake activities of SR membranes were determined by an adaptation of the original procedure of Hawkins *et al* (1994). The various components of the solutions were weighed and combined as follows:

1. Loading buffer: 89.46 g of 1.2 M KCl, 10.165 g of 50 mM $MgCl_2$, 3.25 g 50 of mM Na-azide, plus 118.6 g of 500 mM Tris-maleate.
2. Washing buffer: 1:1 Loading buffer: double-distilled water (DDW).

3. 50 mM of Tris-ATP, pH 6.8 prepared using Tris salt, then 4 mM Tris ATP prepared from the 50 mM Tris ATP.
4. 0.002 g of 250 μM ruthenium red dissolved in 10 ml of DDW.
5. 0.184 g of 100 mM K-Oxalate dissolved in 10 ml of DDW.
6. 0.0038 g of 1 mM EGTA dissolved in 10 mL of DDW, pH 6.8.
7. 0.00147 g of 1mM $CaCl_2$ dissolved in 10 ml of DDW.
8. 1 mM $^{45}CaCl_2$ prepared by adding 10 μL of 2 mCi ^{45}Ca to 10 ml of 1 mM $CaCl_2$.

These reaction cocktails were prepared at room temperature, then stored on ice.

a. Determination of calcium uptake activity

The standard reaction mixture (total volume 250 μl) contained (in mM) 50 Tris-maleate (pH 6.8), 5 NaN_3, 5 ATP, 5 $MgCl_2$, 120 KCl, 5 potassium oxalate, 0.1 EGTA, 0.1 $^{45}CaCl_2$ (12,000 cpm/nmol), and 0.025 ruthenium red. Ruthenium red was added to inhibit Ca^{2+} release channel activity under these conditions. The reaction was initiated by the addition of unphosphorylated SR membranes to the Ca^{2+} uptake reaction mixture. The reaction was terminated after 1 min by filtering a 200 μl aliquot of the reaction mixture. The filters were washed twice with 3 ml washing buffer and promptly dried at 60°C for 1 h. The filters were then placed into scintillation vials, 10 ml of scintillation fluid

was added to each vial, and vials were counted in a beta liquid scintillation counter (Beckman). To measure Ca^{2+} uptake by the SR membranes in the absence of endogenous CaMK activators, namely, Ca^{2+} and endogenous calmodulin, 10 μM of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and 1 mM EGTA were included in the phosphorylation assay medium to inhibit calmodulin, chelate Ca^{2+}, respectively. The exogenous catalytic subunit of PKA was excluded from the phosphorylation assay medium so that Ca^{2+} uptake by the SR membranes could be measured in the absence of PKA activator.

b. Determination of calcium-induced-calcium release activities

Ca^{2+}-release activity of the isolated SR vesicles was measured by a modified procedure as reported previously (Hawkins *et al.*, 1994). SR vesicles were suspended in a reaction mixture with a total volume of 625 μl that included (in mM): 100 KCl, 5 $MgCl_2$, 5 mM potassium oxalate, 5 mM NaN_3, 20 mM Tris-HCL (pH 6.8), and 62.5 μl of 0.5 mg/ml SR. The reaction mixture was then incubated for 45 minutes at room temperature with 10 μM $^{45}CaCl_2$ (20 mCi/L) and 5 mM ATP. Ca^{2+}-induced-Ca^{2+}-release was then carried out through the addition of 1 mM EGTA plus 1 mM $CaCl_2$. The reaction was promptly terminated 15 seconds later using the Millipore filtration technique. The filters were then put in vials and 10 ml of scintillation fluid was added. The vials were then counted in a beta-scintillation counter

(Beckman). The Ca^{2+}-induced Ca^{2+}-release was completely prevented (95% - 97%) by the addition of 20 μM ryanodine to the SR preparations membranes. Uptake and release trials were repeated in triplicate for both male and female samples.

C. Statistical analyses

All values are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc multiple comparison tests. Since male and female samples were analyzed on separate gels (RT-PCR and Western blot products), a two-way ANOVA for diet and gender was deemed inappropriate. Thus, in the current study, male and female results are compared to their respective gender control group. A probability of $P<0.05$ was considered significant. Shapiro-Wilks Tests for Normality were used to confirm normal distribution of data. Where data was not normally distributed, data was log-transformed and one-way ANOVA was repeated. Homogeneity of the data was determined using Levene's Test for Homogeneity of Variance. All statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Using the data for mean and standard error of the mean (SEM) as computed by SAS 9.1, Microcal Origin version 6.5 (Origin Lab Corp., Northampton, MA, USA) was used to create all figures. Appendix 1 shows a sample of the program used for SAS analyses.

VI. RESULTS

A. Effects of CLA isomers on cardiac gene expression

This study investigated the effects of dietary CLA treatment on the expression of six genes involved in cardiac calcium homeostasis through analysis of mRNA levels of left ventricular cardiac tissue in both male and female rats. The results of dietary treatment with *c*9,*t*11 CLA, *t*10,*c*12 CLA, a mixture of both CLA isomers in fatty acid form, or in triglyceride form on the expression of genes encoding for L-type calcium channel, SERCA2a, phospholamban, calsequestrin, Na^+/Ca^{2+}-exchanger and ryanodine receptor proteins are shown in Figures 2 through 7. Representative bands are shown below the figures. As mentioned, between-gender comparison was not possible as PCR and gel electrophoresis for each gene were run separately for male and female samples.

1. Effects of dietary CLA on L-type calcium channel gene expression

As can be seen in Figure 2, only the female groups displayed significant differences in L-type calcium mRNA levels. Specifically, both the *c*9,*t*11 single isomer diet and the fatty acid mixed isomer diet were significantly reduced compared to the *t*10,*c*12, TG mix, and control diets. In the male groups, there were no significant differences observed in L-type Ca^{2+} gene expression for any groups when compared to male control.

2. Effects of dietary CLA on SERCA2a gene expression

Figure 3 depicts the effects of dietary CLA treatment on the expression of SERCA2a. In males, LV SERCA2a was elevated in the *c*9,*t*11 and FA mix compared to *t*10,*c*12, TG mix, and control groups. Of particular significance is the almost 100% elevation in the expression of this gene in male rats fed the fatty acid mixture of isomers, which may indicate a synergistic effect of the two individual isomers, *c*9,*t*11 and *t*10,*c*12. In females, there were no significant differences in SERCA2a mRNA levels for any dietary groups.

3. Effects of dietary CLA on phospholamban gene expression

Figure 4 depicts phospholamban gene expression in male and female rats. In males, only the fatty acid mixed isomer dietary group had significantly reduced mRNA PLB levels compared to the control, *t*10,*c*12 and triglyceride mixed isomer diet groups. In females, only the *t*10,*c*12 diet produced significant changes in PLB mRNA; specifically, PLB mRNA levels in this group were significantly lower than all other female dietary groups, including control.

4. Effects of dietary CLA on calsequestrin gene expression

Similar to the effect of dietary CLA on PLB gene expression, CLA treatment also produced few changes in the expression of genes encoding for the SR storage protein calsequestrin. As shown in Figure 5, there were no significant changes in calsequestrin mRNA in male rats, while in females, only

the *t*10,*c*12 single isomer produced a significant reduction in calsequestrin mRNA levels compared to all other dietary groups.

5. Effects of dietary CLA on Na^+/Ca^{2+}-exchanger gene expression

Figure 6 shows that, in male rats, only the *t*10,*c*12 single isomer produced a significant elevation in Na^+/Ca^{2+}-exchanger mRNA levels compared to all other dietary groups. Conversely, in female rats, the *t*10,*c*12 dietary group had Na^+/Ca^{2+}-exchanger mRNA levels that were significantly lower than the *c*9,*t*11 and control group, but they were not different from either of the mixed isomer dietary groups. Furthermore, both the fatty acid and trigyceride dietary groups had significantly lower Na^+/Ca^{2+}-exchanger mRNA levels compred to the *c*9,*t*11 dietary group.

6. Effects of dietary CLA on ryanodine receptor gene expression

As shown in Figure 7, in male rats, the triglyceride mixed diet resulted in RyR mRNA levels that were significantly reduced in comparison to all other dietary groups, including control. In females, the mRNA levels of RyR were more varied, with the triglyceride mixed isomer group having significantly higher RyR mRNA levels compared to the single isomer groups, and the *t*10,*c*12 single isomer dietary group exhibiting significantly lower mRNA levels compared to both mixed isomer groups as well as control.

