EFFECTS OF CONJUGATED LINOLEIC ACID ON BODY COMPOSITION PARAMETERS IN AGED AND CACHECTIC MICE

By

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DEDICATION

I would like to dedicate this thesis to the doctors, counselors and staff at the UofA's Counseling and Psychological Services. Without the knowledge, advice and understanding from these individuals the completion of this thesis may have not occurred.

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LIST OF ABBREVIATIONS

ACC	=	Acetyl-CoA carboxylase
ADG	=	Average daily gain
AIDS	=	Acquired Immune Deficiency Syndrome
ATP	=	Adenosine triphosphate
BSA	=	Bovine serum albumin
BW	=	Body weight
cDNA	=	Complimentary deoxyribonucleic acid
CLA	=	Conjugated linoleic acid
Cont	=	Control
COX-2	=	Cyclooxygenase-2
DEXA	=	Dual energy X-ray absorptiometry
DNA	=	Dioxyribonucleic acid
EDTA	=	Ethylenediaminetetraacetic acid
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
Epid	=	Epididymal fat depot
Est	=	Eicosatrienoic acid
FAS	=	Fatty acid synthase
Gastroc	=	Gastrocnemius muscle
GC	=	Gas chromatography
GH	=	Growth hormone
GHRH	=	Growth hormone releasing hormone
GI	=	Gastrointestinal
HCL	=	Hydrochloric acid
HIV	=	Human Immunodeficiency Virus
HRPO	=	Horseradish peroxidase
IGF-1	=	Insulin-like Growth Factor-1
IgG	=	Immunoglobulin G
IL-6	=	Interleukin-6

JAK/STAT	=	Janus kinase/Signal transducers and activators of transcription
LCPUFA	=	Long chain polyunsaturated fatty acid
MAFbx	=	Muscle Atrophy F-box protein
MAIDS	=	Murine Acquired Immunodeficiency Syndrome
MCP-1	=	Monocyte Chemoattractant Protein-1
MEM	=	Minimal essential media
mRNA	=	messenger Ribonucleic acid
MuLV	=	Murine Leukemia Virus
MuRF-1	=	Muscle-specific Ring Finger-1
NFκB	=	Nuclear Factor κ B
PAI-1	=	Plasminogen Activator Inhibitor-1
PBSN	=	Phosphate buffered saline/ azide
PGE_2	=	Prostaglandin E_2
Quad	=	Quadricep muscle
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse Transcriptase Polymerase Chain Reaction
SDS	=	Sodium Dodecyl Sulfate
SEM	=	Standard Error of Measurement
SREBP-1	=	Sterol regulatory element-binding protein-1
TNF-α	=	Tumor Necrosis Factor-α
Trt	=	Treatment
TTBS	=	Tris/Tween buffered saline
VLPUFA	=	Very long polyunsaturated fatty acid

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ABSTRACT

Research for this thesis consisted of three parts; determining CLA effects on 1) whole animal body composition in aged mice, 2) whole animal body composition parameters in a murine model of cachexia, 3) specific muscles, adipose depots, and organs as well as the effects on parameters of muscle degradation in aged mice. The first study indicates CLA decreased fat mass and increased whole animal protein mass in aged mice. In the second study, infection resulted in changes in carcass water, fat, and protein. CLA caused an increase in protein mass in the controls but was unable to increase protein mass in cachectic animals. In the third study, no changes in muscle weights were observed with CLA and there was no effect on parameters of muscle degradation. These results demonstrate the complex nature of CLA and the need for further investigation.

CHAPTER 1

LITERATURE REVIEW

Age-Associated Changes in Body Composition

Age-associated changes in body composition are a well documented global phenomenon. Generally in humans and many animals, aging is associated with an increase in fat mass and a decrease in muscle mass. These alterations occur regardless of changes in total body weight, and in humans, begin in the second to third decade of life and progress with advancing age (Villareal et al., 2005). As the average human life expectancy continues to increase, problems associated with body composition in the aging population have become an area of focus.

Changes in body fat parameters with aging not only encompass an increase in total fat but also, and possibly more importantly because of associated health problems, a redistribution of adipose tissue. Aging is associated with an increase in visceral fat (omental and mesenteric adipose tissue), subcutaneous abdominal fat, intramuscular fat, intramyocellular lipid, and intrahepatic fat (St-Onge, 2005). Increases in abdominal, intramuscular and intrahepatic adipose tissue are positively associated with diseases including insulin resistance, type II diabetes and cardiovascular disease (Villareal et al., 2005). Additionally, the prevalence of obesity (defined as a BMI $\geq$ 30) in the aged population has been increasing and is due to both an increase in the total number of aged persons as well as an increase in the percentage of aged persons who are obese ($\geq$ 15% of older persons; Villareal et al., 2005).

The condition of age-associated declines in muscle mass and strength is known as sarcopenia. Sarcopenia affects all individuals to some extent, regardless of nutritive or physical status (Roubenoff, 2000) and individuals over 50 years of age lose approximately 1-2% of muscle mass per year (Dirks and Leeuwenburgh, 2006). Sarcopenia leads to increased frailty, a loss of functional independence and increased morbidity and mortality (Roubenoff, 2004), as well as decreased protein and nitrogen stores, a compromised immune response and reduced ability to fight disease (Roubenoff, 2000). It affects 10-25% of individuals under the age of 70 and 40% of individuals over 80 years old (Dirks and Leeuweenburgh, 2006).

The combination of the aforementioned diseases (sarcopenic-obesity) is thought to exacerbate each condition's deleterious effects, as it increases the amount of physical impairment and markedly increases mortality (Roubenoff, 2004). The continuing increase in the number of older individuals and the prevalence of sarcopenic-obesity has raised concern about possible strains on healthcare resources. In 2000 the direct costs of sarcopenia alone were estimated at $18.5 billion (Janssen, 2004), and with obesity itself being an expensive disease ($117 billion annually; Stein and Colditz, 2004), sarcopenic-obesity is a serious health care and economic concern.

Age-related changes in body composition are numerous and it is clear the mechanisms behind these changes are considerably complex. Additionally, the problems of obesity and sarcopenia are not independent, as fat and muscle mass, and their respective metabolic systems, affect each other (Roubenoff, 2004), making the condition even more convoluted and multi-factored. Below are brief descriptions of some of the

changes that have been shown to contribute to body composition changes associated with aging.

Energy Balance

The pathogenesis of age-induced increased fat mass is not fully elucidated but it is clear that energetic changes are involved. An increase in fat mass is generally caused by an imbalance in energy intake and expenditure, leading to a positive energy balance. Frequently, there is little change in energy intake with aging (it may actually decrease) and it is more likely that energy expenditure decreases with age (Villareal et al., 2005). Total energy expenditure is multi-factorial, and includes resting metabolic rate, thermic effect of food, and physical activity. Each of these aspects have been shown to decrease with aging, with decreased physical activity accounting for about 50% (Villareal et al., 2005). In addition, resting fat oxidation decreases with advancing age and this is thought to be a product of a decreased energy demand in aged individuals. The combination of decreased fat oxidation capacity and decreased quantity of fat oxidizing tissue (i.e. muscle) leads to an increase in fat mass (Levadoux et al., 2001).

Satellite Cells

Muscle mass is a function of the rate of protein anabolism versus the rate of protein catabolism. Muscle from aged humans and animals accumulates more contractile damage and the rate of muscle repair is reduced as compared to younger subjects. This impaired regeneration is thought to be due to a decrease in satellite cell activation by factors within the muscle's micro environment (Carlson and Faulkner, 1989). Although regeneration and satellite cell activation's role is important in sarcopenia, it will not be

extensively covered. For an excellent review on the aforementioned topic see Machida and Narusawa, 2006.

Somatotropic Axis

Age associated decreases in growth hormone and the growth hormone/insulin-like growth factor (GH-IGF) axis (termed somatopause) have been linked to skeletal muscle loss and increased adiposity. Decreased growth hormone secretion from the pituitary gland, and a subsequent decrease in hepatic IGF secretion, begins in the third decade (Leow and Loh, 2006). Growth hormone deficiency may begin upstream of the pituitary with possible alterations in growth hormone releasing hormone (GHRH), somatostatin or ghrelin pulsatility. Additionally, cells become GH resistant with increasing age, possibly through alterations in JAK/STAT signaling pathways (Leow and Loh, 2006). As muscle cells primarily respond indirectly to GH via IGF, the decline in IGF levels has important negative impact on the muscles' anabolic capability (Florini et al., 1996).

Growth hormone replacement therapy has been a major area of investigation as a possible treatment of sarcopenia, though this has been controversial as a decline in the GH/IGF axis may be an adaptive response to aging and there is concern that exogenous GH may increase certain tumor proliferation (Leow and Loh, 2006). Additionally, rodent studies indicate decreased GH levels actually results in increased longevity (Leow and Loh, 2006). As a consequence, the GH-IGF axis' role in sarcopenia remains ambiguous and further research is required to delineate its importance.

Insulin Action

Aging is associated with deterioration in systemic glucose tolerance and there is strong evidence that this is the result of an increase in peripheral insulin resistance due to a post-receptor defect (Fink et al., 1984). However, age accounts for only a small portion of age-related insulin resistance (1.1%; Ferrannini et al., 1996) and other age-related changes in body composition and hormonal status may have an important role. Intra-abdominal adipose tissue tends to increase with age and this depot is associated with insulin resistance, likely due to an increase in adipokine secretion (i.e. leptin). Muscle is the principle tissue with regards to glucose uptake and therefore sarcopenia and a decrease in physical activity may contribute to insulin resistance. Furthermore, circulating IGF-I decreases with age and this may lead to insulin resistance because the IGF-I receptor is involved in glucose uptake (Paolisso et al., 1999).

Cytokines

Aging is associated with an increase in circulating levels of inflammatory markers, including pro-inflammatory cytokines, anti-inflammatory cytokines, cytokine antagonists and acute phase proteins (Dirks and Leeuwenburgh, 2006). However, how these changes in inflammatory variables impact skeletal muscle is still uncertain. *In vitro* studies have demonstrated an increase in the production of inflammatory cytokines in monocytes from elderly donors (Fagiolo et al., 1993) and *in vivo* measurements indicate elevated levels of circulating inflammatory mediators (Ershler and Keller, 2000). Elevated inflammatory cytokines have led to the supposition that a chronic, low-grade inflammation is associated with ageing and causes attributed to this condition include a

decreased production of sex steroids, sub-clinical disorders, asymptomatic bacteriuria and a higher relative/absolute amount of fat tissue (Licastro et al., 2005).

TNF-α has been implicated in sarcopenia, as elevated levels have been positively associated with a decrease in muscle mass and strength in human studies (Licastro et al., 2005). Additionally in animal models, TNF-α injection results in a decrease in body weight (Pariza et al., 1999), a decrease in skeletal muscle mass (Goodman, 1991) and an increase in muscle proteolysis (Flores et al., 1989). There is evidence that the loss of muscle fibers (hypoplasty) that is a characteristic of sarcopenia, is the result of apoptosis with TNF-α possibly being the pro-apoptotic signal (Dirks and Leeuwenburgh, 2006).

Cachexia

Cachexia is a condition of accelerated skeletal muscle loss that occurs with a chronic inflammatory response, as in patients with cancer, HIV, sepsis and renal failure, to name a few (Kotler, 2000). The biology of cachexia is different from calorie deficiency (although anorexia is one characteristic of cachexia) as pair-fed hypocaloric animals did not loose muscle mass to the same extent of the cachectic animals (Medina et al., 1995). Muscle protein mobilization during early cachexia is thought to play an important role in providing amino acids for an acute-phase response, an immune response that demands high amounts of energy and essential amino acids for hepatic protein synthesis (Kotler, 2000). However, prolonged cachexia results in depletion of protein and energy reserves, muscle wasting and weakness and impedes recovery from the initial illness. Therefore, cachexia is associated with a poor disease outcome and increased mortality (Baracos, 2006).

Along with changes in skeletal muscle metabolism, cachexia is characterized by changes in hepatic lipid metabolism including hypertriglyceridemia, increased secretion of very-low-density lipoproteins, increased *de novo* triglyceride synthesis and esterification, and changes in adipocyte lipid metabolism including decreased lipoprotein lipase activity and increased release of free fatty acids (Feingold et al., 1989). These changes are believed to be mediated, in large part, by cytokines and may be a host defense adaptation, rather than reflecting nutritional status and energetic state (Kotler, 2000). Additionally, cachexia is associated with peripheral insulin resistance (thought to be mediated by inflammatory cytokines) which would partition glucose towards the liver and away from skeletal muscle (Kotler, 2000).