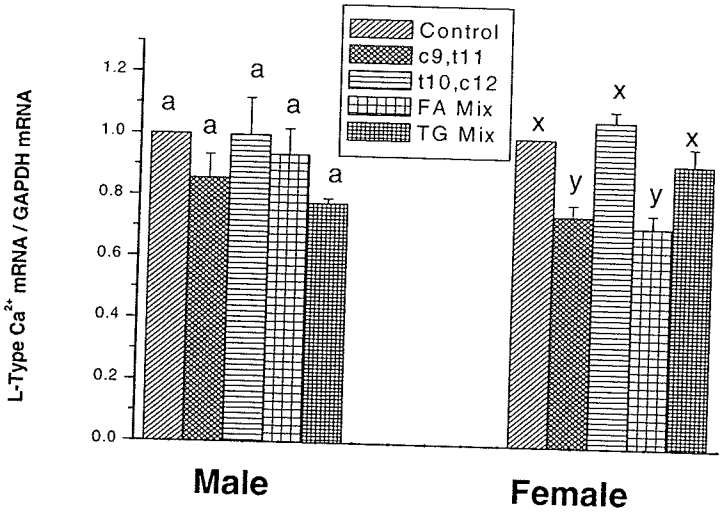
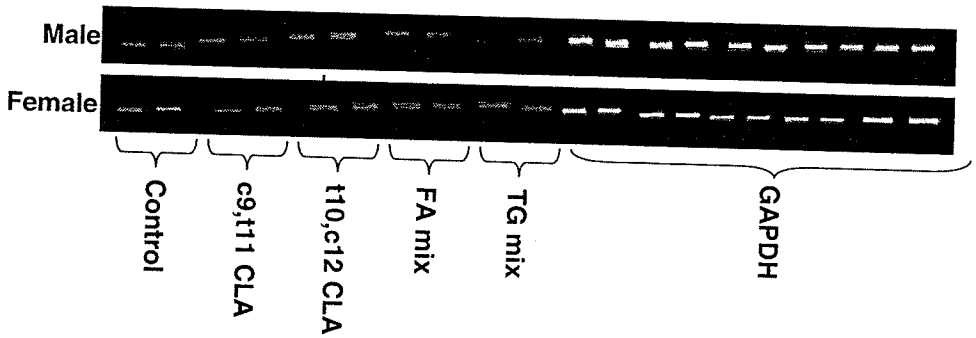


Figure 2. Relative L-Type Ca^{2+} channel mRNA levels

Values from RT-PCR are means ±SEM for male and female, n=6/group . Control mean was set to 1 for each gender. Columns with different letters are significantly different ($p<0.05$) from each other. Representative gel electrophoresis photographs below.



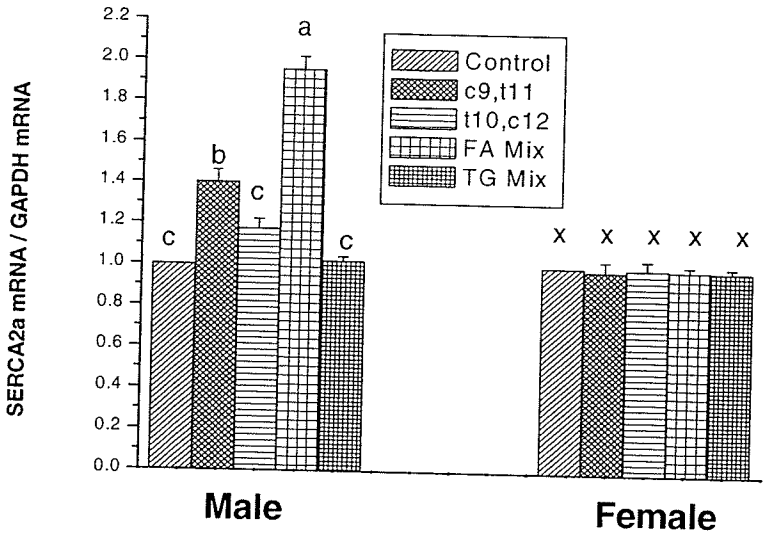
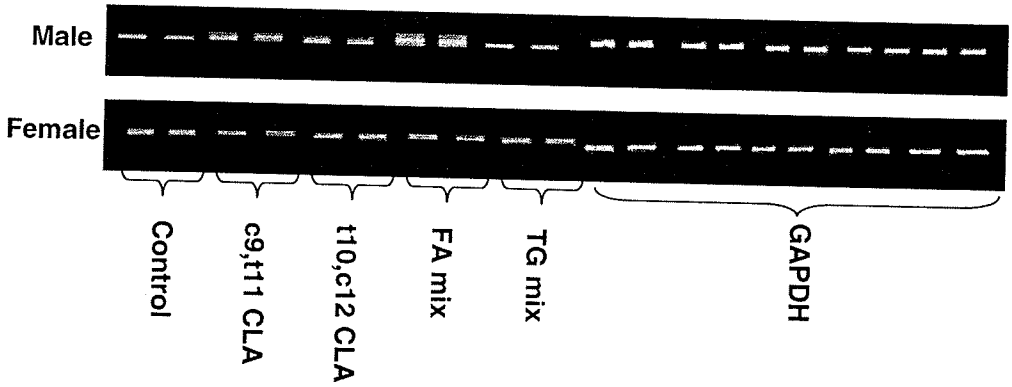


Figure 3. Relative SERCA2a mRNA levels

Values from RT-PCR are means ±SEM for male and female, n=6/group. Control mean was set to 1 for each gender. Within each gender, columns with different letters are significantly different ($p<0.05$) from each other. Representative gel electrophoresis photographs below.



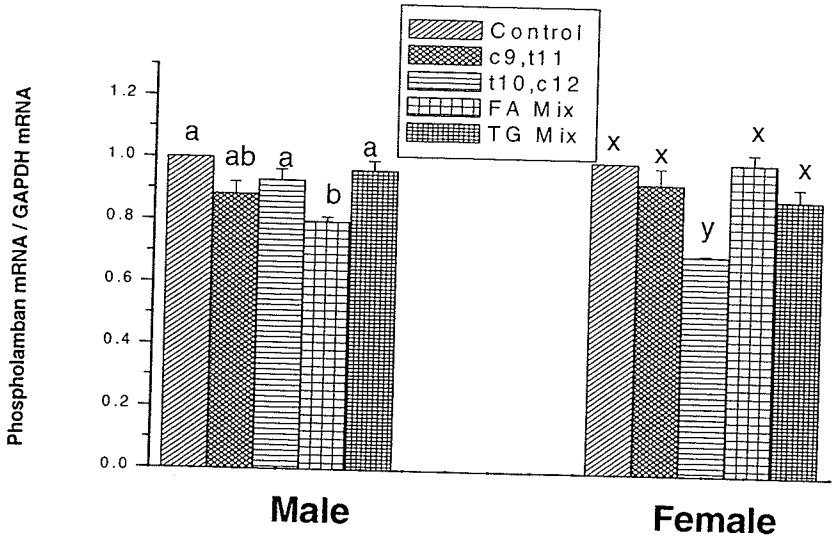
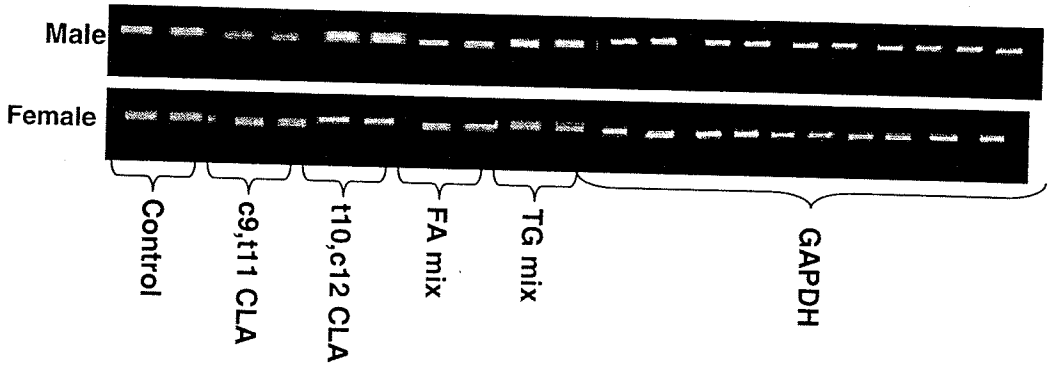


Figure 4. Relative phospholamban mRNA levels

Values from RT-PCR are means ±SEM for male and female, n=6/group. Control mean was set at 1 for each gender. Within a gender, columns with different letters are significantly different ($p<0.05$) from each other. Representative gel electrophoresis photographs below.



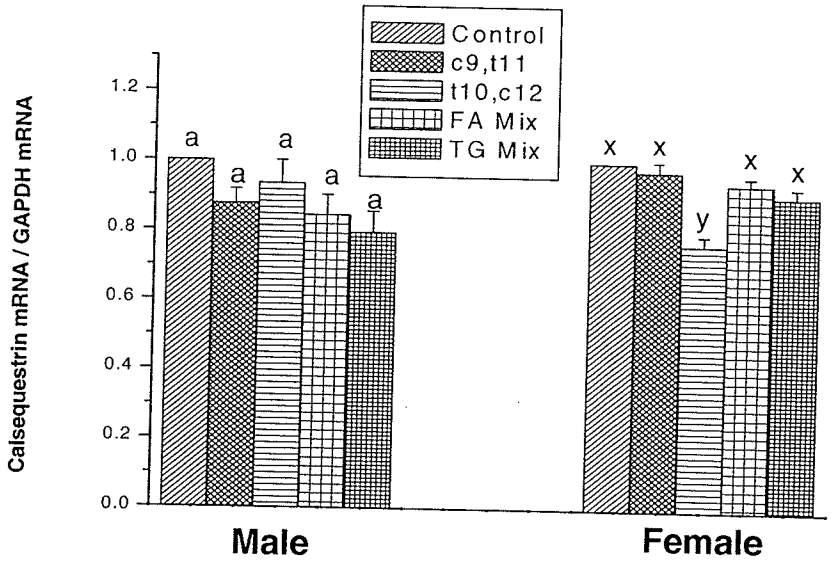
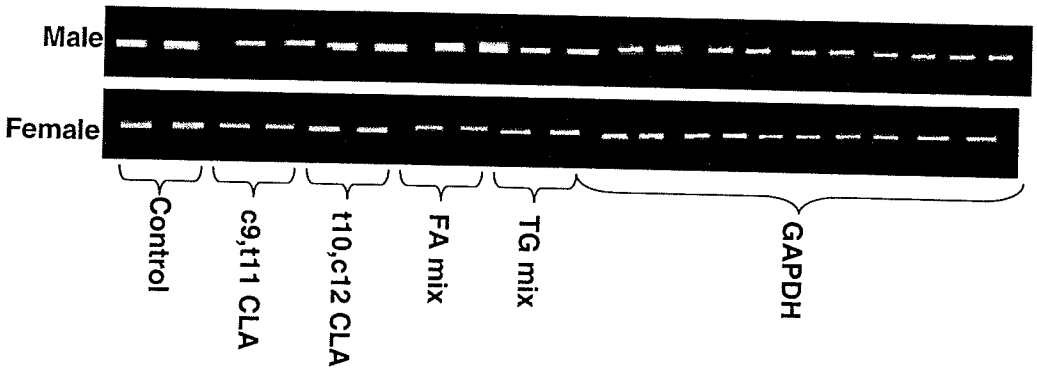


Figure 5. Relative calsequestrin mRNA levels

Values from RT-PCR are means ±SEM for male and female, n=6/group. Control mean was set at 1 for each gender. Within a gender, columns with different letters are significantly different ($p<0.05$) from each other. Representative gel electrophoresis photographs below.



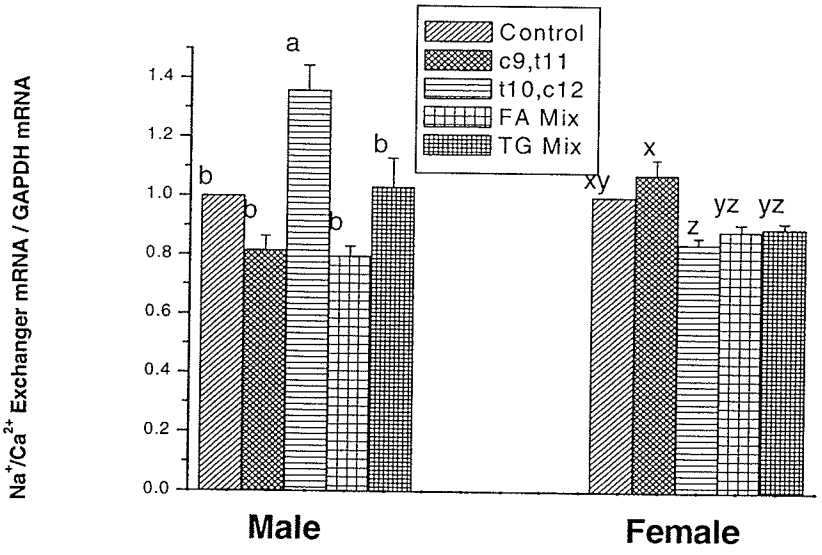
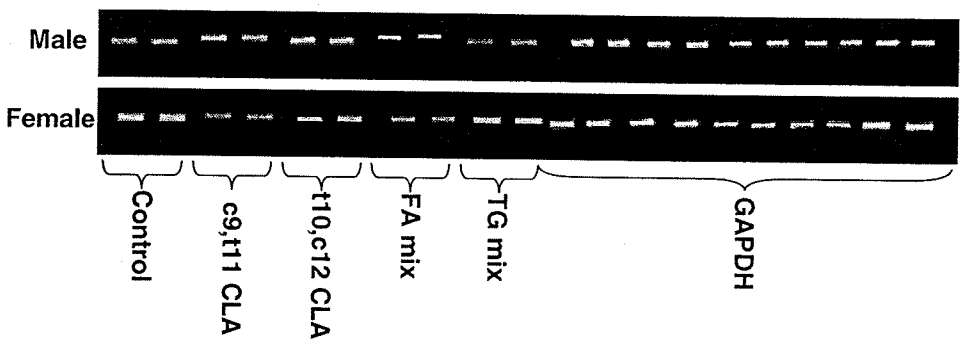


Figure 6. Relative Na^+/Ca^{2+} exchanger mRNA levels

Values from RT-PCR are means ±SEM for male and female, n=6/group. Control mean was set to 1 for each gender. Within a gender, columns with different letters are significantly different ($p<0.05$) from each other. Representative gel electrophoresis photographs below.



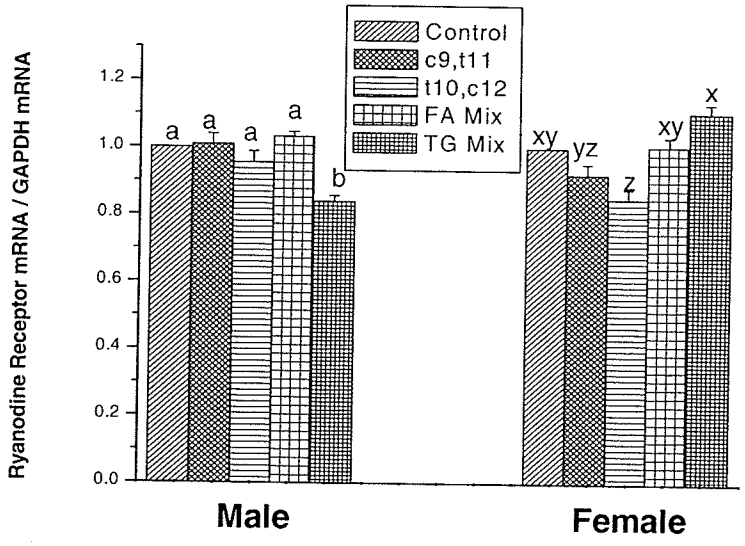
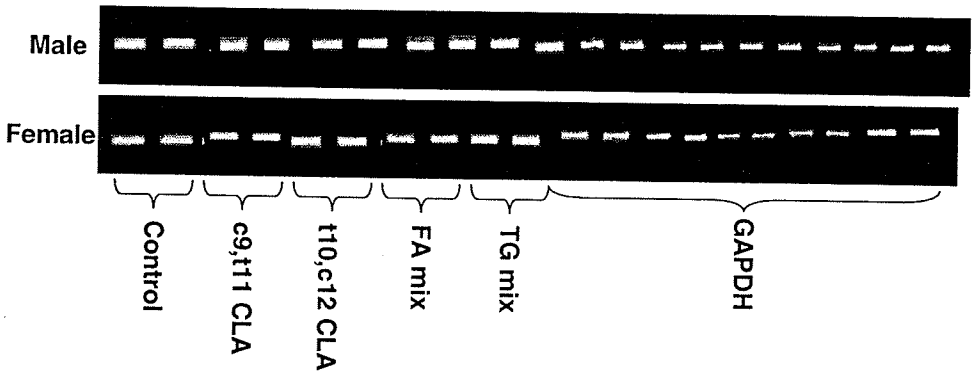


Figure 7. Relative ryanodine receptor mRNA levels

Values from RT-PCR are means ±SEM for male and female, n=6/group. Control mean was set at 1 for each gender. Within a gender, columns with different letters are significantly different ($p<0.05$) from each other. Representative gel electrophoresis photographs below.



7. Possible effects of dietary CLA on cardiac function

Table 5 summarizes the results of the gene expression assays. Assuming that the changes observed for mRNA levels would be translated into similar changes in protein levels, the following observations can be made regarding possible changes to cardiac function:

a. *c*9,*t*11 CLA isomer

In male rats, the *c*9,*t*11 CLA isomer resulted in an elevation in SERCA2a expression with a reduction in PLB expression. This could translate into increased Ca^{2+} uptake and enhanced cardiac relaxation. In the *c*9,*t*11 dietary group in female rats, there was a reduction in L-Type calcium channel, meaning possible decreased stimulation of RyR and therefore decreased contractility.

b. *t*10,*c*12 CLA isomer

In male rats, the *t*10,*c*12 CLA isomer resulted in an elevation in NCX expression which would probably have no significant effect on cardiac function. In female rats, the *t*10,*c*12 CLA isomer produced a significant reduction in PLB expression, meaning possible decreased inhibition of SERCA2a activity and therefore enhanced uptake of Ca^{2+} and consequent enhanced cardiac relaxation. At the same time, the *t*10,*c*12 CLA isomer produced a significant reduction in RyR expression, meaning possible decreased Ca^{2+} release from the SR and therefore decreased cardiac contractility.

c. Fatty acid mixed CLA isomer

In male rats, the fatty acid mixed CLA isomer diet resulted in a significant elevation in SERCA2a expression with a reduction in PLB expression. This could translate into increased Ca^{2+} uptake and enhanced cardiac relaxation. In female rats, there may be decreased Ca^{2+} release by RyR due to reduced expression of L-type channels and therefore decreased RyR stimulation, translating into decreased cardiac contractility.

d. Triglyceride mixed CLA isomer

In male rats, the TG CLA group exhibited reduced RyR expression, which may translate into decreased Ca^{2+} release by the SR, and therefore decreased cardiac contractility. In female rats, the triglyceride mixed CLA isomer diet resulted in a no significant changes in cardiac gene expression, therefore one would assume that no changes in cardiac function would occur.