There are many factors contributing to cachexia (see review: Morley et al., 2006), but an increase in skeletal muscle protein degradation seems to play a central role. The increase in protein degradation is through increases in calpain proteolysis (Hasselgren and Fischer, 2001) and the ubiquitin-proteasome system (described below) and inflammatory cytokines (with TNF-α being the most important) are key mediators of this process (Kotler, 2000). Numerous studies (both *in vitro* and *in vivo*) indicate TNF-α has significant catabolic effects on muscle (see Table 1.1). TNF-α is believed to contribute to cachexia by inhibiting the activation of messenger RNA for myosin synthesis and by stimulating myosin proteolysis (Morley et al., 2006) as well as increasing ubiquitin mRNA production (Garcia-Martinez et al., 1994; Llovera et al., 1994).

TABLE 1.1: The effect of TNF-α on muscle

Author	Model	Treatment	Effect
Acharyya et al., 2004	C2C12 cultures	TNF & IFNγ	↓ myosin heavy chain expression
Flores et al., 1989	Rats	TNF-α	↑ muscle proteolysis
Garcia-Martinez et al., 1994	Rats	TNF-α	↑ ubiquitin expression
Goodman, 1991	Rats	TNF-α	↑ proteolysis
Li et al., 2005	C2C12 and primary Cultures	TNF	↑ binding of NFkB to DNA ↑ ubiquitin conjugation ↑ urea, creatine, 3-methylhistidine excretion
Llovera et al., 1994	Rats	TNF-α	↑ ubiquitin expression
Llovera et al., 1996	Tumor-bearing rats	Anti-TNF	prevention of increase in ubiquitin expression

There is no described treatment for cachexia, however many interventions have been suggested. Though hypercaloric feeding and exercise cannot reverse cachectic muscle loss, deficiencies in these areas exacerbate the disease (Kotler, 2000). Appetite stimulants are another treatment to increase caloric intake. Therapies under examination to either increase protein synthesis or decrease protein degradation rates include anabolic treatments using growth hormone, anticytokine therapies and anti-inflammatory agents (Kotler, 2000).

Intracellular Protein Degradation

It is a fairly recent discovery that cellular proteins are in a constant dynamic state of synthesis and degradation (Schoenheimer, 1942). Since this finding there has been extensive work on the mechanisms of cellular protein degradation and this area has become an important target for drug development. Cellular proteolysis plays a major role in the cell cycle, tissue development, differentiation, transcription regulation, antigen presentation, signal transduction, receptor-mediated endocytosis, removal of damaged

and misfolded proteins, and modulation of diverse metabolic pathways (Ciechanover, 2004). The study of proteolysis began with the discovery of the lysosome, but shortly after it was realized that lysosomal protein degradation could not explain all cellular proteolysis. Further research led to the detection of the ATP-dependent ubiquitin-proteasome system of protein degradation, which now has been shown to be responsible for the majority of intracellular proteolysis (Ciechanover, 2004).

Proteolysis through the ubiquitin-proteasome system begins with the covalent linkage of ubiquitin (a small protein cofactor) to the desired protein. The process is multienzymatic and the ubiquitin molecule must first be activated through the conversion of its carboxyl end to a thiol ester by an ATP-dependent enzyme, E1. The activated ubiquitin is then transferred to a carrier protein, E2 and the carboxyl group of the ubiquitin molecule is coupled to the ε-amino groups of lysine residues in the desired protein substrate via the action of an ubiquitin conjugating ligase, E3. This step is repeated until a chain of five or more ubiquitin molecules are conjugated to the protein substrate. At this point the protein is then rapidly degraded by the 26S proteasome (Mitch and Goldberg, 1996).

The 26S protesome is a proteolytic complex consisting of a 20S core, where proteolysis occurs, and a 19S subunit at either end. The 20S subunit is barrel shaped and consists of four stacked rings with each ring containing 7 subunits around a central cavity containing the proteolytic activity (Mitch and Goldberg, 1996). Protein substrates enter the core of the proteasome through the 19S subunit, which is believed to be responsible for the obligatory unfolding of the protein substrate and is an ATP-dependent step.

Proteins entering the proteasome core are cleaved into small peptides which are further degraded by cytosolic exopeptidases (Mitch and Goldberg, 1996).

The proteasome functions through a novel proteolytic mechanism, in which peptide-bond cleavage is catalyzed by the hydroxyl group of threonine at the N terminal of inner-ring subunits. The proteolytic active sites of the proteasome subunits have different substrate specificity; one preferentially cleaving peptides after large hydrophobic amino acids, one after basic amino acids, and one after acidic residues and these different specificities work together to degrade the protein (Mitch and Goldberg, 1996).

Although the ubiquitin-proteasome system is involved in many areas of cellular function, the focus of this section will be its role in muscle protein degradation and muscle wasting. It has been demonstrated that the ubiquitin-proteasome system is responsible for degrading myofibrillar proteins (actin and myosin) during catabolic states (Costelli and Baccino, 2003). The level of ubiquitin-conjugated proteins and the expression of components of the pathway, including ubiquitin ligases and proteasome subunits, are increased in muscle wasting conditions (Costelli and Baccino, 2003). To date, two striated muscle specific ligases have been described; Muscle-specific RING-finger-1 (MuRF-1) and muscle atrophy F-box protein (MAFbx/atrogin-1). The expression of these enzymes are increased in several conditions of muscle wasting (Lecker et al., 2004) and knock-out mice for either gene are partially protected against muscle wasting (Bodine et al., 2001).

The Calpain System

Myofibrillar protein degradation is a signature of skeletal muscle wasting and it is believed to occur through the ubiquitin-proteasome system (Raynaud et al., 2005). However, the sarcomere structure containing these proteins is very large and therefore intact actin and myosin cannot enter the proteasome. For this reason it is believed that the myofibrils must first be removed from the sarcomere and calpains may be the best candidate responsible (Raynaud et al., 2005).

Calpains are a family of intracellular Ca^{+2} –regulated cysteine proteases involved in many cellular functions including cytoskeleton organization, the cell cycle, signal transduction, apoptosis, and growth and tissue regeneration (Benyamin, 2006). Currently there are 14 identified calpain members and the two ubiquitous members, calpain 1 and 2 (mu- and m-calpain, respectively) are the most well described. Muscle tissue expresses mainly three calpain members; the ubiquitous members and a muscle specific member calpain 3 (also known as p94; Bartoli and Richard, 2005).

In their native form, mu- and m-calpain are heterodimers consisting of an 80-kDa and a 28-kDa subunits. The 80-kDa subunit of mu- and m-calpain are different gene products, though they share a 55-65% sequence homology within a given species (Goll et al., 2003). Based on the amino acid sequence, the 80-kDa subunit was divided into four domains. Domain I (N-terminal region) contains no homology to other identified proteins. Domain IIa and b are structurally similar to the catalytic domain of other cysteine proteases. Domain III binds phospholipids and interacts with the membrane. Its three-dimensional topology is similar to a C2 domain (Bartoli and Richard, 2005).

Domain IV contains 5-EF-hand domains, a conserved domain responsible for binding Ca^{+2}, though in calpain, the 5th and probably the 4th EF-hand do not bind Ca^{+2}. The recent identification of the crystallographic structure of m-calpain shows the 80-kDa domain to have 6, rather than 4 domains. Whether this is true for mu-calpain as well is yet to be determined. The 28-kDa subunit of mu- and m-calpain are identical and consist of Domain V, an unordered structure that may serve as a tether and Domain VI which also contains 5 EF-hands and is similar to Domain IV (Goll et al., 2003).

M- and mu-calpain are distinguishable and were named based on their *in vitro* calcium sensitivities. Mu-calpain binds Ca^{+2} in the range of 5-50 μM and m-calpain binds in the range of 250-1000 μM (Bartoli and Richard, 2005). These concentrations however, are higher than physiological intracellular calcium concentrations and a mechanism that would lower the concentration for binding is currently being investigated. For a detailed review of calpains Ca^{+2} binding properties see the recent review by Goll et al., 2003.

Calpains are responsible for intracellular proteolysis and tight regulation is required. Calpastatin has been identified as a specific inhibitor of calpain activity and has not been shown to inhibit any other tested protease (Friedrich and Bozoky, 2005). Calpastatin contains an N-terminal L-domain and 4 inhibitory domains, each of which have been shown to inhibit m- and mu-calpain activity (Friedrich and Bozoky, 2005), suggesting that one calpastatin molecule may inhibit four calpain molecules (Goll et al., 2003). Each domain contains three subfragments: A, B and C with subfragments A and C being binding elements and subfragment B containing all of the inhibitory activity

(Friedrich and Bozoky, 2005). Calpastatin inhibition involves binding to calpain at three sites and this binding requires Ca^{+2}. The regulation of calpain by calpastatin *in vivo* as well as its effect on other members of the calpain family has yet to be fully elucidated (Goll et al., 2003).

There is a growing body of evidence that the ubiquitous calpains are involved in muscle degradation by dissembling the sarcomere at the Z-disk (Bartoli and Richard, 2005). Mu- and m-calpain are concentrated in the myofibrils at the Z-disk and also at the I-band. Additionally, proteins within this region, including titin, α-actinin and γ-filamin, bind mu-calpain with increasing affinity in the presence of Ca^{+2} (Lebart and Benyamin, 2006). Calpain expression and activity are increased in atrophic conditions (Hong and Forsberg, 1995; Voisin et al., 1996) and overexpression of calpastatin in transgenic mice confirmed that calpains are involved in muscle wasting (Tidball and Spencer, 2002). Furthermore, m-calpain inhibition stabilizes sarcomeric proteins in atrophic muscle and myofibrils are released from ATP-depleted cells post mortem, a process that is reduced in calpastatin overexpressing animals (Tidball and Spencer, 2002).

Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) is a descriptor for geometric and positional conjugated isomers of linoleic acid. Since 1987 when CLA was isolated from grilled beef and demonstrated to have anticarcinogenic properties (Ha et al., 1987) there has been extensive research on CLA isomers. In addition to altering carcinogensis, CLA alters atherogenesis, diabetes, body composition and immune function (House et al., 2005). The majority of CLA research focuses on 18:2 *cis*-9, *trans*-11 and 18:2 *trans*-10,

cis-12 isomers and therefore these two isomers will be the emphasis of this thesis. CLA is synthesized via biohydrogenation of unsaturated fatty acids by bacteria in the rumen of ruminant animals. The *cis*-9, *trans*-11 isomer is the product of isomerization of the 12 position double bond of linoleic acid which is further hydrogenated to stearic acid. However, a portion of the *cis*-9, *trans*-11 isomer escapes hydrogenization and is absorbed by the small intestine (Bauman et al., 1999). Additionally, within the tissues of the ruminant animal, vaccenic acid (18:1 *trans*-11) can be dehydrogenated at the 9 position by Δ^9 -desaturase, resulting in the *cis*-9, *trans*-11 isomer and this isomer is the principle isomer found in ruminant food products (Corl et al., 2001; Kay et al., 2004). The *trans*-10, *cis*-12 isomer is synthesized when a change in the rumen pH causes isomerization at the 9 position of linoleic acid. This isomer is also further hydrogenated to stearic acid, though some gets absorbed and it is found at much lower concentrations in ruminant food products (Bauman et al., 1999). CLA can also be artificially synthesized through alkaline isomerization of linoleic acid-enriched vegetable oils (House et al., 2005).

A plethora of animal studies (see Table 1.2) indicate that feeding CLA causes significant changes in body composition, particularly in adipose depots. Many of the CLA studies on body composition have used a 1:1 ratio of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers though the *trans*-10, *cis*-12 isomer is responsible for the changes in lipid metabolism and body composition (Park et al., 1999; Baumgard et al., 2000). The vast majority of studies have utilized growing animals though a few have used mature, non-growing models. In a study of adult (15 wk) rats, CLA supplementation had no effect on body composition (Patureau Mirand et al., 2004). These results may be due to

the species of animal studied (rats are less sensitive to CLA; Wahle et al., 2004) rather than the age of the animals as studies in mature mice have shown significant decreases in fat mass (Pariza et al., 2001; Rahman et al., 2006).