Table 5: Summary of gene expression results[1]

Males	*c9,t11*	*t10,c12*	Fatty Acid Mix	Triglyceride Mix
L-type Ca^{2+} Channel	↓ 15 %	NC	NC	↓ 22 %
SERCA2a	↑ 40 % *	↑ 17 %	↑ 95 % *	NC
Phospholamban	↓ 12 %	NC	↓ 20 % *	NC
Calsequestrin	↓ 12 %	NC	↓ 16 %	↓ 20 %
Na^{+}/Ca^{2+} Exchanger	↓ 18 %	↑ 36 % *	↓ 20 %	NC
Ryanodine Receptor	NC	NC	NC	↓ 16 % *

Females	*c9,t11*	*t10,c12*	Fatty Acid Mix	Triglyceride Mix
L-type Ca^{2+} Channel	↓ 25 % *	NC	↓ 28 % *	NC
SERCA2a	NC	NC	NC	NC
Phospholamban	NC	↓ 29 % *	NC	↓ 11 %
Calsequestrin	NC	↓ 23 % *	NC	NC
Na^{+}/Ca^{2+} Exchanger	NC	↓ 16 % *	↓ 11 % *	↓ 10 %
Ryanodine Receptor	NC	↓ 15 % *	NC	↑ 11 %

[1] Percentages are calculated from values for fraction of same gender control. ↑ represents an increase in expression (mRNA level), ↓ represents decrease in expression (mRNA level). Changes <10% compared to control depicted as NC = No Change.
* denotes significant difference ($p<0.05$) according to one-way ANOVA and means testing with Tukey's.

B. Protein levels as detected by Western blot analysis

1. Protein levels of SERCA2a

As seen in Figure 8, the level of SERCA2a protein in males was reduced in the *t*10,*c*12 single isomer and fatty acid mix groups compared to control. Of particular note is the single *t*10,*c*12 isomer diet, where SERCA2a protein levels were reduced by almost 70% compared to control. Conversely, the other single isomer diet (i.e. *c*9,*t*11 CLA) produced SERCA2a levels almost double that of control. In females, the effects of dietary CLA treatment on SERCA2a protein levels were notably different, particularly with regard to the *c*9,*t*11 single isomer diet, where SERCA2a levels were reduced by close to 50% compared to control. Unlike the significant reduction observed in male rats fed the *t*10,*c*12 single isomer diet, SERCA2a protein levels were reduced in the *t*10,*c*12 CLA female group compared to trigyceride mix, but not compared with control. In female rats, the fatty acid mixed isomer diet also reduced SERCA2a protein levels by approximately 60% compared to the control group. It is interesting to note that the triglyceride mix showed no significant difference in SERCA2a levels as compared to control in either male or female rats.

2. Protein levels of phospholamban

Figure 9 illustrates Western Blot results for protein levels of phospholamban (unphosphorylated). In male rats, the *c*9,*t*11 CLA treatment

did not affect PLB levels as compared to control. PLB protein in the *t*10,*c*12 CLA dietary group was reduced approximately 28% compared to control, while the two mixed isomer diets (fatty acid and triglyceride mix) had PLB levels that were reduced approximately 80% compared to control. In females, all dietary treatments reduced PLB protein levels significantly compared to control. PLB protein levels were reduced approximately 21% in the *c*9,*t*11 group, approximately 40% in the *t*10,*c*12 CLA and fatty acid groups, and approximately 70% in the triglyceride group. Table 6 summarizes the observed changes in protein levels for SERCA2a and PLB.

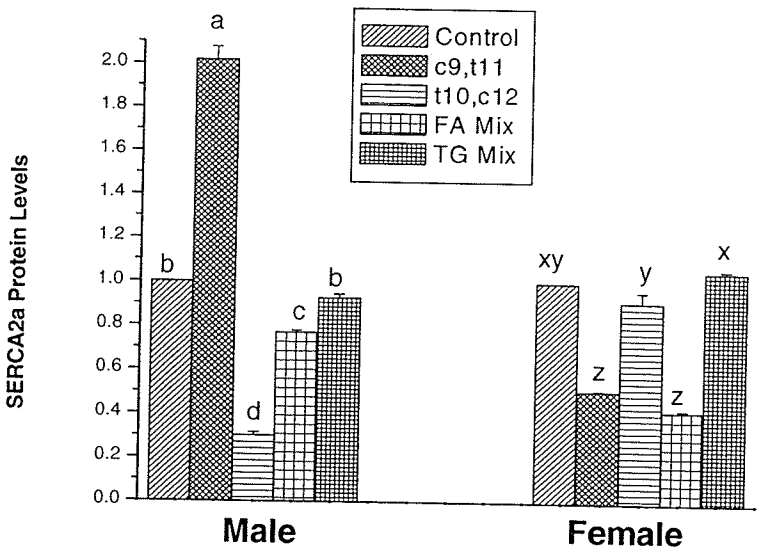
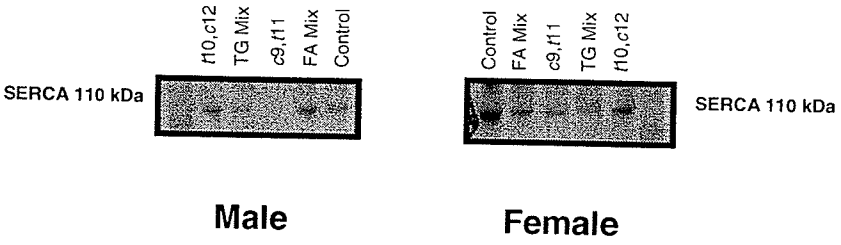


Figure 8. SERCA2a Protein Levels

Values for Western blot analysis are means ±SEM for male and female, n=6/group. Mean for Control was set at 1 for each gender. Within a gender, columns with different letters are significantly different ($p<0.05$) from each other. Representative fluorescence detection photographs are shown below.



Note: Order for Western blot lanes differ from figure. Order for Western blot SERCA2a protein level Male: *t*10,*c*12 isomer, TG Mix, *c*9,*t*11 isomer, FA mix, Control. Order for Western Blot SERCA2a protein level Female: Control, FA mix, *c*9,*t*11 isomer, TG Mix, *t*10,*c*12 isomer.

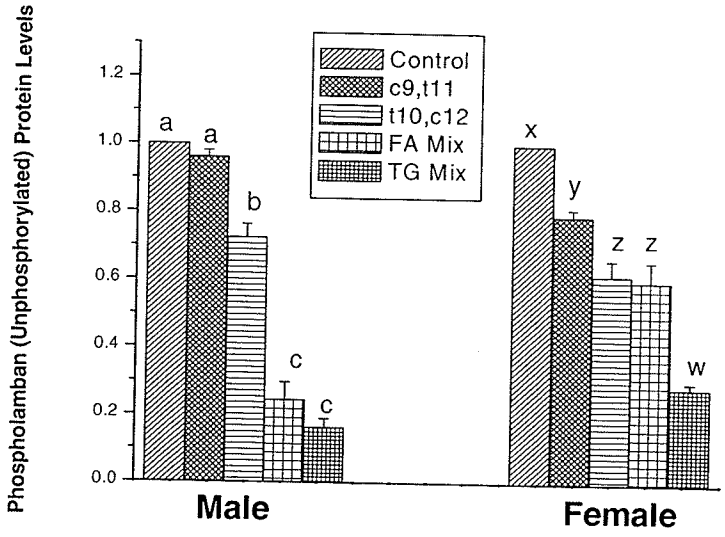
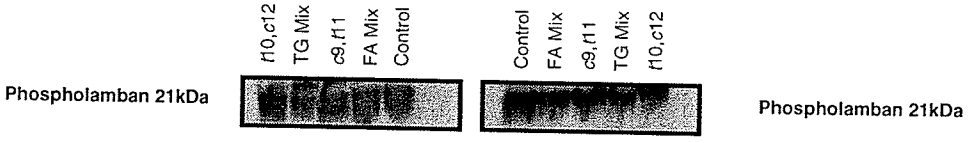


Figure 9. Phospholamban Protein Levels

Values for Western blot analysis are means ±SEM for male and female, n=6/group. Mean for Control was set at 1 for each gender. Within a gender, columns with different letters are significantly different ($p<0.05$) from each other. Representative fluorescence detection photographs are shown below.



Note: Order for Western Blot lanes differ from figure. Order for Western blot phospholamban protein level Male: *t*10,*c*12 isomer, TG Mix, *c*9,*t*11 isomer, FA mix, Control. Order for Western Blot Phospholamban protein level Female: Control, FA mix, *c*9,*t*11 isomer, TG Mix, *t*10,*c*12 isomer

Table 6: Summary of changes observed in SERCA2a and phospholamban protein levels by Western Blot analysis[1]

Males	*c9,t11*	*t10,c12*	Fatty Acid Mix	Triglyceride Mix
SERCA2a	↑ 102 % *	↓ 60 % *	↓ 23 % *	NC
Phospholamban	NC	↓ 28 % *	↓ 76 % *	↓ 84 % *

Females	*c9,t11*	*t10,c12*	Fatty Acid Mix	Triglyceride Mix
SERCA2a	↓ 49 % *	NC	↓ 58 % *	NC
Phospholamban	↓ 21 % *	↓ 38 % *	↓ 40 % *	↓ 71 % *

[1] Percentages are obtained from values calculated as fraction of same-gender control. ↑ denotes an increase in protein level as compared to same-gender control, ↓ denotes a decrease in protein level as compared to same-gender control. NC denotes no change to protein level compared to same-gender control. * denotes significant difference ($p<0.05$) compared to control as assessed by one-way ANOVA and means testing by Tukey's.

C. Calcium uptake and release activity

Calcium uptake by SR vesicles isolated from left ventricular tissue of male and female rats fed different CLA-enriched diets was studied by an adaptation of the method established by Hawkins *et al* (1994), as described in the Materials and Methods section. As mentioned, all trials were repeated in triplicate. However, there was large variation in values for uptake and release trials, and it as was not possible (due to unforeseen circumstances) to perform further trials, thus one-way ANOVA was not applied to this data set and significance at $p<0.05$ was not established. Rather, means of trials ± SEM were calculated using Microcal Origin version 6.5 (Origin Lab Corp., Northampton, MA, USA), and presented in tabular form. Table 7 shows the results for uptake and release trials for male and female rats.

1. Calcium uptake and release activities in male rats

Calcium uptake was not significantly changed in any male CLA treatment groups when compared to control values. However, calcium release activities were depressed in all dietary treatment groups for male rats. Calcium release activities correlate with systolic pressure measurements and maximum pressure development ($+dP/dt_{max}$) which were decreased for most treatment groups in male rats. This is not surprising, since calcium release is associated with ventricular contraction (SP and $+dP/dt_{max}$ hemodynamic measurements).

2. Calcium uptake and release activities in female rats

Unlike male rat data, where calcium uptake activities in CLA-treated groups were unchanged compared to control, the data for female dietary groups showed some other differences. Specifically, the triglyceride mix produced a reduction in uptake activity. In females, calcium release was more markedly changed, with a reduction in the *t*10,*c*12 CLA dietary group, a matched reduction in the *c*9,*t*11 CLA group, and an even more marked reduction in release activity in the triglyceride dietary group. It is interesting to note that, unlike the hemodynamic data seen in male groups, hemodynamic data for females does not correlate as closely with uptake and release activity results, particularly in the triglyceride mix diet, where the most substantial reduction were observed for both uptake and release activities, yet the hemodynamic changes were either mild (i.e. <10% decrease in DP, +dP/dt and –dP/dt) or unchanged when compared to control (i.e. no change in SP).

Table 7: Ca^{2+} uptake and release data for male and female rats[1,2,]

Male	Control	*c*9,*t*11	*t*10,*c*12	Fatty acid mix	Triglyceride mix
Ca^{2+}-uptake, $nmol \cdot mg^{-1} \cdot 2\ min^{-1}$	134 ± 16.0	97 ± 11.6	125 ± 23.4	105 ± 44.5	103 ± 37.5
Ca^{2+}-release, $nmol \cdot mg^{-1} \cdot 2\ min^{-1}$	15.0 ± 0.6	5.0 ± 0.3	8.0 ± 0.1	11.0 ± 3.5	8.3 ± 0.5
Female					
Ca^{2+}-uptake, $nmol \cdot mg^{-1} \cdot 2\ min^{-1}$	77 ± 19.2	76 ± 31.2	68 ± 28.8	77 ± 5.3	50 ± 7.8
Ca^{2+}-release, $nmol \cdot mg^{-1} \cdot 2\ min^{-1}$	4.4 ± 1.8	2.7 ± 1.7	2.7 ± 1.9	4.1 ± 0.7	0.5 ± 0.1

[1] For each group, n=3. Each trial performed in triplicate. Trials performed by Andrea Babick, Institute of Cardiovascular Sciences, St. Boniface Research Centre, Winnipeg, Manitoba, Canada.
[2] Data represented in table are means ±SEM. Significance was not established for this data.

VII. DISCUSSION

To our knowledge, this is the first study to examine the effects of dietary CLA isomer and form on the expression of key genes coding for sarcolemmal and sarcoplasmic reticulum proteins involved in cardiac calcium homeostasis. Our previous study demonstrated that CLA had direct cardiac effects as evidenced by changes in hemodynamic parameters (Tappia *et al.*, 2006). In this present study, it has been demonstrated that dietary CLA affects the expression of genes encoding proteins involved in cardiac calcium homeostasis, particularly SERCA2a in male rats. Furthermore, our results indicate that these effects are isomer specific, influenced by the form of CLA (i.e. fatty acid or triglyceride), and exhibit gender differences. Our results also show that dietary CLA had an effect on protein levels of SERCA2a and phospholamban, with a tendency to decrease protein levels relative to control in both male and female rats. Additionally, dietary CLA supplementation tended to decrease sarcoplasmic reticulum calcium release.

A. Effects of CLA as single isomers and fatty acid versus triglyceride

This study examined the effects of the two most biologically active CLA isomers, *c*9,*t*11 and *t*10,*c*12 CLA. It was apparent from the hemodynamic measurements in our initial CLA study that these two isomers had different effects on cardiac performance (i.e. in male rats *c*9,*t*11 CLA decreased heart rate and *t*10,*c*12 CLA increased heart rate, while in female rats,

*c*9,*t*11 CLA increased heart rate but *t*10,*c*12 CLA had no effect), and thus differences in single isomer effects on cardiac gene expression were also anticipated. As shown in the Results section of this thesis, the type of isomer did have an effect on cardiac gene expression. Since these effects are described fully in the Results section, they will not be further discussed here.

In addition to the isomer examined, however, we were also interested in the form of CLA used in the experimental diets. There were two reasons for this: 1) previous research with other LCPUFA has demonstrated that only the fatty acid form has an effect on gene expression (Pegorier *et al.*, 2004); and 2) while CLA from food sources is predominantly delivered in the triglyceride form, CLA in commercially available supplements is in fatty acid form (Nagao and Yanagita, 2005). Thus, if CLA does have cardiac effects that are form-dependent, it is important to determine the form that has the beneficial/detrimental effect. Our expermiments established that the form of CLA consumed in male and female rats had little difference in cardiac gene expression, but clear differences in effect were seen on protein levels, (See Table 6 for summary of these results).

The results of this study demonstrated that the cardiac effects of CLA are both isomer- and form-dependent. Nonetheless, although both triglyceride and fatty acid forms of CLA are evidently capable of getting CLA isomers incorporated into cardiac cells where they have an effect at the nuclear transcription level, our results also suggest that gender is more important in

determining the cardiac effects of CLA than are either isomer or form of CLA. This interpretation is supported by the clear differences in cardiac gene expression in response to dietary CLA in male and female rats (Figures 2-7), in protein levels of SERCA2a and PLB (Figures 8-9), and in calcium uptake and release (Table 7).

B. CLA effects on cardiac gene expression

Dietary fat is an essential macronutrient in the diets of all animals, and it provides a source of energy and hydrophobic components for biomolecules (Jump and Clarke, 1996). Beyond its importance as a macronutrient, dietary fat has recently gained prominence for its role in regulating gene expression. The current study provides evidence that dietary CLA affects the expression of several genes that encode proteins involved in cardiac calcium homeostasis, and demonstrates that these effects are different between genders. In male rats, the most striking changes were observed in the effect of the mixed isomer CLA in fatty acid form on the mRNA levels of SERCA2a, where levels almost doubled, and in the effect of the *t*10,*c*12 single isomer on NCX, which showed an elevation of almost 40%. In female rats, the most notable changes in cardiac gene expression occurred in the *t*10,*c*12 CLA group, where PLB mRNA levels were reduced approximately 30%, and CSQ mRNA levels were reduced by approximately 20%. Also, in female rats, L-type calcium channel

mRNA levels were reduced by almost 30% in both the *c*9,*t*11 CLA and fatty acid mixed isomer dietary groups.

Research has demonstrated that PUFAs can affect gene expression through various mechanisms, including changes in membrane composition (Clarke and Jump, 1994; Jump *et al.*, 2005), eicosanoid production (Sampath and Ntambi, 2005), and changes to intracellular calcium levels (Xiao *et al.*, 1997; Honen *et al.*, 2003). Additionally, PUFAs and their various metabolites can act at the nuclear level, working in conjunction with specific nuclear receptors and transcription factors to affect the transcription of a number of genes (Jump and Clarke,1999; Pégorier *et al.*, 2004). Several of these nuclear receptors are present in cardiac myocytes, including the fatty acid sensitive PPARs α, -β and -γ (Braissant *et al.*, 1996; Gilde *et al.*, 2003), and the RXR (Clarke and Jump, 1996; Kahn and Vanden Heuvel, 2003). It is conceivable that dietary CLAs act as ligands of the PPARs present in cardiac cells, allowing them to dimerize with RXR and bind to DNA sequences on the cardiac genes studied, thereby affecting transcription of those target genes. This process has been described previously for n-3 and n-6 fatty acids, which have been shown to activate all three PPAR isoforms (Sampath and Ntambi, 2004; Tai *et al.*, 2005). More importantly, it has been shown that various PUFAs differ in their affinities for these receptors; this might explain the differences in gene expression observed in our study, whereby CLA as a dietary PUFA effected changes in gene expression that differed between

isomers as well as between fatty acid and triglyceride forms. The different CLA isomers used in our study may also vary in their affinities for the various cardiac PPARs and RXRs, and thus they had different effects on transcription of the various genes studied.

CLA has also been shown to be a potent ligand for PPARα (Vanden Heuvel, 2004), a nuclear receptor involved in the regulation of lipid transport proteins, including fatty acid binding protein and acyl-CoA binding protein, as well as affecting genes involved in fatty acid transport. It is possible that CLA may also have an indirect effect on cardiac gene expression by altering the cellular transport of other fatty acids important in the expression of the cardiac genes studied. As activators of PPARβ, CLA isomers may also indirectly affect cardiac gene expression by altering the myocyte lipid environment through changes to cellular lipid metabolism.