The mechanism behind CLAs fat lowering property has been thoroughly studied and many explanations have been developed. CLA increases energy expenditure, increases adipocyte apoptosis, increases fatty acid oxidation, increases adipocyte lypolysis, decreases adipocyte lipogenesis and fatty acid uptake, and increases adipocyte differentiation (House et al., 2005).

TABLE 1.2: The effect of CLA on body fat and adipose depots in animal studies

Author	Species	Sex	Age	Fat source	%Decrease
Akahoshi et al., 2002	Mice	Male	3 wk	Epidydimal	80%
				Perirenal	60%
Battacharya et al., 2005	Mice	Male	8wk	Total	75%
Clement et al., 2002	Mice	Female	Not reported	Periuteral	85%
de Deckere et al., 1999	Hamsters	Male	13 wk	Epidydimal	16%
DeLany et al., 1999	Mice	Male	6 wk	Inguinal	35%
				Epidydimal	21%
				Retroperitoneal	33%
				Mesenteric	46%
				Perirenal	47%
Faulconnier et al., 2004	Rats	Male	17 wk	Epidydimal	47%
Hargrave et al., 2002	Mice	Male & female	12 wk	Total	20%
Kang et al., 2004	Mice	Not reported	7 wk	Epidydimal	40%
				Retroperitoneal	60%
Kelley et al., 2006	Mice	Female	8 wk	Retroperitoneal	59%
Meadus et al., 2002	Pigs	Male	Not reported	Subcutaneous	9%
Miner et al., 2001	Mice	Male	12 wk	Brown adipose tissue	29%
				Epidydimal	30%
				Retroperitoneal	50%
Nakanishi et al., 2004	Mice	Male	Not reported	Epidydimal	71%
				Perirenal	54%
Ostrowska et al., 2003	Pigs	Female	Not reported	Total	12%
Park et al., 1997	Mice	Female	6 wk	Total	60%
Rahman et al., 2006	Mice	Female	12 mo	Total	43%
Terpstra et al., 2002	Mice	Male	6 wk	Total	67%
Tsuboyama-	Mice	Female	7 wk	Parametrial	73%
Kasaoka et al., 2000				Perirenal	93%
				Retroperitoneal	100%
				Abdominal subcutaneous	96%
				Dorsal subcutaneous	97%
				Brown adipose tissue	100%
Warren et al., 2003	Mice	Female	8 wk	Retroperitoneal	60%
Zabala et al., 2006	Hamsters	Male	9 wk	Epidydimal , Perirenal & Subcutaneous	24%

The considerable affect of CLA on adiposity in animal models has led to human studies where CLA was hypothesized to act as an anti-obesity compound. However, these studies have provided conflicting results. Relatively few studies have shown CLA

to reduce fat mass in humans (Blankson et al., 2000; Thom et al., 2001) while many others have shown no affect of CLA (Atkinson, 1999; Berven et al., 2000; Zambell et al., 2000; Benito et al., 2001). Furthermore, it is difficult to analyze these results due to the differences is CLA dose and isomers, duration of supplementation, differences in age and sex, and differences in the method of body composition detection (Tricon and Yaqoob, 2006).

The effect of CLA on lean tissue has been less clear. Though many animal studies show an increase in the content of lean tissue with CLA supplementation, this can largely be explained by the congruent decrease in the quantity of carcass fat. Few studies have measured lean/muscle *mass* directly. However, some studies have demonstrated an increase in lean tissue mass with CLA supplementation (Ostrowska et al., 1999; Bhattacharya et al., 2005; Rahman et al., 2006; See Table 1.3). The results of studies on lean mass in humans have produced conflicting results, similar to those results for fat mass (Tricon and Yaqoob, 2006). Although few human studies indicate an increase in lean mass with CLA supplementation (Kamphuis et al., 2003; Gaullier et al., 2004), the vast majority report no effect of CLA (Kreider et al., 2002; Riserus et al., 2002; Malpuech-Brugere et al., 2004; Whigham et al., 2004). Again, these inconsistencies in the literature are likely due to differences in CLA dose and length of supplementation, differences in age, sex and physical condition of the subjects and differences in how lean mass was measured. For a thorough evaluation of the effects of CLA supplementation in humans see review by Salas-Salvado et al., 2006.

TABLE 1.3: The effect of CLA on lean tissue

Author	Species	Sex	Age	Detection method	Change
Bhattacharya et al., 2005	Mice	Male	8 wk	DEXA	↑
DeLany et al., 1999	Mice	Male	6 wk	N determination (%)	↑
				N determination (g)	↔
Kang et al., 2004	Mice	Not reported	7 wk	Leg muscle wt	↔
Meadus et al., 2002	Pigs	Male	Not reported	Dissection	↑
Ostrowska et al., 2003	Pigs	Female	Not reported	N determination	↔
				Carcass evaluation	↑
Park et al., 1997	Mice	Female	6 wk	N determination (%)	↑
				N determination (g)	↔
Rahman et al., 2006	Mice	Female	12 mo	Gastroc. dry muscle wt	↔
				Quad. dry muscle wt.	↑
				DEXA	↔
Terpstra et al., 2002	Mice	Male	6 wk	N determination (g)	↔
Tsuboyama-.	Mice	Female	7 wk	Gastroc. wet muscle wt	↔
Kasaoka et al., 2000				Quad. wet muscle wt.	↔
Zabala et al., 2006	Hamsters	Male	9 wk	Gastroc. wet muscle wt.	↑

A consistent phenomenon that occurs with feeding CLA (particularly *trans*-10, *cis*-12) is an increase in carcass water (Ostrowska et al., 1999; Sanders et al., 2004). This may be a consequence of an increase in lean mass as muscle is composed of approximately 70% water. Due to the large concentration of water in muscle, a significant change in water content may be more easily detected before a change in protein mass (Sanders et al., 2004).

In addition to its effects on adipose and lean tissue, there is some evidence that CLA increases bone formation and mass. In mice, CLA supplementation caused a significant increase in bone mineral density (Rahman et al., 2006) and studies in a murine osteoblast cell line showed CLA to increase the secretion of bone formation markers (Watkins et al., 2004). The effects of CLA on bone are thought to be due to CLA's inhibition of COX-2 mediated formation of PGE_2 (Watkins et al., 2004). For further

information on CLA's effects on bone metabolism see review by Watkins and Seifert, 2000.

Summary

In recent history, medicine has made enormous strides in alleviating and eliminating what were once certainly fatal diseases, allowing us to live longer lives. However, body composition changes associated with advancing age and chronic disease continue to negatively impact our quality of life and longevity. Increases in body fat and decreases in muscle mass diminish our functional independence and autonomy, as well as increase our susceptibility to disease. These conditions affect anyone who experiences advancing age and research is crucial to determine the mechanisms behind these ubiquitous changes and develop therapeutic treatments utilizing drugs or nutritional modifications.

CHAPTER 2

EFFECTS OF CONJUGATED LINOLEIC ACID (CLA) ON BODY COMPOSITION PARAMETERS IN AGED MICE

Abstract

CLA decreases the fat content in growing rodents, but its effectiveness in aged animals has not been evaluated. Female aged (25 mo old; n=17) C57BL/6 mice were fed iso-energetic diets containing 0 or 1.68% CLA methyl esters (50/50 *cis*-9, *trans*-11 and *trans*-10, *cis*-12) for 42 d. Mice fed CLA lost, while controls gained weight (-2.6 vs. 1.6 g). Compared to controls, CLA increased carcass water (56 vs. 71%) and decreased dry matter whole body carcass (minus heart, spleen and liver) fat content by 51% and increased carcass protein and ash content by 49 and 38%. CLA decreased the quantity of carcass fat by 73% (8.1 vs. 2.4 g) and increased the amount of carcass protein by 14% (4.4 vs. 3.8 g), but did not alter ash mass (1.0 g). CLA did not alter heart or spleen weight but increased (38%) liver weight (1.9 vs. 3.0 g) that was only partially accounted for by increased liver fat content (25.5 vs. 34.7%). Dietary CLA increased circulating PAI-1 (71%), and tended to increase insulin (87%) and IL-6 (155%) levels, decreased systemic leptin (86%) levels, but had no effect on either MCP-1 or resistin concentrations. Dietary CLA markedly reduced the fat content and improved muscle mass in aged female mice but whether it can be utilized as a dietary tool to manage tissue balances in the elderly human population is unknown.

Introduction

Body composition changes associated with aging typically include a reduction in lean tissue mass (muscle) with a corresponding increase in adipose tissue (Karakelides and Sreekumaran Nair, 2005). Increased adipose tissue accretion can lead to obesity, which is a major health concern in all age groups. Although the incidence of obesity is less in the elderly population the frequency is increasing. For example, it is estimated the prevalence of obesity in adults aged 60 and older will increase to almost 40% by 2010 (Arterburn et al., 2004). Obesity, specifically intra-abdominal visceral fat accumulation, is strongly associated with a plethora of human health disorders including diabetes mellitus, cardiovascular disease, dyslipidemia, osteoarthritis and certain cancers (Wyatt et al., 2006). Although the exact mechanism linking obesity and these diseases remains unclear, recent studies suggest that dysregulation of adipocyte derived cytokines (adipokines) may play an important role in their development (Trayhurn and Wood, 2005).

The loss of lean tissue mass associated with human aging (known as sarcopenia) starts gradually during the fourth decade of life with a continual loss of skeletal muscle thereafter. Sarcopenia is associated with metabolic changes that may lead to type II diabetes, dislypidemia and hypertension, as well as physical impairments (Karakelides and Sreekumaran Nair, 2005). Muscle mass and functional loss occurs with advancing age in all humans, regardless of physical activity, and is associated with increased dependency, poor health and increased mortality (Roubenoff and Hughes, 2000). Although diet (increased protein quality and quantity), and exercise (resistance training

and aerobic exercise) can decrease the extent of muscle wasting, sarcopenia is a consistent and predictable phenomenon (Karakelides and Sreekumaran Nair, 2005). Reduced motor neuron input, decreased production of or sensitivity to anabolic molecules (sex steroids, growth hormone, insulin, etc.) and increased catabolic signals (inflammatory cytokines) have all been hypothesized as possible contributors to sarcopenia. Although not completely understood, it is clear that sarcopenia is a multi-factorial and complex disorder (Roubenoff and Hughes, 2000).

The combination of increasing adipose and decreasing muscle tissue (sarcopenic-obesity) represents a condition that probably compounds the deleterious effects of both syndromes (Pierson, 2003). Paradoxically, obese individuals aged 50-75 years are at a significantly higher risk of involuntary weight loss (a characteristic of sarcopenia) than non-obese persons (Sahyoun et al., 2004). Therefore, identifying a dietary food component that beneficially affects both lipid and protein metabolism to combat sarcopenic-obesity is of interest.

Conjugated linoleic acids (CLA) are geometrically unique isomers of linoleic acid and are found naturally in dairy products and ruminant meat as a result of polyunsaturated fatty acid rumen biohydrogenation (Bauman et al., 1999). Dietary CLA have been shown to have beneficial effects on human disorders ranging from atherosclerosis to cancer. CLA also dramatically decreases the body fat content and mammary fat synthesis in a number of growing and lactating species, respectively (Bauman et al., 1999; Belury, 2002). Most trials have utilized a CLA supplement containing a variety of isomers, but using purified preparations it was determined that

trans-10, *cis*-12 CLA reduces fat accretion and mammary fat synthesis while *cis*-9, *trans*-11 CLA has little or no effect on lipid metabolism (Park et al., 1999; Baumgard et al., 2000).

The effect of CLA on protein metabolism is less clear, as most rodent studies report an increase in carcass protein content but little or no effect on carcass protein yield (Pariza et al., 2001). However, recent reports suggest dietary CLA increased lean tissue accretion rates in growing pigs (Ostrowska et al., 2003), a model that may be more appropriate for human comparisons (Miller and Ullrey, 1987). In addition to increasing lean tissue mass in healthy growing animals, CLA also reduces the extent of muscle wasting during immune-induced cachexia (Cook et al., 1993). Because sarcopenia and cachexia share some similarities with regards to their effects on protein metabolism, we hypothesized that CLA may be an effective dietary intervention to help maintain or increase muscle mass in an aged animal model.