Researchers have reported that downregulation of gene expression by fatty acids is restricted to fatty acids having >18 carbons and at least two double bonds (Clarke *et al.,* 1997), whereas upregulation of gene expression is independent of the degree of saturation of the carbon chain (Pegorier *et al.,* 2004). CLA contains 18 carbons and 2 double bonds. It should be noted that although much remains to be learned about the specific metabolism of CLA *in vivo*, research has shown that CLA undergoes metabolic conversion by elongation, desaturation and oxidation (Brugengraber *et al.,* 1978; McGarry and Foster, 1980; Neat *et al.,* 1980; Nilsson *et al.,* 1986; Flatmark *et al.,*

1988; Fitzpatrick and Murphy, 1989; Karara *et al.*, 1989; Oliw, 1994; Goetzl *et al.*, 1995; Jump *et al.*, 1996; DuBois *et al.*, 1998; Leneuville *et al.*, 1995; Luthria *et al.*, 1996; Makita *et al.*, 1996). It is possible, therefore, that the metabolites of dietary CLA (i.e. their acyl-CoA derivatives) are responsible for the changes in gene expression observed in the current study.

C. CLA effects on protein content of SERCA2a and phospholamban

SERCA2a plays a crucial role in calcium cycling and the beat-to-beat function of the heart (Babu and Persiamy, 2005). Cardiac muscle contraction is initiated when Ca^{2+} enters the cell via the L-type Ca^{2+} channels and triggers the release of a much larger amount of Ca^{2+} from the SR via the ryanodine receptors. The free cytosolic Ca^{2+} concentration determines the extent of muscle activation and regulates force development (i.e. +dP/dt). SERCA2a pumps Ca^{2+} back into the SR and is therefore responsible for cardiac muscle relaxation and for replenishing the Ca^{2+} stores needed for the next contraction (Schwinger *et al.*, 1995; Dutta *et al.*, 2002; Babu and Persiamy, 2005). SERCA pump activity is regulated by the small phosphoprotein phospholamban, which, when in its unphosphorylated state, lowers the affinity of SERCA for Ca^{2+} (Simmermann and Jones, 1998). The SERCA pump is a transmembrane protein belonging to a family of proteins encoded by three genes – SERCA1, SERCA2 and SERCA3, each with two isoforms, *a* and *b*

(Arai *et al.*, 1994). SERCA2a is the primary isoform expressed in the heart, with lesser amounts of SERCA1a expressed as well (Wu *et al.*, 1995). Although expression of SERCA1a was not examined in the current study, expression of SERCA2a was studied. Results of this study showed significant elevation in SERCA2a expression in male rats the fed *c*9,*t*11single isomer diet (approximately 40% elevation), and mixed isomers in fatty acid form (approximately 95% elevation). In females, one-way ANOVA revealed no significant changes in SERCA2a expression as the result of dietary CLA treatment.

As mentioned, PLB is a negative regulator of SERCA2a pump activity (Li *et al.*, 2005). Thus, a decrease in PLB gene expression may translate into increased SR SERCA2a pump activity, more efficient uptake of Ca^{2+} into the SR, and conceivably, improved LV contractile performance, particularly if decreased PLB is accompanied by increased SERCA2a levels. Analyses in this study show that in male rats, SERCA2a mRNA levels were elevated significantly in the *c*9,*t*11 CLA and fatty acid mixed diet groups when compared to male control (Figure 3). Examination of PLB mRNA in males revealed a reduction in the fatty acid group compared to control (Figure 4). It is possible, therefore, that in male rats, treatment with *c*9,*t*11 CLA and fatty acid mixed isomer CLA will increase LV contractile performance. Conversely, female rats had no significant changes in SERCA2a gene expression in any of the dietary treatment groups, and only the *t*10,*c*12 single

isomer diet resulted in reduced PLB gene expression (29% reduction). Thus, in female rats, it is possible that a slight improvement in LV contractile function may occur with dietary *t*10,*c*12 CLA.

However, it must be noted that elevation or reduction in gene expression (i.e. mRNA levels) do not necessarily translate into functional changes of the proteins for which the genes encode, as post-transcriptional events may circumvent the effects of gene expression changes (Backs and Olson, 2006). For instance, an increased SERCA2a gene expression may not result in increased levels of SERCA2a protein (Fu *et al.*, 2004; Lehrmann *et al.*, 2002; Menon *et al.*, 2005). Therefore, in order to assess whether the changes in the expression of the SERCA2a and PLB genes observed in this project resulted in changes in the amount of SERCA2a and PLB proteins, Western blot analysis was performed to assess SERCA2a and PLB (unphosphorylated) protein levels.

In comparing the results of the gene expression assays to those of the Western blot analyses, it is clear that changes in gene expression do not correlate with protein levels of SERCA2a or PLB. In males, the most striking discrepancies with regard to SERCA2a are seen in the single isomer *t*10,*c*12 CLA dietary group, where gene expression was unchanged (Figure 3), but protein level of SERCA2a was reduced by almost 60% (Figure 8), and in the mixed isomer fatty acid diet, where gene expression was elevated almost 90% (Figure 3), but protein levels were found to be reduced approximately 20%

compared to control (Figure 8). In female rats, considerable dispcrepancies were also seen. For instance, while there were no significant changes to SERCA2a gene expression in any of the dietary groups as compared to female control (Figure 3), Western blotting showed a significant reduction in SERCA2a protein level in the *c*9,*t*11 CLA and fatty acid mixed isomer dietary groups (approximately 50% and 60%, respectively, Figure 8).

Substantial differences were also noted between PLB gene expression and PLB protein levels. In male rats, RT-PCR analysis showed that only the fatty acid mixed isomer dietary group led to a a significant reduction in PLB gene expression (approximately 20%, Figure 4), but analysis of PLB protein levels showed significant reduction in the *t*10,*c*12 CLA, fatty acid mixed isomer and triglyceride mixed isomer dietary groups (approximately 30%, 75% and 85%, respectively, Figure 9). As mentioned, in female rats only the *t*10,*c*12 single isomer diet resulted in significant changes in PLB gene expression (approximate 29% reduction, Figure 4). However, Western blot analysis demonstrated a significant reduction in PLB protein levels for all dietary groups compared to control (Figure 9).

While we cannot fully explain the reason for these discrepancies, a review of existing literature offers some interesting data that may shed some light on this inconsistency. In two transgenic studies of independent mouse lines, SERCA2a was overexpressed 1.2-fold (He *et al.,* 1997) and 1.5-fold (Baker *et al.,* 1998). The researchers reported that despite much higher mRNA

levels for SERCA2a (2.6-fold and 8-fold, respectively), the increase in SERCA2a protein level was only modestly elevated above normal endogenous expression. Although there is as yet no concrete explanation for this phenomenon, it has been speculated that: 1) there may be competition between the exogenous and the endogenous protein, and 2) there are powerful post-transcriptional mechanisms working to maintain a level of SERCA within a specific physiological range (Periasamy and Huke, 2001). Another study examined transgenic mice expressing SERCA1a (Ji *et al.*, 1999). These researchers found that overexpression of this pump increased SERCA levels 2.5-fold, and hemodynamic parameters were enhanced, but only slightly. Furthermore, the expression of SERCA1a led to a 50% downregulation of the SERCA2a pump, suggesting a competition of SERCA2a and 1a for functional sites in the SR.

Schwinger *et al* (1995) examined protein levels of SERCA2a and PLB in patients with dilated cardiomyopathy. Their analyses indicate that protein levels may be regulated independent of the encoding mRNA levels, and therefore mRNA levels cannot be assumed to be a certain predictor of protein content or even of function. According to Schwinger *et al* (1995) these differences may be related to mRNA processing, mRNA translation, posttranslational modifications, and rates of protein synthesis and degradation. Clarke and Jump (1994) demonstrated that dietary PUFA can inhibit transcription of genes, possibly by regulating the phosphorylation state of

specific transcription factors. Although it is not possible to definitively explain the discrepancy observed between gene expression of SERCA2a and PLB and their respective protein levels, it is possible that dietary CLA acted by one or more of the mechanisms outlined above and thus affected protein levels of both SERCA2a and PLB.

D. Effect of CLA on SR Ca^{2+} uptake and release

The final goal of this study was to assess the functional changes, if any, that resulted from dietary CLA treatment. Just as changes in gene expression may not result in matching changes in levels of the protein for which that gene encodes, it is also possible that changes in gene expression and/or protein levels may not equate to increased functionality of a particular protein. This could be due to post-translational effects, where proteins may be produced, but where post-transcriptional and/or post-translational modifications may occur that render the protein non-functional (Bendig, 1988; Blanchong *et al.*, 2001; Kaufman, 1990).

As mentioned, the sarco(endo)plasmic reticulum Ca^{2+}ATPase pump plays a pivotal role in calcium cycling and the beat-to-beat function of the heart (Babu and Persiamy, 2005). Ca^{2+} uptake is regulated by several factors, but essentially it is controlled mainly by SERCA2a. Conversely, Ca^{2+} release is controlled by the ryanodine receptor. This study examined the activity of Ca^{2+} uptake and release using an adaptation of Hawkins *et al.* (1994).