CLA effects on body composition in mature or older animals have not been intensively studied, and a recent report suggests that CLA is ineffective at altering body composition in mature rats (Patureau Mirand et al., 2004). To our knowledge, there have been no reports of CLA effectiveness in improving body composition in an aged animal model. Therefore, study objectives were to determine if dietary CLA could decrease the fat content and either maintain or increase the amount of lean tissue mass in female aged mice. Furthermore, it was of interest to determine CLA effects on organ weight and fatty acid profiles in hepatic tissue and carcass lipids, and systemic biomarkers of metabolism and inflammation.

Materials and Methods

Animals

All protocols and procedures were approved by the University of Arizona Institutional Animal Care and Use Committee. Seventeen female aged (25 mo) C57BL/6NHsd mice (mean initial BW was 34.6 ± 6.1 g) were utilized to determine dietary CLA supplementation effects on whole animal body composition parameters and biomarkers of metabolism and inflammation.

Experimental Protocol and Treatment

Mice were randomly divided into two groups and fed a standard iso-energetic and iso-nitrogenous diet (Dyets Inc., Bethlehem, PA) with the control diet containing no CLA and the supplemented diet containing 1.68% of a 50/50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Table 2.0; BASF AG, Ludwigshafen, Germany). The control diet was supplemented with soybean oil to remain iso-energetic and contained a similar amount of fat as the CLA treatment (Table 2.1). Mice were housed 4 to a cage and were fed the respective diets for 42d.

TABLE 2.0: Fatty acid profile of CLA supplement

Fatty acid	% of supplement
C14:0	0.10
C16:0	6.96
C16:1	0.12
C18:0	5.32
C18:1 *c*-9	25.93
C18:2 *c*-9,*c*-12	2.42
C18:2 *c*-9,*t*-11	26.27
C18:2 *t*-10,*c*-12	25.78
Other CLA isomers	2.09
Unknown[1]	5.01

[1]Unidentifiable fatty acids

TABLE 2.1: Chemical composition and fatty acid profile of control and CLA supplemented diet

	Treatment	
	Control	CLA
Component	%	
Protein[1]	18.3	18.3
Carbohydrate[2]	67.1	67.1
Fat[3]	5.0	5.0
Fiber[4]	5.0	5.0
Ash[5]	4.5	4.5
Fatty Acid	%	
14:0	<0.1	0.1
16:0	10.2	6.0
16:1 c-9	<0.1	0.1
18:0	4.5	4.5
18:1 c-9	22.7	22.4
18:2 c-9,c-12	54.8	2.4
18:2 c-9,t-11	ND[6]	31.0
18:2 t-10,c12	ND	30.8
Other CLA isomers	ND	1.2
18:3 c-9,c-12,c-15	7.8	ND
Unknown[7]	<0.1	1.6

[1]Vitamin-free casein (86%) and DL-Methionine (19%)
[2]Sucrose (5%) and cornstarch (50%)
[3]Control (100% soybean oil); CLA (40% soybean oil and 60% CLA supplements)
[4]Cellulose (100%)
[5]Mineral and vitamins (100%)
[6]Non-detectable
[7]Unidentifiable fatty acids

At the end of the trial, animals were euthanized using CO_2 asphyxiation and blood was collected by cardiac puncture and serum was separated by centrifugation and stored at –80°C until analysis. Hearts, spleens and livers were removed and immediately weighed. Livers were stored at –80°C for fatty acid analysis and hearts and spleens were utilized for a different experiment. Gastrointestinal (GI) food contents were removed and the GI tracts were added back to the carcass and animals were freeze-dried (Virtis, Gardiner, NY). Livers from all animals and spleens from infected animals were freeze-dried separately. Freeze-dried carcasses were homogenized, along with dry ice, using a

food processor (Cuisinart Mini-Prep Plus Processor, East Windsor, NJ) and freeze-dried livers were ground using a mortar and pestle. The carcass homogenate was used to determine dry matter, ash, protein and fat percent.

Body Composition Analysis

A detailed description of these techniques has been described earlier (Sanders et al., 2004) but briefly, for ashing, tissue (~ 0.5 g) was dried overnight in a 100^{o}C oven, weighed, placed in a muffle furnace for 6 h at 550^{o}C, then re-weighed. Protein analysis was performed using a FP-528 Nitrogen Determinator (Leco Corp, St. Joseph, MI) using ~ 0.1 g of tissue. Total N content was multiplied by a correction factor of 6.25 to obtain protein content. Both ash and protein analyses were performed in triplicate. Percent fat was determined using a modified Folch method (Folch et al., 1956). Briefly, a 2:1 chloroform:methanol solution was added to tissue (0.75 g), vortexed for 5 min and centrifuged (400 x g). Supernatant was filtered through a Buchner funnel using a #1 Whattman paper. To filtrate, 0.58% NaCl solution was added, mixed, re-centrifuged (400 x g) and the top layer was removed and discarded. The lower layer was dried under N until less than 8 ml remained, then transferred to a pre-weighed extraction tube and dried down completely. Fat extractions were performed in duplicate. A separate extraction was performed for fatty acid analyses.

Fatty Acid Analysis

Fatty acid methyl esters from carcass and liver lipids were prepared and transmethylated as previously described (Sanders et al., 2004). Fatty acid methyl esters were quantified using a gas chromatograph (Hewlett Packard GC system 6890;

Wilmington, DE) equipped with a flame ionization detector and a CP-7420 fused silica capillary column (100 m ± 0.25 mm i.d. with 0.2-μm film thickness; Varian, Walnut Creek, CA). Besides a split ratio of 50:1, gas chromatograph temperature, ramping, inlet and detector temperatures, along with peak identification were as previously described (Sanders et al., 2004).

Serum Analysis

Serum concentrations of insulin, leptin, resistin, IL-6, plasminogen activator inhibitor-1 (PAI-1) and monocyte chemoattractant protein-1 (MCP-1) were quantified by LINCO Research (St. Charles, MO.) using the mouse serum adipokine panel of a LINCO*plex*™ Multiplex Immunoassay Kit. Although multiplex assays are comparable in sensitivity to other detection methods (i.e. ELISAs) caution should be used when comparing cytokine absolute values between assays (Khan et al., 2004).

Statistical Analyses

Data were statistically analyzed using the PROC-MIXED procedure of SAS. Data are presented as least square means ± SEM and considered significant when main effects were less then $P < 0.05$. Correlations were completed using a Pearson analysis in Minitab.

Results

Although similar at the onset (34.3 ± 2.2 g) and at the end of the experiment (33.8 ± 1.4 g), control mice had gained weight while CLA fed mice lost weight (1.6 vs. -2.6 g; $P < 0.05$). CLA fed mice had increased carcass water (56 vs. 71%; $P < 0.01$), decreased ($P < 0.01$) whole carcass (minus the heart, spleen and liver) fat content (26.9 vs. 55.0%),

increased (P <0.01) protein content (56.4 vs. 28.5%) and increased (P <0.01) ash content (12.4 vs. 7.6%). On a carcass yield basis, CLA decreased (P <0.01) the quantity of carcass fat by 73% (8.1 vs. 2.4 g) and increased (P <0.01) the amount of carcass protein by 14% (3.8 vs. 4.4 g), but did not alter the amount of ash (1.0 g). CLA did not alter heart or spleen weight but did increase (P <0.01) liver weight by 38% (1.9 vs. 3.0 g; Table 2.3).

The CLA supplemented diet increased (P <0.01) the content of *cis*-9, *trans*-11 (<0.1 vs. 3.7%) and *trans*-10, *cis*-12 CLA (<0.1 vs. 2.3%; Table 2.4) in carcass lipids. CLA decreased the carcass content of 18:2 (P =0.02) and 18:3 (P <0.01; 21 and 56%, respectively). CLA increased (P <0.01) the liver lipid content of *cis*-9, *trans*-11 (<0.1 vs. 1.5%; Table 2.5) and *trans*-10, *cis*-12 CLA (<0.1 vs. 0.5%).

Serum leptin concentrations were decreased (P <0.05) in CLA fed mice compared to controls (1.3 vs. 9.6 ng/ml; Table 2.6). PAI-1 (P <0.05) and insulin (P <0.08) levels were increased (3.8 vs. 6.4 ng/ml and 1.2 vs. 2.3 ng/ml, respectively) and IL-6 tended (P =0.137) to be increased (52.4 vs. 133.4 pg/ml) in CLA fed mice compared to control. Treatment had no effect on circulating resistin and MCP-1 levels (Table 2.6).

TABLE 2.2: Body composition of aged mice treated with control or CLA supplemented diet

Variable		Treatment		SEM	*P*
		Control	CLA		
Water	%	55.54	71.14	2.29	<0.01
	g	19.54	22.38	2.29	<0.01
Protein[1]	%	28.52	56.36	3.04	<0.01
	g	3.81	4.40	0.13	<0.01
Fat[1]	%	54.97	26.87	3.66	<0.01
	g	8.93	2.37	1.07	<0.01
Ash[1]	%	7.64	12.37	0.79	<0.01
	g	1.02	0.96	0.03	0.20

[1]Values are on a dry matter basis

TABLE 2.3: Wet organ weights and liver composition of aged mice treated with control or CLA supplemented diet

Organ (g)	Treatment		SEM	*P*
	Control	CLA		
Heart	0.16	0.16	0.01	0.57
Spleen	0.27	0.38	0.07	0.22
Liver	1.89	3.04	0.14	<0.01
Lipid[1] %	25.51	34.68	3.34	0.07
% Dry Matter	31.52	35.76	1.61	0.08
% BW	5.34	9.70	0.44	<0.01

[1]Values are on a dry matter basis

TABLE 2.4: Carcass fatty acid profile of aged mice treated with control or CLA supplemented diet

	Treatment			
Fatty acid	Control	CLA	SEM	*P*
	……g/100 g fatty acid…….			
C14:0	1.27	0.93	0.06	<0.01
C16:0	17.00	16.36	0.71	0.54
C16:1	6.84	2.28	0.53	<0.01
C18:0	4.14	5.16	0.63	0.27
C18:1*c*9	38.50	35.76	1.68	0.27
C18:1*c*11	2.84	3.32	0.24	0.18
C18:2	21.51	17.03	1.27	0.02
C18:3	1.01	0.45	0.07	<0.01
C20:0	0.15	0.13	0.03	0.61
C20:1	0.56	0.75	0.05	0.02
C20:2	0.15	0.14	0.02	0.80
C20:3	0.28	0.28	0.06	0.92
C20:4	1.58	2.66	0.49	0.14
C22:6	1.67	2.50	0.65	0.39
CLA				
*c*9,*t*11	0.11	3.72	0.34	<0.01
*t*10,c12	<0.01	2.31	0.27	<0.01
Unknown	2.74	6.38	0.69	<0.01

TABLE 2.5: Liver fatty acid profile of aged mice treated with control or CLA supplemented diet

	Treatment			
Fatty acid	Control	CLA	SEM	*P*
	…….g/100 g fatty acid…….			
C14:0	0.57	0.59	0.03	0.78
C16:0	28.62	25.74	1.89	0.30
C16:1	2.39	3.18	0.26	0.04
C18:0	12.43	7.54	1.43	0.03
C18:1*c*9	29.85	35.25	3.04	0.23
C18:1*c*11	3.41	5.75	0.52	<0.01
C18:2	8.84	7.03	1.01	0.22
C18:3	0.31	0.13	0.04	<0.01
C20:0	0.32	0.11	0.23	<0.01
C20:1	0.40	0.94	0.09	<0.01
C20:2	0.20	0.14	0.04	0.34
C20:3	0.40	0.58	0.14	0.38
C20:4	5.37	3.94	0.79	0.22
C22:6	3.02	2.14	0.52	0.24
CLA				
*c*9,*t*11	0.11	1.53	0.20	<0.01
*t*10,c12	<0.01	0.45	0.52	<0.01
Unknown	4.36	5.30	0.59	0.28

TABLE 2.6: Metabolic hormone and adipokine concentrations in serum of aged mice treated with control or CLA supplemented diet

	Treatment			
Variable	Control	CLA	SEM	*P*
Insulin (ng/ml)	1.2	2.3	0.4	0.07
Leptin (ng/ml)	9.6	1.3	2.2	0.02
Resistin (ng/ml)	4.6	5.2	7.0	0.56
IL-6 (pg/ml)	52.4	133.4	36.0	0.13
MCP-1 (pg/ml)	95.7	86.7	13.7	0.65
PAI-I (ng/ml)	3.8	6.4	8.6	0.04

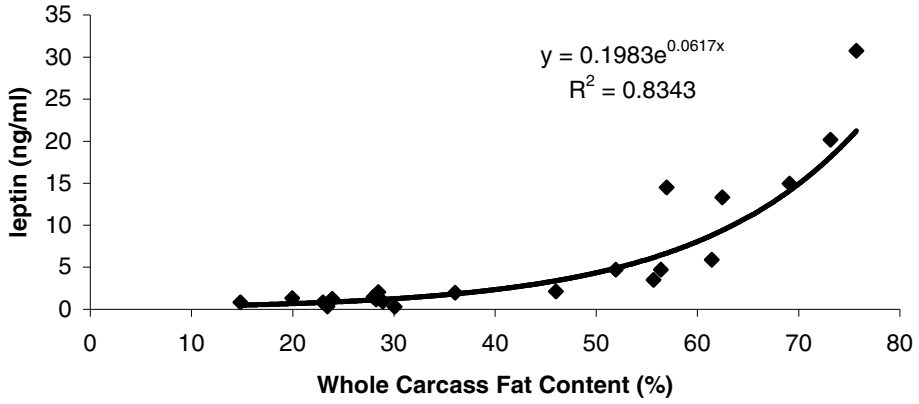

FIGURE 2.1: Correlation between carcass percent fat and circulating leptin levels in geriatric mice fed a control or CLA supplemented diet. Points represent values for individual animals. Regression line is shown for analyses that include all data points. Pearson's correlation coefficient, based on all data points, is shown.