Triplicate trials revealed a trend for all forms of dietary CLA to reduce Ca^{2+} release in male rats, and either a decrease or no change in Ca^{2+} uptake and Ca^{2+} release in female rats. Several observations from studies of dietary PUFAs may explain the results obtained in our study. (The majority of these studies have looked at the antiarrhythmic effects of PUFAs in Ca^{2+}-overloaded hearts, but their findings are also germaine to our study). Negretti *et al.* (2000) looked at the inhibition of SR function by PUFAs in rat ventricular muscle. Among their key findings were the following: 1) PUFAs had a negative inotropic effect, due entirely to inhibition of the L-type calcium current. This translates to smaller amounts of Ca^{2+} entering the myocyte to trigger Ca^{2+}-induced Ca^{2+} release from the RyR; 2) PUFAs reduce the availability of calcium for uptake; 3) PUFAs inhibit the release mechanism of the SR (i.e. RyR release of Ca^{2+}), and 4) PUFAs increase SR content of Ca^{2+}. This would mean that, with more Ca^{2+} held in the SR, less would be made available for Ca^{2+} release, and as a result, less would also be available for the SERCA2a pump to pull back into the SR following ventricular systole (hence a decrease in Ca^{2+} uptake and/or Ca^{2+} release). In a more recent study, Swan *et al.* (2003) demonstrated that the LCPUFA eicosapentanoic acid caused a reduction in P_0, the RyR open probability (i.e. eicosapentanoic acid increased the mean closed time of RyR channels and reduced mean opened times of the channels). Logically, if the channels are open for shorter periods of time, measurements of Ca^{2+} release would show that this function is decreased. Swan *et al.* (2003) also postulates

that PUFAs may also interact more locally with the RyR channel complex itself to cause a reduction in the sensitivity of the complex to cytosolic Ca^{2+} levels produced by the L-type calcium channels.

It is true that the mechanisms by which PUFAs reduce excitability of cardiac cells and inhibit Ca^{2+} release are poorly understood, and it is currently unknown whether PUFAs interact directly and selectively with certain channels to modify function, whether they change the properties of cell membranes and indirectly modify channel behaviour, or whether they activate intracellular pathways to produce the effects on SR Ca^{2+} uptake and release. It is possible that the inhibitory effect on SR Ca^{2+} uptake and Ca^{2+} release by CLA observed in our study could have been produced by more than one mechanism.

E. CLA: Potential therapeutic agent for heart disease?

Cardiovascular disease accounts for the death of more Canadians than any other disease (Heart and Stroke Foundation, 2006). In 2002, the latest year for which Statistics Canada has data, cardiovascular disease accounted for 74,626 Canadian deaths (Statistics Canada, 2004). During the past five years, death rates from heart failure have more than doubled; currently 500,000 individuals in Canada are living with heart failure, and 50,000 more people will be diagnosed with it every year (Heart and Stroke Foundation, 2006). Heart failure is difficult to define because it more than simply an inability of

the heart to pump blood; rather, it is the final common pathway for numerous cellular and molecular defects caused by many instigating factors (Dhalla *et al.*, 2000; Cox *et al.*, 2005).

Recent progress in molecular cardiology makes it possible to envision a new therapeutic approach to heart failure, particularly through targeting key molecules involved in intracellular Ca^{2+} handling, including SERCA2a, RyR and PLB. Heart failure is an attractive candidate for gene therapy, since a number of protein targets have been identified as defective or functionally impaired during progression to end-stage heart failure (Hajjar *et al.*, 1997). A number of recent studies have focused on restoring SERCA2a pump activity, as a decrease in SERCA2a expression and/or activity seems to be a major defect that is pivotal in the impaired function of the failing heart (Schwinger *et al.*, 1995; Dutta *et al.*, 2002; Lowes *et al.*, 2002).

One of the initial hypotheses of this study was that dietary CLA would increase the expression of several of the genes encoding for proteins involved in cardiac calcium homeostasis, particularly SERCA2a. This research has demonstrated that dietary CLA does have an effect on the expression of these genes. However, the changes observed in the expression of these cardiac genes do not translate into corresponding changes in protein levels, nor do they correlate directly with analyses of SR Ca^{2+} uptake and release activities. Thus, even though significant increases were observed for SERCA2a expression in male rats fed the *c*9,*t*11 single CLA isomer and the fatty acid mixture of CLA

isomers, these changes did not result in identifiable functional increases in SERCA2a protein levels. In female rats, none of the dietary CLA treatments had any discernable effect on SERCA2a expression, but SERCA2a protein was reduced in the *c*9,*t*11 CLA and fatty acid mix groups. Nonetheless, it was evident that CLA may have antiarrhythmic effects similar to those observed in the LCPUFA eicosapentanoic acid, as evidenced by both the hemodynamic measurements in our previous study (i.e. decreased inotropic response in male rats fed *c*9,*t*11, FA mix and TG mix), as well as the decreased Ca^{2+} release observed in all treatment groups in males rats, and in the *c*9,*t*11 CLA, *t*10,*c*12 CLA, and TG mix groups in female rats. Perhaps then, CLA has potential to be an effective agent for the arrhythmic problems common to post-ischemic events, rather than the potential to be an accessory agent for gene therapy in heart failure.

VIII. CONCLUSIONS

1. CLA affects expression of genes encoding proteins responsible for cardiac calcium homeostasis in both male and female rats. The effects of CLA on cardiac gene expression differ between genders.
2. The changes to gene expression are isomer specific and appear to be dependent upon the form of CLA, i.e. fatty acid vs. triglyceride.
3. SERCA2a and phospholamban gene expression did not correlate with SERCA2a or phospholamban protein levels in either male or female rats, suggesting that post-transcriptional or translational changes are occurring in response to dietary CLA.
4. While *c*9,*t*11 CLA, *t*10,*c*12 CLA and the fatty acid mixture of CLA significantly increased the expression of SERCA2a, further studies with an appropriate model are required to determine if CLA might serve as an adjunct agent to gene-therapy in the treatment of heart failure.
5. Supplementation with all forms of dietary CLA depressed calcium release activities in male rats, but only the *c*9,*t*11 CLA, *t*10,*c*12 CLA and triglyceride mix reduced calcium release in female rats.
6. It is possible that CLA may serve as an anti-arrhythmic agent similar to other LCPUFA, and thus may have a role in the treatment of post-ischemic arrhythmic events.

IX. SUMMARY OF MAJOR FINDINGS

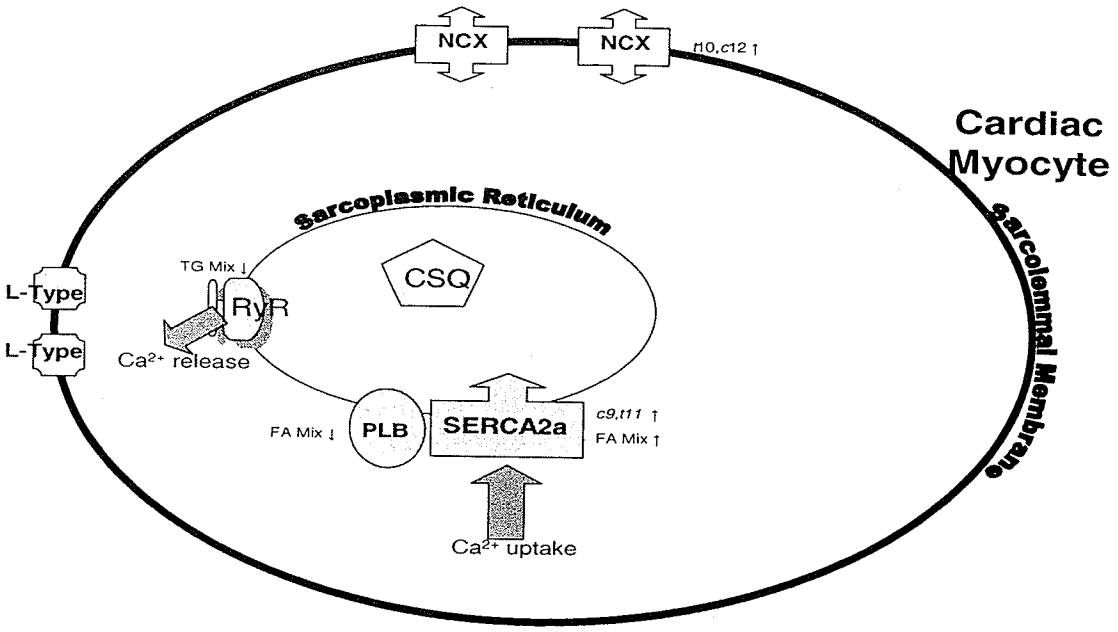


Figure 10: Calcium movement within the cardiomyocyte: Effects of dietary CLA in male rats

Abbreviations: L-Type = L-type calcium channels; RyR = ryanodine receptors; CSQ = calsequestrin; PLB = phospholamban; SERCA2a = sarco(endo)plasmic reticulum ATPase pump 2a; NCX = sodium-calcium exchanger. Arrows for Ca^{2+} uptake and Ca^{2+} release indicate sarcopsmic reticulum management of myocyte calcium homeostasis via uptake of Ca^{2+} by SERA2a and Ca^{2+} release by RyR.

Summary of major findings in male rats:

1. *c*9,*t*11 CLA, *t*10,*c*12 CLA and fatty acid mix increased gene expression of SERCA2a 40%, 17% and 95% respectively.
2. Protein levels of SERCA2a for these groups were not consistent with gene expression – Protein levels for these same groups were: *c*9,*t*11 increase 102%, *t*10,*c*12 CLA decrease 60%, fatty acid mix decrease 23%. This indicates that CLA, as a PUFA, may have post-transcriptional effects or may be affecting protein enzyme systems

involved in protein synthesis. Calcium release decreased for all dietary groups in males.