Discussion

Sarcopenia is a well documented phenomenon that occurs to some degree in all aging individuals independent of activity level, dietary habits or disease state (Roubenoff and Hughes, 2000). In 2000, the estimated healthcare costs attributed to sarcopenia alone was $18.5 billion (Janssen et al., 2004). Additionally, the prevalence of obesity, a disease with healthcare costs of approximately $117 billion/year (Stein and Colditz, 2004), is

increasing in the elderly population (Arterburn et al., 2004). The combination of these conditions, termed sarcopenic-obesity, exacerbates the debilitating effects, and likely the costs of each condition alone.

Similar to previous CLA trials in growing rodents (see reviews, Pariza et al., 2001; House et al., 2005), dietary CLA markedly (51%) decreased the fat content in these extremely old female mice (Table 2.2). A recent report indicated that CLA was ineffective at reducing body fat in adult (4 mo) male rats and the authors suggested that CLA effectiveness may be age dependent (Patureau Mirand et al., 2004). Clearly, adipose tissue in the aged female mice in our study was extremely sensitive to CLA (Table 2.2). Reasons for the discrepancies between the two trials are not entirely clear as the dose of mixed CLA isomers, length of feeding and determination of body composition were similar among trials. One methodological difference that may contribute to the inconsistency is the difference in animal gender and species, as it has been suggested that females are more sensitive to CLA than males (Park et al., 1997) and mice are more sensitive than rats (Wahle et al., 2004).

Although most rodent studies report an increase in carcass protein content with CLA feeding (Park et al., 1997; Park et al., 1999; Peters et al., 2001), the actual amount of carcass lean tissue is frequently unaffected. This increase in protein content is an artifact of arithmetic as the percentage of carcass fat is markedly reduced. However, in addition to an increase in carcass protein content (Table 2.2), we also detected an impressive increase (14%) in the quantity of carcass protein (Table 2.2). Unfortunately we did not determine body composition prior to experiment initiation, so we are unable to

conclude whether CLA caused an increase in lean tissue mass, as has been shown in growing pigs (Ostrowska et al., 2003), or prevented muscle wasting as has been shown in cachectic models (Yang and Cook, 2003; Graves et al., 2005). Regardless, the improvement in muscle mass in this aged model is impressive and warrants further investigation.

Consistent with previous trials (Park et al., 1997; Peters et al., 2001; Sanders et al., 2004) we measured marked CLA-induced increases in both the content and quantity of carcass water (Table 2.2). A large portion of body water is associated with muscle (~70%) and this increase agrees with the increase in the quantity of carcass protein (Table 2.2). Feeding CLA also dramatically (62%) enhanced the carcass ash content and this agrees with others (Park et al., 1997). Presumably, the carcass ash content closely reflects carcass bone percentage; however the actual quantity of carcass ash did not differ between treatments and the increase in ash content was a consequence of arithmetic.

CLA had no effect on either heart or spleen weight (Table 2.3) and this agrees with previous reports (Sanders et al., 2004; Kelley et al., 2006). CLA increased the wet weight (37.8%) and fat content (26.4%) of the liver (Table 2.3) and this is consistent in the rodent literature (Takahashi et al., 2003; Sanders et al., 2004; Kelley et al., 2006). Increased liver lipid in the CLA fed mice accounted for ~ 50% of the increase in weight and water content (~ 43%) accounted for much of the rest. It has been hypothesized that an increase in hepatic lipogenesis, and subsequently liver steatosis, is the result of CLA induced hyperinsulinemia (Poirier et al., 2005) as insulin is a potent stimulator of lipogenesis (Saltiel and Kahn, 2001). However, we previously demonstrated that CLA

fed Zucker rats had reduced insulin levels, but enhanced liver lipid content, suggesting the increase in hepatic fat content is insulin-independent (Sanders et al., 2004). It appears CLA directly increases hepatic lipogenesis through enhanced expression of the mitochondrial citrate carrier and key lipogenic enzymes (i.e. ACC and FAS; Ferramosca et al., 2006). This is paradoxical, as a consistent mechanism in which CLA decreases adipose and mammary lipid synthesis includes a coordinated reduction in key lipogenic enzyme expression (Baumgard et al., 2002; House et al., 2005). Although not entirely clear, the tissue specific responses may be due to differences in nuclear transcription factors and CLA's affinity for them. For example, the primary peroxisome proliferator-activated receptor (PPAR) in adipose is PPARγ while PPARα predominates in hepatic tissues (Ferre, 2004) and CLA has been shown to moderate both differently (Khan and Vanden Heuvel, 2003).

Both *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers were incorporated into whole carcass and liver lipids; however, despite being fed in equal quantities, the *trans*-10, *cis*-12 isomer accumulated less in carcass and liver lipids (38 and 71%, respectively) than the *cis*-9, *trans*-11 CLA isomer, and this agrees with previous reports (Sanders et al., 2004; Kelley et al., 2006). The exact reason why the two isomers are different with regards to incorporation is not known but may be due to preferential metabolism of the *trans*-10, *cis*-12 CLA isomer because of their distinct structural dissimilarities (Kramer et al., 1998). Evaluating the effects of CLA on whole carcass fatty acid profiles is difficult, as this includes lipids from different adipose depots and different tissues (i.e. muscles, skin, organs, etc.), but consistent with hepatic lipids, linoleic acid content was decreased

(Table 2.5; 20.5%). In contrast to previous reports (Sanders et al., 2004; Kelley et al., 2006) we did not detect changes in hepatic very long PUFAs (VLPUFA), including arachidonic acid. *Trans*-10, *cis*-12 is a potent down regulator of the enzymes (desaturation and elongation) necessary for VLPUFA production (Bretillon et al., 1999; Eder et al., 2002) and the lack of response with regard to the VLPUFA content in the current experiment is difficult to explain. However, aging is associated with decreased activity of desaturation and elongation enzymes in aged animals (Hrelia et al., 1989) and natural reductions in these systems may have limited our ability to detect CLA's typical effects.

Although dietary CLA and specifically the *trans*-10, *cis*-12 isomer improves a variety of parameters associated with glucose homeostasis in diabetic models (Ryder et al., 2001; Henriksen et al., 2003) it has been shown to cause mild insulin resistance in non-diabetic models (DeLany and West, 2000). This is consistent with our data as serum insulin levels tended to increase (48%) with CLA supplementation (Table 2.6). Incidentally, the CLA-induced hyperinsulinemia may partially explain the enhanced carcass protein mass (Table 2.2) as insulin is a well-known anabolic molecule with regards to skeletal muscle (Allen, 1988).

Despite the increased insulin levels, resistin concentration did not differ between treatments suggesting the CLA induced hyperinsulinemia is independent of the resistin system, as has been shown in recent reports (Axelsson et al., 2006; Haluzik et al., 2006). CLA decreased leptin concentrations (Table 2.6), which is consistent with a decrease in fat mass (Figure 2.1) and has been previously reported (Bhattacharya et al., 2005). PAI-1

is an adipokine involved in haemostasis and is elevated in obese animals (Trayhurn and Wood, 2005). However, despite the decrease in carcass fat mass, CLA fed mice had increased PAI-1 levels (Table 2.6) and PAI-1 was negatively correlated with carcass fat ($y= -108.8x + 9584.1$; $R^2= 0.46$; $P <0.01$). This may be explained by the fact that fatty livers, a result from feeding CLA (Table 2.3), overproduce PAI-1 (Yki-Jarvinen, 2005). We detected a trend ($P= 0.13$) for CLA supplemented animals to have higher IL-6 levels (Table 6) though this is difficult to interpret as previous studies have shown CLA to both increase (Poirier et al., 2005; LaRosa et al., 2006) and decrease (Yu et al., 2002; Changhua et al., 2005) IL-6 expression and secretion. Additionally, CLA had no effect on MCP-1, an adipokine potentially involved in atherosclerosis (Chen et al., 2005), and this agrees with previous CLA research (Schleser et al., 2006).

Cachexia, or muscle wasting associated with a disease state, shares some biological similarities with sarcopenia but is thought to be attributed primarily to the catabolic action of inflammatory cytokines, including TNF-α (Argiles et al., 2005). CLA reduces TNF-α expression *in vitro* (Yu et al., 2002) and *in vivo* (Roche et al., 2002) and decreases circulating levels (Yang and Cook, 2003). This TNF-α lowering effect is thought to partially explain CLA's protective properties against muscle wasting in cachectic models (Graves et al., 2005). Although not measured in the current study, CLA may have decreased TNF-α concentration (via a variety of potential mechanisms) and this may partially explain why CLA fed mice had more lean tissue (Table 2.2) in this aged model.

In conclusion, dietary CLA decreased carcass fat and increased carcass protein mass in aged female mice. The seemingly beneficial changes CLA had on body composition warrant further investigation of CLA's potential to alleviate sarcopenia and sarcopenic-obesity in the elderly. Further research is required to examine CLA's effect on skeletal muscle specifically and investigate possible mechanisms of action.

CHAPTER 3

EFFECTS OF CONJUGATED LINOLEIC ACID (CLA) ON BODY COMPOSITION PARAMETERS IN A MURINE AIDS CACHEXIA MODEL

Abstract

CLA alleviates cachectic symptoms in some experimental models. In a 2x2 factorial design, 24 female 1 mo old C57BL/6 mice were injected with either LP-BM5 murine leukaemia retrovirus (n = 12; to induce cachexia) or saline (n = 12) as a control. Mice were fed diets containing 0 or 1.68% CLA (50/50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12) for 88 d. There was no effect of CLA on average daily gain, however, MuLV infection increased body weight. CLA increased the content (41%) and mass (14%) of carcass protein in control mice but had no effect on the protein yield in infected mice. Both CLA and infection markedly reduced the content (72 and 51%, respectively) and amount (83 and 60%, respectively) of carcass fat and CLA decreased total carcass fat (>50%) in infected mice. Carcass water content and yield were increased by the main effects of infection and CLA, but in infected mice CLA had no effect on water variables. Infection decreased heart weight (34%), kidney weight (29%) and increased spleen weight (94%) while CLA had no effect on the mass of these organs. CLA increased liver weight by 69% in controls and 33% in infected mice and increased hepatic lipid content by 65% in controls and 37% in infected mice. Feeding CLA increased the carcass and liver content of *cis*-9, *trans*-11 (<0.1 vs. 2.3% and <0.1 vs. 1.7%, respectively) and *trans*-10, *cis*-12 CLA (<0.1 vs. 1.6% and <0.1 vs. 0.5%, respectively). CLA decreased 18:2 content in carcass (46%) and liver (66%) lipids but differentially regulated 20:4 levels in carcass and liver lipids (66 % increase and 65% decrease, respectively). Infection

increased the content of most LCPUFA (i.e. ≥20:2) in both carcass and liver lipids. In conclusion, CLA reduced body fat in infected mice but was unable to increase carcass protein as it did in controls.

Introduction

Cachexia is a syndrome of muscle atrophy that is associated with a variety of diseases, including cancer, congestive heart failure, and Acquired Immunodeficiency Syndrome (AIDS; Freeman et al., 1994). It is characterized by a decrease in skeletal muscle and adipose tissue mass and this differs from starvation where adipose is markedly mobilized and muscle mass is relatively conserved (Tisdale 1997). In mammals, skeletal muscle is the principal amino acid reservoir which can be oxidized for energy or used as precursors to support the synthesis of immune system molecules, particularly acute phase proteins (Kotler, 2000). A severe decrease in skeletal muscle mass, as is observed in cachexia, contributes to the morbidity of the underlying disease state (Lecker and Goldberg, 2002). Although not clearly defined, cachexia is thought to reduce muscle mass by increasing muscle proteolysis mediated via inflammatory cytokines (i.e. TNF-α; Lecker and Goldberg, 2002).

Conjugated linoleic acids (CLA) are geometrically unique isomers of linoleic acid that are found naturally in dairy products and ruminant meat as a result of polyunsaturated fatty acid rumen biohydrogenation (Bauman et al., 1999). Dietary CLA has been shown to modulate the immune system, as well as prevent cachexia in some experimental animal models. For example, dietary CLA prevented muscle wasting in tumor-bearing mice (Graves et al., 2005) and in endotoxin induced cachectic mice, chicks

and rats (Cook et al., 1993; Yang and Cook, 2003). In addition, dietary CLA reduced weight loss in a rodent model for systemic lupus erythematosus (Yang et al., 2000).

Current study objectives were to determine if dietary CLA could attenuate cachexia variables in mice infected with a retrovirus (LP-BM5 Murine Leukemia Virus; MuLV), a model that shares some symptom similarities with human AIDS (Sklan et al., 2000).

Materials and Methods

Animals

All protocols and procedures were approved by the University of Arizona Institutional Animal Care and Use Committee. Twenty-four female growing (1 mo) C57BL/6 mice were utilized to determine dietary CLA supplementation effects on whole animal body composition parameters. Twelve mice were inoculated intraperitoneally with either LP-BM5 murine leukaemia retrovirus (MuLV), which produces pathologies that are similar to HIV infection, including lymphadenopathy, splenomegaly, hypergammaglobulinemia, T-and B-cell dysfunctions, late appearance of B-cell lymphomas, and opportunistic infections (Sklan et al., 2000) or with saline (0.1 ml) as a control. The LP-BM5 MuLV mixture was administered in 0.1 ml minimal essential medium (MEM) with an esotropic titre of 4.5 $\log_{10}$ plaque forming units per 10^{-3}/l, which induces disease as previously described (Wang and Watson, 1994; Liang et al., 1996).

Experimental protocol and treatments

Mice were divided into four groups; non-infected mice fed a control diet (n=6), non-infected mice fed a diet supplemented with CLA (n=6), infected mice fed a control

diet (n=7) and infected mice fed a diet supplemented with CLA (n=5). All mice were fed a standard iso-energetic and iso-nitrogeous diet (Dyets Inc., Bethlehem, PA) with the control diet containing no CLA and the supplemented diet containing 1.68% of a 50/50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA methyl esters (Table 3.0; BASF AG, Ludwigshafen, Germany). The control diet was supplemented with soybean oil to remain iso-energetic and contained a similar amount of lipid as the CLA treatment (Table 3.1). Mice were housed 3 or 4 to a cage and fed respective diets for 88 d. Feed intake was recorded three times per week and body weights weekly.

After 88 days on treatment, animals were euthanized using CO_2 asphyxiation and hearts, spleens and livers were removed and weighed. Gastrointestinal (GI) food contents were removed from the entire tracts and the GI tract and carcass were freeze-dried (Virtis, Gardiner, NY). Livers and the spleens were freeze-dried separately. Freeze-dried carcasses were homogenized, along with dry ice, using a food processor (Cuisinart Mini-Prep Plus Processor, East Windsor, NJ) and freeze-dried livers were ground using a mortar and pestle. The carcass homogenate was used to determine dry matter, ash, protein and fat percent.

TABLE 3.0: Fatty acid profile of CLA supplement diet

Fatty acid	% of supplement
C14:0	0.10
C16:0	6.96
C16:1	0.12
C18:0	5.32
C18:1 *c*-9	25.93
C18:2 *c*-9,*c*-12	2.42
C18:2 *c*-9,*t*-11	26.27
C18:2 *t*-10,*c*-12	25.78
Other CLA isomers	2.09
Unknown[1]	5.01

[1]Unidentifiable fatty acids

TABLE 3.1: Chemical composition and fatty acid profile of control and CLA supplemented diet

	Treatment	
	Control	CLA
Component	%	
Protein[1]	18.3	18.3
Carbohydrate[2]	67.1	67.1
Fat[3]	5.0	5.0
Fiber[4]	5.0	5.0
Ash[5]	4.5	4.5
Fatty Acid	%	
14:0	<0.1	0.1
16:0	10.2	6.0
16:1 c-9	<0.1	0.1
18:0	4.5	4.5
18:1 c-9	22.7	22.4
18:2 c-9,c-12	54.8	2.4
18:2 c-9,t-11	ND[6]	31.0
18:2 t-10,c12	ND	30.8
Other CLA isomers	ND	1.2
18:3 c-9,c-12,c-15	7.8	ND
Unknown[7]	<0.1	1.6

[1]Vitamin-free casein (86%) and DL-Methionine (19%)
[2]Sucrose (5%) and cornstarch (50%)
[3]Control (100% soybean oil); CLA (40% soybean oil and 60% CLA supplements)
[4]Cellulose (100%)
[5]Mineral and vitamins (100%)
[6]Non-detectable
[7]Unidentifiable fatty acids

Body composition analysis

A detailed description of these techniques has been described earlier (Sanders et al., 2004; Baker et al., 2005). Briefly, for ashing, homogenate tissue (0.5 g) was dried overnight in a 100^{o}C oven, weighed, placed in a muffle furnace for 6 h at 550^{o}C, then re-weighed. Protein analysis was performed in triplicate using a FP-528 Nitrogen Determinator (Leco Corp, St. Joseph, MI) using 0.1 g of tissue. Total N content was multiplied by a correction factor of 6.25 to obtain protein concentrate. Both ash and protein analyses were performed in triplicate. Percent fat was determined using a modified Folch method (Folch et al., 1956). Briefly, a 2:1 chloroform:methanol solution was added to tissue (1.0 g), vortexed for 5 min and centrifuged (400 x *g*). Supernatant was filtered through a Buchner funnel using a #1 Whattman paper and a NaCl solution (0.58%) was added to the filtrate which was re-centrifuged (400 x *g*) and the top layer was removed and discarded. The lower layer was dried under N until less than 8 mL remained, then transferred to a pre-weighed extraction tube and dried down completely. Fat extractions were performed in duplicate. A separate extraction was performed for fatty acid analyses.

Fatty acid analysis

Fatty acid methyl esters from carcass and liver lipids were prepared by the transmethylation procedure described by Christie (1982) as previously described (Sanders et al. 2004). Briefly, hexane (2 ml, HPLC grade) was added to 40 mg of lipid followed by 40 µl of methyl acetate. After vortexing, 40 µl of methylation reagent (1.75 ml methanol and 0.4 ml of 5.4 mol/L sodium methylate) was added, the mixture was re-

vortexed and then allowed to react for 10 min; then 60 μl termination reagent (1 g oxalic acid in 30 ml diethyl ether) and ~ 200 mg of calcium chloride was added and allowed to stand for 60 min. Samples were centrifuged at 2,400 x g at 4^{o} C for 5 min. Following centrifugation, the liquid portion was transferred to a labeled GC vial and stored at –20^{o} C. Fatty acid methyl esters were quantified using a gas chromatograph (Hewlett Packard GC system 6890; Wilmington, DE) equipped with a flame ionization detector and a CP-7420 fused silica capillary column (100 m ± 0.25 mm i.d. with 0.2-μm film thickness; Varian, Walnut Creek, CA). Gas chromatograph temperature, ramping, inlet and detector temperatures, along with peak identification procedures were as previously described (Sanders et al., 2004; Baker et al., 2005) with a change in the split ratio to 50:1.

Statistical analyses

Data were statistically analyzed using the PROC-MIXED procedure of SAS (1992; Gary, NC). Data are presented as Least Square Means ± SEM and considered significant when main effects were less then $P < 0.05$.

Results

MuLV infection increased (P <0.01; 57.3%) average daily gain and CLA had no effect on body weight and there was an interaction (P <0.01) as CLA had no effect on BW in controls but decreased weight gain in infected mice. Both infection and CLA increased (P <0.01) carcass water (content and grams; Table 3.2) and protein (content and grams; Table 3.2) and there was an interaction (P <0.01) as CLA was much more effective at enhancing water and protein variables in controls compared to infected mice. Infection and CLA decreased (P <0.01) carcass fat (content and grams; Table 3.2) and

there was an interaction (P <0.01) as the effects were much more pronounced in controls. Neither treatment effected carcass ash variables. CLA had no effect on spleen, heart or kidney weight, but did increase (P <0.01) wet liver weight (55.1%) and liver fat content (55.9%; Table 3.3). Infection decreased (P <0.01) heart weight (34.0%), kidney weight (29.2%) and increased (P <0.01) spleen weight (94.1%). Infection also increased (P <0.01) liver wet weight (17.8%) and the liver fat content (41.9%; Table 3.3).

The CLA supplemented diet increased (P <0.01) the content of carcass *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Table 3.4). Feeding CLA decreased (P <0.01) the carcass content of 18:2 (10.3 vs. 19.0%) and increased (P <0.01) the carcass content of 20:4 (9.3 vs. 3.2%). Feeding CLA increased (P <0.01) the liver content of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Table 3.5). CLA decreased (P <0.01) the liver content of 18:2, 18:3 and 20:4 (65.8, 71.0 and 65.4%, respectively).

MuLV infection did not have an impact on carcass fatty acid profiles, but increased (P <0.01) the liver content of arachidonic acid (20:4; 66.9%) and decreased 18:1 *c*-9 (75.5%; Table 3.5).

TABLE 3.2: Growth rate and body composition parameters in control vs. MuLV infected mice treated with control and CLA supplemented diet

		Non-infected		MuLV			*P*-values		
		Cont	CLA	Cont	CLA	SEM	CLA	MuLV	CxM
ADG (g/d)[1]		0.04[c]	0.06[c]	0.13[a]	0.10[b]	<0.1	0.57	<0.01	<0.01
Body Composition									
Water	%	60.29[a]	71.90[b]	73.01[b,c]	74.87[c]	0.9	<0.01	<0.01	<0.01
	g	11.62[c]	14.98[b]	18.31[a]	17.66[a]	0.5	0.01	<0.01	<0.01
Protein[2]	%	44.33[c]	75.26[a]	65.83[b]	76.10[a]	1.8	<0.01	<0.01	<0.01
	g	3.04[b]	3.55[a]	3.71[a]	3.59[a]	0.1	0.05	<0.01	<0.01
Fat[2]	%	35.18[a]	9.74[c]	16.99[b]	8.32[c]	1.3	<0.01	<0.01	<0.01
	g	2.46[a]	0.46[c]	0.99[b]	0.39[c]	0.1	<0.01	<0.01	<0.01
Ash[2]	%	10.85[d]	18.32[a]	14.63[c]	16.51[b]	0.6	<0.01	0.10	<0.01
	g	0.74[c]	0.86[a]	0.82[a,b]	0.77[b]	<0.1	0.17	0.80	<0.01

[1]Average daily body weight gain
[2]Values are on a dry matter basis

TABLE 3.3: Wet organ weights of control vs. MuLV infected mice treated with control and CLA supplemented diet

	Non-infected		MuLV			*P*-values		
Organ(g)	Cont	CLA	Cont	CLA	SEM	CLA	MuLV	CxM
Heart	0.14	0.13	0.09	0.08	<0.1	0.43	<0.01	0.90
Kidneys	0.30	0.30	0.22	0.20	<0.1	0.52	<0.01	0.64
Spleen	0.09	0.11	1.78	1.61	0.2	0.65	<0.01	0.53
Liver	1.13[d]	3.59[a]	1.53[c]	2.35[b]	0.1	<0.01	<0.01	<0.01
Lipid%[1]	16.83[c]	48.23[a]	14.64[c]	23.19[b]	1.8	<0.01	<0.01	<0.01
%BW[1]	5.86[c]	17.19[a]	6.29[c]	10.16[b]	0.4	<0.01	<0.01	<0.01