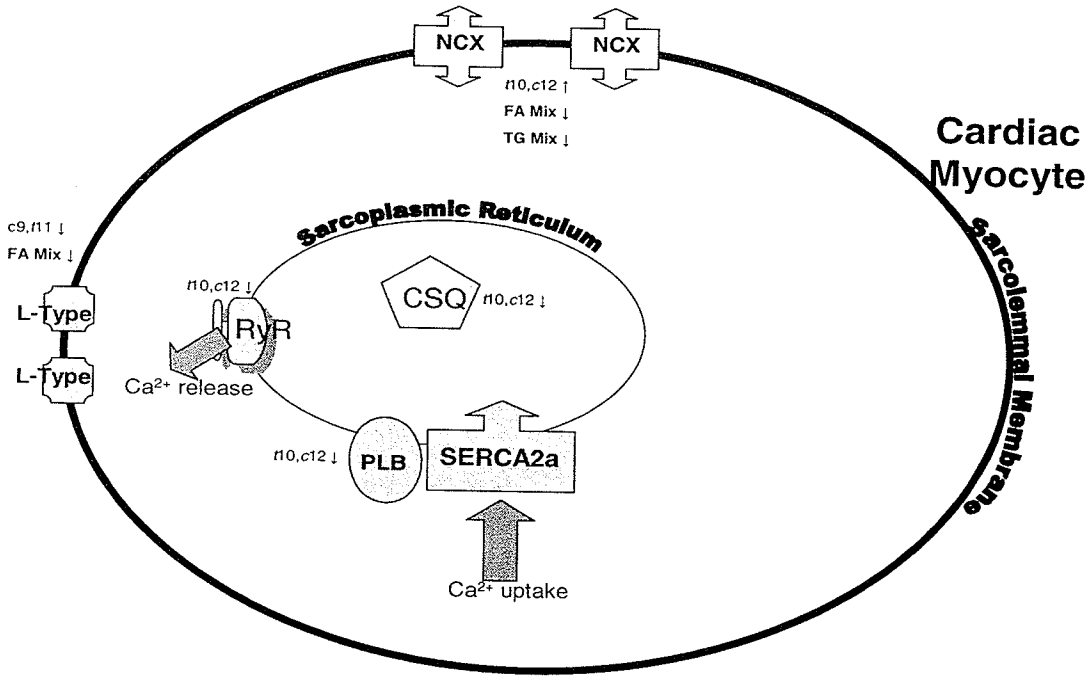


Figure 11: Calcium movement within the cardiomyocyte: Effects of dietary CLA in female rats

Abbreviations: L-Type = L-type calcium channels; RyR = ryanodine receptors; CSQ = calsequestrin; PLB = phospholamban; SERCA2a = sarco(endo)plasmic reticulum ATPase pump 2a; NCX = sodium-calcium exchanger. Arrows for Ca^{2+} uptake and Ca^{2+} release indicate sarcopsmic reticulum management of myocyte calcium homeostasis via uptake of Ca^{2+} by SERA2a and Ca^{2+} release by RyR.

Summary of major findings in female rats:

1. There was no observed dietary effect of CLA on levels of SERCA2a gene expression.
2. Protein levels of SERCA2a in females were not consistent with gene expression – protein levels decreased 49% in the *c*9,*t*11 CLA dietary group, decreased 58% in fatty acid mixture. This indicates that CLA, as a PUFA, may have post-transcriptional effects or may be affecting protein enzyme systems involved in protein synthesis.

3. Calcium uptake decreased in *t*10,*c*12 and fatty acid mix diets, but was unchanged in *c*9,*t*11 and triglyceride diets. Calcium release decreased in all diets except for the fatty acid diet, where release was unchanged.

X. FUTURE DIRECTIONS

1. Our study has demonstrated that CLA affects cardiac gene expression in a healthy animal model. It is possible that the effects of CLA may be more pronounced when an animal is exposed to the stress of calcium overload; therefore, future studies should look at the effects of dietary CLA on myocytes from failing hearts, or the effect of dietary CLA treatment in animals where heart failure has been surgically-induced.
2. Our study examined the effects of CLA at a single time point. Future research should also look at the transition in changes of cardiac gene expression over time in order to better understand: 1) How long it takes for CLA to have an effect on cardiac gene expression; 2) If the changes in cardiac gene expression are transient only, (i.e. whether they have a maximum effect then decline); 3) If continued CLA supplementation might have adverse effects on cardiac gene expression.
3. Our study used a single, sustained dose of CLA. Future studies should examine possible dose-dependent relationships of CLA on cardiac gene expression in order to: 1) Establish an upper and lower limit for CLA intake in regard to the health of the heart in both genders; 2) Evaluate any toxicological effects of CLA on cardiac health. This is important as it is still unclear at present to what extent CLA's effects on cardiac gene expression may become detrimental.

4. Future studies should establish whether there are sex-related differences in the activity of other calcium-handling proteins, whether these differences are mediated by sex hormones, (including the genes in our study), and whether these differences are important with regard to the effect of CLA on cardiac function.

XI. LIMITATIONS

1. This study examined only the genes and proteins involved in cardiac calcium homeostasis, but did not examine cytosolic or SR calcium levels in response to CLA treatment.
2. This study is limited because it addresses only differences in mRNA gene expression and protein abundance in select locations.
3. Our study looked at healthy animals, and thus it is not possible to know how CLA might act as an agent in the treatment of existing heart disease.
4. The study design could have been improved so that more powerful conclusions could be drawn from the data. For instance, PCR and gel electrophoresis trials were run separately for male and female samples, and therefore 2-way ANOVA could not be used to fully compare gender responses to CLA treatment. Additionally, no baseline measurements were made, and thus it is more difficult to define the magnitude of the changes observed for all groups.
5. This study could have examined the phosphorylation state of phospholamban to elucidate its regulation of SERCA2a pump activity.

XII. STUDY STRENGTHS AND CONTRIBUTIONS

1. This study examined both the effects of isomer and form of CLA on cardiac gene expression, levels of cardiac proteins, and cardiac calcium uptake and release in male and female rats, and has provided valuable information in regard to the effects of dietary CLA in cardiac gene expression, protein translation, and cardiac calcium homeostasis.
2. This study demonstrated that CLA does have cardiac effects at the cellular level, and that these effects differ between genders.
3. The results of this study have provided initial data indicating that dietary CLA is not a potential adjunctive agent to gene therapy for progressive heart failure, but does have potential as an antiarrhythmic agent for use in treatment of post-ischemic cardiac events.

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XIV. APPENDIX 1
Sample SAS Program

```
Data data;
Title 'One-Way ANOVA L-type gene expression Females';
Input group PAR;
lPAR=log(PAR);
*gender;
*1=females;
*2=males;
*diet groups;
*1=A=t10c12;
*2=B=2 isomer mix TG;
*3=C=c9t11;
*4=D=2 isomer mix FFA;
*5=E=Control;
*groups = 1 to 10;
* L-type gene expression as % control/GAPDH;
Cards;
        1    1.0323
        1    0.9677
        1    1.1935
        1    0.9932
        1     1.101
        1    1.0733
        2     1.129
        2     0.887
        2    0.7097
        2    0.8577
        2    0.9611
        2    0.9903
        3    0.7857
        3    0.5714
        3    0.8214
        3    0.7955
        3    0.8013
        3    0.7312
        4    0.6786
        4    0.6429
        4    0.9286
        4    0.6944
        4    0.7001
        4    0.6606
        5         1
        5         1
        5         1
        5         1
        5         1
        5         1
Proc glm Data=data;
 Class group;
 Model PAR=group/SS3;
 Output out=PARout residual=PARR predicted=PARP;
 Means group/Tukey;
Proc Print Data=PARout;
Proc univariate Data=PARout plot normal;
 var PARR;
Proc Plot Data=PARout;
 Plot PARR*PARP;

Proc GLM Data=data;
 Class group;
 Model PAR=group/ss3;
 means group/Tukey hovtest=levene;

Proc glm Data=data;
 Class group;
 Model lPAR=group/SS3;
 Output out=lPARout residual=lPARR predicted=lPARP;
 Means group/Tukey;

Proc Print Data=lPARout;
Proc univariate Data=lPARout plot normal;
 var lPARR;
Proc Plot Data=lPARout;
 Plot lPARR*lPARP;
```

```
Data data;
Title 'One-Way ANOVA L-type gene expression Males';
Input group PAR;
lPAR=log(PAR);
*gender;
*1=females;
*2=males;
*diet groups;
*1=A=t10c12;
*2=B=2 isomer mix TG;
*3=C=c9t11;
*4=D=2 isomer mix FFA;
*5=E=Control;
*groups = 1 to 10;
* L-type gene expression as % control/GAPDH;
Cards;
        6       1.1
        6       1.4
        6      1.15
        6    0.7222
        6         1
        6    0.6111
        7    0.7143
        7    0.7857
        7    0.7667
        7    0.7667
        7       0.8
        7    0.8333
        8    0.9565
        8    0.6522
        8    1.1739
        8    0.8333
        8    0.7778
        8    0.7222
        9         1
        9      0.75
        9      1.25
        9     0.722
        9    0.8333
        9    1.0556
       10         1
       10         1
       10         1
       10         1
       10         1
       10         1
Proc glm Data=data;
 Class group;
 Model PAR=group/SS3;
 Output out=PARout residual=PARR predicted=PARP;
 Means group/Tukey;
Proc Print Data=PARout;
Proc univariate Data=PARout plot normal;
 var PARR;
Proc Plot Data=PARout;
 Plot PARR*PARP;

Proc GLM Data=data;
 Class group;
 Model PAR=group/ss3;
 means group/Tukey hovtest=levene;

Proc glm Data=data;
 Class group;
 Model lPAR=group/SS3;
 Output out=lPARout residual=lPARR predicted=lPARP;
 Means group/Tukey;

Proc Print Data=lPARout;
Proc univariate Data=lPARout plot normal;
 var lPARR;
Proc Plot Data=lPARout;
 Plot lPARR*lPARP;
```