[1]On a dry matter basis

TABLE 3.4: Carcass fatty acid profile of control vs. MuLV infected mice treated with control and CLA supplemented diet

	Non-infected		MuLV			*P*-values		
Fatty Acid	Cont	CLA	Cont	CLA	SEM	CLA	MuLV	CxM
	……g/ 100g fatty acid ……							
C12:0	0.10	0.02	0.14	0.03	<0.1	0.03	0.58	0.76
C14:0	1.31[a]	0.47[c]	1.11[a]	0.64[b]	0.1	<0.01	0.82	0.01
C14:1	0.13	0.03	0.10	0.15	0.1	0.83	0.66	0.49
C15:0	0.08	0.12	0.09	0.16	<0.1	<0.01	0.01	0.29
C16:0	14.70	18.69	16.49	19.40	0.4	0.01	<0.01	0.24
C16:1	7.00[a]	1.96[c]	4.40[b]	2.71[c]	0.4	<0.01	0.03	<0.01
C17:0	0.13	0.22	0.19	0.24	<0.1	0.02	0.14	0.38
C18:0	3.37	10.39	7.85	11.22	1.0	<0.01	0.02	0.09
C18:1c9	39.16	21.55	30.22	18.08	2.5	<0.01	0.03	0.30
C18:1c11	3.17[c]	3.96[b]	2.89[c]	4.57[a]	0.1	<0.01	0.26	<0.01
C18:2	20.65	11.11	17.40	9.46	0.7	<0.01	<0.01	0.28
C18:3	1.26	1.13	1.19	1.37	0.1	0.87	0.56	0.31
C20:0	0.10	0.18	0.20	0.33	<0.1	<0.01	<0.01	0.29
C20:1	0.25	0.25	0.25	0.21	0.1	0.85	0.87	0.86
C20:2	0.30	0.13	0.41	0.38	0.1	0.19	0.03	0.37
C20:3	0.20	0.16	0.13	0.28	<0.1	0.24	0.58	0.07
C20:3est[1]	1.09	0.92	0.41	0.66	0.2	0.83	0.03	0.29
C20:4	1.32	9.09	4.99	9.46	1.1	<0.01	0.10	0.17
C22:0	nd	nd	nd	nd	-	-	-	-
C22:1	nd	0.38	0.14	0.15	0.1	0.93	0.04	-
C22:4	0.16	1.04	0.83	1.81	0.2	<0.01	<0.01	0.78
C22:5n6	0.10[c]	0.65[b]	0.37[b,c]	1.36[a]	0.1	<0.01	<0.01	0.04
C22:5n3	0.12	0.75	0.53	1.01	0.2	<0.01	0.05	0.63
C22:6	2.01[c]	8.79[a]	5.50[b]	7.64[a,b]	1.1	<0.01	0.29	0.05
C24:0	nd	0.38	0.21	0.56	0.1	<0.01	0.04	-
CLA								
*c*9*t*11	0.07	2.53	0.08	2.00	0.3	<0.01	0.33	0.33
*t*10*c*12	nd	1.85[a]	<0.01[b]	1.26[c]	0.2	<0.01	<0.01	-
unknown	4.22	3.72	4.27	5.00	0.8	0.89	0.41	0.45

[1]eicosatrienoic acid

TABLE 3.5: Liver fatty acid profile of control vs. MuLV infected mice treated with control and CLA supplemented diet

	Non-infected		MuLV			*P*-values		
Fatty Acid	Cont	CLA	Cont	CLA	SEM	CLA	MuLV	CxM
	……g/ 100g fatty acid ……							
C12:0	0.02	0.02	0.07	0.11	<0.1	0.46	<0.01	0.34
C14:0	0.51[a]	0.61[a]	0.17[b]	0.55[a]	0.1	<0.01	<0.01	0.02
C15:0	0.07	0.04	0.09	0.07	<0.1	<0.01	<0.01	0.06
C16:0	21.04	23.95	21.35	24.79	0.6	<0.01	0.35	0.67
C16:1	2.77[a]	3.86[c]	0.57[a]	2.15[b]	0.3	<0.01	<0.01	0.44
C17:0	0.18	0.04	0.29	0.15	<0.1	<0.01	<0.01	0.95
C18:0	9.87	2.89	18.13	8.90	1.1	<0.01	<0.01	0.31
C18:1c9	31.29	50.22	7.66	26.83	2.2	<0.01	<0.01	0.96
C18:1c11	3.29	6.29	1.84	5.53	0.4	<0.01	0.01	0.40
C18:2	15.15	2.47	14.41	7.64	0.7	<0.01	<0.01	<0.01
C18:3	0.57	1.59	0.21	1.08	0.1	<0.01	<0.01	0.39
C20:0	0.05[a]	0.02[c]	0.03[b]	0.05[a]	<0.1	0.27	0.56	<0.01
C20:1	0.04[c]	0.12[b]	0.18[a]	0.09[b]	<0.1	0.41	<0.01	<0.01
C20:3est[1]	0.84	0.25	0.93	0.53	.06	<0.01	<0.01	0.16
C20:4	6.24[b]	1.29[c]	16.26[a]	6.49[b]	0.9	<0.01	<0.01	0.01
C22:0	0.02[b,c]	0.03[b]	0.04[a]	0.02[c]	<0.1	0.03	0.33	<0.01
C22:4	0.19	0.03	0.66	0.60	0.1	0.31	<0.01	0.61
C22:5n6	0.17[b]	0.05[b]	0.43[b]	1.09[a]	0.1	0.07	<0.01	0.01
C22:5n3	0.15	0.02	0.72	0.84	0.2	1.00	<0.01	0.55
C22:6	4.28[b]	0.65[c]	12.80[a]	3.73[b]	0.5	<0.01	<0.01	<0.01
CLA								
*c*9*t*11	0.06[c]	0.99[b]	0.06[c]	2.33[a]	0.2	<0.01	<0.01	<0.01
*t*10*c*12	nd	0.27	nd	0.69	0.1	-	<0.01	-
unknown	3.22	4.32	3.29	5.89	0.7	0.02	0.24	0.29

[1]eicosatrienoic acid

Discussion

Cachexia has been described as accelerated loss of skeletal muscle in the context of a chronic inflammatory response (Kotler, 2000) and affects patients suffering from a wide variety of chronic diseases including cancer and AIDS. The mobilization of amino acids from muscle for gluconeogenesis, acute-phase protein synthesis, and support of the immune system is beneficial to the patient initially, however prolonged mobilization leads to a delay of recovery and ultimately increased mortality (Hasselgren and Fischer, 2001). Although cachexia is a long and well recognized condition, an effective treatment has yet to be developed. Several studies indicate CLA alleviates cachexia in animal models of diseases including cancer (Graves et al., 2005) and lupus (Yang et al., 2000) and our objective was to investigate the CLA effects on cachexia in a murine AIDS model.

Infection with the LP-BM5 MuLV retrovirus, a retrovirus that shares some symptoms with human AIDS (Sklan et al., 2000), dramatically altered body composition in young mice. Infected animals gained weight during the experimental period which may partially be attributed to increased spleen weight (Table 3.3) and carcass water content (Table 3.2). The increase in carcass water is consistent with the murine AIDS (MAIDS) literature and is a consequence of the lymphadenopathy that is associated with this infection (Araghi-Niknam et al., 1998). CLA had no effect on body weight, which is inconsistent with results from Yang et al. (2000), who reported a decrease in body weight when CLA was fed to mice with systemic lupus erythematosus, and Graves et al. (2005), who reported that CLA increased body weights in tumor-bearing animals. The variability

is likely due to differences in the pathogenesis of the disease model. CLA fed animals also had increased water content (Table 3.2), which is consistent with previous reports (Park et al., 1997; Sanders et al., 2004; Baker et al., 2005) and may be due to enhanced lean tissue deposition.

CLA fed mice had an increased quantity of whole carcass protein (Table 3.2), which suggests that CLA caused an increase in muscle mass as compared to controls, and this is consistent with previous data from our lab (Baker et al., 2005). Other studies have reported a protective effect of CLA against cachexia associated weight and muscle loss (Yang and Cook, 2003; Graves et al., 2005; McCarthy and Graves, 2006). It is hypothesized that CLA decreases the production of inflammatory cytokines, via inhibiting NF-κB and cyclooxygenase-2 (COX-2; Ringseis et al., 2006; Hwang et al., 2007), and the reduction in inflammatory cytokines removes their catabolic effect on muscle protein (Kotler et al., 2000). MuLV infection also increased the total amount of carcass protein (Table 3.2) and this is surprising as we expected infected animals to loose muscle mass as has been previously demonstrated (Morley et al, 2006). Many markers of nutrient status can be affected by the disease itself (Shenkin, 1997) and one explanation for an increase in carcass protein in infected mice, although unlikely, may be due to the increase in circulating cytokines, c-reactive proteins and immune cells, although this was not measured in the current study. Additionally, cachexia results from a chronic inflammatory disease and MuLV infected mice developed MAIDS and were harvested relatively quickly (88 d) after infection. Therefore there may not have been a chronic infection long enough to produce detectable negative effects on muscle mass.

CLA caused a dramatic decrease in carcass fat and this is consistent with studies in growing (House et al., 2005), as well as aged (Baker et al., 2005) mice. MuLV infection also decreased fat mass which is observed in cachectic animals as well as AIDS patients (Wanke, 2004). There was an interaction between CLA and MuLV infection as CLA caused an 81% decrease in fat in control mice but only a 61% decrease in infected mice (Table 3.2).

Both CLA isomers were incorporated into carcass and liver tissue of control and infected mice (Table 3.4 and 3.5, respectively). Although the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers were fed in equal amounts, the *cis*-9, *trans*-11 isomer was incorporated at a higher amount (~ 64% higher in carcass and ~ 200% in the liver). This is consistent with previous studies (Sanders et al., 2004; Baker et al., 2005). The exact reason for the variability is unknown, however, it may be due to preferential metabolism of the *trans*-10, *cis*-12 CLA isomer because of their distinct structural dissimilarities (Kramer et al., 1998).

By inhibiting COX-2, CLA may decrease the production of prostaglandin-E_2 (PGE_2), in turn, decreasing the production of inflammatory cytokines, particularly TNF-α (Graves et al., 2005). Additionally it is suggested that CLA decreases PGE_2 synthesis by decreasing the concentration of arachidonic acid (the precursor for prostaglandin synthesis) in the cell membrane (Ringseis et al., 2006). However, we found CLA to increase the content of arachidonic acid in the carcass (Table 3.4) and decrease the content in the liver (Table 3.5) and this may be due to regional differences in the

expression and/or activity of the elongases and desaturases involved in arachidonic acid synthesis.

CLA caused an increase in liver size and fat content and this is consistent with previous studies (Sanders et al., 2004; Baker et al., 2005; Kelley et al., 2006). There was in interaction between CLA and infection, as the infection reduced the increase in liver weight with feeding CLA (218% increase in controls vs. 54% increase in infected mice). CLA had no effect on spleen, kidney or heart weight which is consistent with rodent studies with growing (Sanders et al., 2004; Kelley et al., 2006) and aged (Baker et al., 2005) animals. MuLV infection decreased heart and kidney weight, and dramatically increased spleen weight (1.78 vs. 0.09 g), and this is a typical consequence of the acute viral infection and immune response (Sklan et al., 2000).

Based on the parameters measured (body weight and body composition), MuLV infection did not induce severe cachexia in these growing mice. Therefore, it is difficult to determine whether CLA was able to alleviate MuLV-induced cachexia. However, feeding CLA resulted in decreased carcass fat and increased carcass protein in growing mice. Also, MuLV retrovirus infection caused significant changes in body composition and organ weights.

CHAPTER 4

EFFECT OF CONJUGATED LINOLEIC ACID (CLA) ON SPECIFIC ADIPOSE DEPOTS AND MUSCLES AND THE CALPAIN SYSTEM IN AGED MALE MICE

Abstract

Conjugated linoleic acid (CLA) slightly increases carcass protein in aged mice (Baker et al., 2005), but the biological mechanism by which this occurs and the specific muscles which are effected are unknown. Aged (22 mo) male C57BL/6 mice were fed isoenergetic diets containing 0 or 1.68% methyl esters of CLA (50/50 *cis*-9, *trans*-11 and *trans*-10, *cis*-12) and mice were harvested at 1, 2, 4, 20 (6/trt) and 30 d (5/trt) post treatment feeding. Six mice were harvested 1 d prior to treatment initiation. CLA reduced (18%) feed intake for the first 7 d, but did not alter intake thereafter. CLA decreased (12%) BW at d 4, but BW did not differ at other time points. Neither time nor CLA affected the wet weight (%BW) of the pectoral, gastrocnemius and soleus muscles nor the gastrocnemius muscle protein content. CLA had no effect on the gastrocnemius muscle m-calpain content or on total calpain activity. CLA decreased (~23%) the wet weight (%BW) of subcutaneous and perirenal fat by d 2 and these depots were reduced (>70%) by d 30. CLA reduced (76%) the epididymal fat pad starting at d 20 and it was nearly immeasurable by d 30. There was no dietary effects on spleen or kidney mass, but CLA increased liver wet weight (%BW) starting on d 20 (87%) and increased heart weight on d 2 (23%), 4 (17%) and 30 (20%). CLA had no effect on muscle weights or components of the skeletal muscle calpain system, but markedly and quickly reduced the mass of adipose depots in these aged male mice. Further investigation is required to

better understand the apparent inconsistencies with regards to CLA's effects on protein and muscle metabolism.

Introduction

Age-associated muscle wasting (sarcopenia) is a serious condition that affects all aging individuals despite their quality of nutrition or activity level. The disease reduces physical ability, decreases autonomy and increases morbidity and mortality (Roubenoff, 2000). Sarcopenia is likely caused by changes in skeletal muscle protein synthesis, protein degradation or both and many mechanisms governing these changes have been proposed, including increased inflammatory cytokines, decreased growth hormone action and decreased insulin sensitivity (Argiles et al., 2005).

Studies evaluating skeletal muscle protein degradation in growing and disease models have demonstrated that the ubiquitin-proteasome system plays a key role in sarcopenia (Kotler, 2000). Muscle loss is positively associated with increases in the muscle specific ubiquitin conjugating enzymes, including atrogin-1 (Costelli and Baccino, 2003). Although the proteasome degrades myofibrillar proteins, it is unclear how the myofibrillar proteins detach from the sarcomere unit, but the calcium dependent protease, calpain, is proposed to be responsible (Bartolli and Richard, 2005).

Conjugated linoleic acids (CLA) are geometrically unique isomers of linoleic acid and are found naturally in dairy products and ruminant meat as a result of polyunsaturated fatty acid rumen biohydrogenation (Bauman et al., 1999). In experimental animals, dietary CLA has beneficial effects on diseases such as cancer, atherosclerosis and diabetes and also consistently reduces body fat (Belury, 2002). The

effects of CLA on lean tissue mass (muscle) however have been less consistent (Patureau Mirand et al., 2006). In cachectic animals (those experiencing disease-induced muscle loss), CLA either prevents muscle wasting or increases muscle mass (Yang and Cook, 2003; Graves et al., 2005) and therefore may have a beneficial effects in sarcopenic animals.

Study objectives were to determine the effect of dietary CLA on the mass of specific skeletal muscles in aged mice. Furthermore it was of interest to determine the effects of CLA on the calpain system as well as atrogin-1 expression in skeletal muscle and the temporal effect on specific adipose depots.

Materials and Methods

Animals

All protocols and procedures were approved by the University of Arizona Institutional Animal Care and Use Committee. Seventy-two aged (22 mo) male C57BL/6 mice (mean initial BW was 28.18 ± 2.8g) were utilized to determine dietary CLA supplementation effects on skeletal muscle and adipose depot mass. The maximum age for male C57BL/6 mice is ~36 mo and the 50% survival age is 26 mo (Turturro et al., 1999).

Experimental protocol and treatments

Mice were randomly divided into two groups and fed a standard iso-energetic and iso-nitrogenous diet (Harlan Teklad, Bethesda, MD) with the control diet containing no CLA and the supplemented diet containing 1.68% of a 50/50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Table 4.0; BASF AG, Ludwigshafen, Germany). The control

diet was supplemented with soybean oil to remain iso-energetic and contained a similar amount of lipid as the CLA treatment (Table 4.1). Mice were housed individually and were fed the respective diets for 30 d. Feed intake was measured every day or every other day and body weights were measured weekly. Mice were euthanized via CO_2 asphyxiation on days 0 (prior to treatment initiation; n=6), 1, 2, 4, 20 (n=6/trt) and 30 (n=5/trt) of the trial.

Tissue collection

Live body weights were recorded immediately prior to sacrifice. Blood was collected immediately post mortem via cardiac puncture, added to a heparinized tube and placed on ice. To collect plasma, blood was centrifuged (3,000 rpm for 10 min at 40˚C) and the plasma fraction was frozen at -80˚C. Mice were skinned and subcutaneous and epididymal fat pads were removed and immediately weighed. The right and left pectoral, soleus and gastrocnemius muscles were removed and remaining hind limb muscles (including the gluteus group, biceps femoris, vastus group, adductor group, semimembranosus, semitendinosus, extensor digitorum longus, tibialis group, flexor digitorum longus and rectus femoris) were removed, pooled and immediately weighed. The liver, spleen, heart and kidneys were also dissected and weighed. All tissues/organs were snap frozen in liquid nitrogen. Adipose tissues and organs were stored at -80˚C and muscles were stored at -140˚C, except for the soleus muscles which were stored in RNAlater® solution (Ambion, Foster City, CA).

TABLE 4.0: Fatty acid profile of CLA supplement

Fatty acid	% of supplement
C14:0	0.10
C16:0	6.96
C16:1	0.12
C18:0	5.32
C18:1 *c*-9	25.93
C18:2 *c*-9,*c*-12	2.42
C18:2 *c*-9,*t*-11	26.27
C18:2 *t*-10,*c*-12	25.78
Other CLA isomers	2.09
Unknown[1]	5.01

[1]Unidentifiable fatty acids

TABLE 4.1: Chemical Composition and fatty acid profile of control and CLA supplemented diet

	Treatment	
	Control	CLA
Component	%	
Protein[1]	18.3%	18.3%
Carbohydrate[2]	67.0%	67.0%
Fat[3]	5.0%	5.0%
Fiber[4]	5.0%	5.0%
Ash[5]	4.5%	4.5%
Fatty Acid	%	
14:0	0.24	0.14
16:0	11.29	7.21
16:1 *c*-9	0.11	0.11
18:0	3.82	4.37
18:1 *c*-9	19.49	21.52
18:2 *c*-9, *c*-12	51.27	14.24
18:2 *c*-9, *t*-11	ND	23.19
18:2 *t*-10, *c*-12	ND	23.12
Other CLA	ND	1.24
18:3 *c*-9, *c*-12, *c*-15	8.46	ND
Unknown	5.32	4.86

[1]Vitamin free casein (98.4%) and DL-Methionine (1.6%)
[2]Sucrose (50.1%), cornstarch (35.0%) and maltodextrin (14.9%)
[3]Control (100% soybean oil); CLA (40% soybean oil and 60% CLA supplements)
[4]Cellulose (100%)
[5]Mineral and vitamins (100%)

Muscle Homogenization

All procedures were conducted in a cold room (40˚C). Left gastrocnemius muscles were placed in liquid nitrogen then pulverized using a BioPulverizer (Biospec Products Inc., Bartlesville, OK) that was pre-cooled in liquid nitrogen. Powdered muscle was collected, weighed and placed in 2.5 ml of homogenizing buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.1% mercaptoethanol, 1 mM Pefabloc). Tissue was homogenized using a Polytron PT 3,000 tissue homogenizer at 30,000 rpm for 30 sec, placed on ice for 30 sec then homogenized for another 30 sec. 250 µl of the homogenate was saved for total protein assay. Homogenate was centrifuged at 10,000 rpm at 2˚C for 20 min to separate the insoluble protein fraction. The supernatant was poured off through glass wool in order to filter any lipids. Supernatant was stored at -140˚C until analyzed.

Protein Assay

To measure the homogenate total protein content, 250 µl of 1x urea sample buffer (8 M urea, 2 M thiourea, 3% SDS, 0.7 M mercaptoethanol, 50 mM tris-HCl pH 6.8) was added to the 250 µl muscle homogenate aliquot. Samples were boiled for 10 min then diluted 1000x. Protein was measured using a Coomassie Plus Assay Reagent (Pierce, Rockford IL) and absorbance was read at 595 nm. To measure the protein content of the supernatant, samples were diluted 5x, 240 µl of Coomassie Plus Assay Reagent was added and absorbance was read at 595 nm.

Calpain Activity

Calpain activity was measured using a Calpain-Glo Protease Assay according to the manufacturer's protocol (Promega, Madison, WI). Briefly, 50 µl of muscle

supernatant was added to a white NUNC 96-well plate then 50 µl of the luciferase reagent was added to each well. Samples were incubated at room temp for 12 min then 10 µl of EDTA was added to stop the reaction. Luminescence was measured using a luminometer (BioTek Instruments Inc., Winooski, VT).

ELISA

To create a standard curve partially purified human calpain was added at 50 ng/µl and was serially diluted until the lowest concentration was 0.391 ng/µl. Standards and samples were diluted using PBSN. The sample (0.2 µg) was loaded into individual wells and plates were incubated for 12 h at 40˚C. Plates were washed 3x by adding 250 µl of 1X TTBS (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) to each well, then liquid was removed. Excess liquid was removed by tapping plates onto a paper towel. 250 µl of blocking solution (1% BSA in 1X TTBS) was added to each well and plates were incubated at 37˚C for 1 hr. After incubation, blocking solution was removed, and plates were washed 4x with 1x TTBS. 100 µl of rabbit anti-human m-calpain (1/1,000 dilution; Sigma, St. Louis, MO) was added to each well, and plates were incubated at room temperature for 3 hrs. After incubation, antibody was removed, and plates were washed 4x with 1x TTBS. 100 µl of goat anti-rabbit IgG+HPRO (1/1,000 dilution; American Qualex, San Clamente, CA) was added to each well and plates were incubated at room temperature for 2 hrs. Plates were washed 4x with 1x TTBS, then 100 µl of QuantaBlu Flourogenic Peroxidase Substrate Kit (Pierce, Rockford, IL) reagent was added to each well, and plates were incubated at room temp for 40 min. The reaction was terminated by adding 100 µl of QuantaBlu Stopping Solution to each well. Flourescence

was read with an excitation filter at 325 nm and an emission filter at 420 nm (SpectraFlour plate reader, Tecan Trading AG, Switzerland).

RNA extraction and RT-PCR

Left and right soleus muscles were pooled for RNA extraction. To disrupt and homogenize the tissue, muscles were placed in 300 µl of homogenizing buffer (Buffer RLT from RNeasy Fibrous Tissue Mini Kit; Qiagen, Montgomery County, MD) and a Tissue-Tearor was used at maximum rpm at 30 sec intervals until muscle was completely homogenized. Samples were placed on ice between intervals for a minimum of 30 sec. The rest of the extraction was performed as described by the manufacturer (Qiagen, Montgomer County, MD). RNA concentration was estimated by measuring the absorbance of a 1/50 sample dilution at 260 nm and using the equation A260 x RNA constant (40) x dilution factor. RNA quality was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Total RNA (1 µg) was reverse transcribed using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The primer set for mouse Fbxo32 was obtained from SuperArray Bioscience Corporation (Frederick, MD). Reactions (25 µl) were prepared according to the manufacturer's protocol using the iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA), 0.1µM of primer, and 1µg cDNA. PCR quantification of each sample was performed in triplicate, and SYBER Green flourescence was quantified with the iQ™5 Multicolor Real-time PCR Detection System (Bio-Rad). The assay plate contained a standard curve (five serial dilutions of a pool cDNA sample) to determine amplification efficiency of the respective primer pair. For

the assay, 40 PCR cycles were run, with each cycle consisting of 3 stages (95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec). Analyses of amplification plots were performed with the iQ™5 Optical System Software (Bio-Rad).

Statistical analyses

Data were analyzed using the PROC MIXED procedure of SAS and are presented as least square means ± SEM and considered significant when main effects were less then $P< 0.05$. For tissue weights, model = trt day trt*day. For gene expression, model = day or model = trt. For protein content and calpain parameters, model = trt day trt*day. To analyze feed intake, daily data were grouped into periods of 5 days (d 1-5, 6-10, 11-15, 16-20, 21-25, 26-30) where model = trt period trt*period.

Results

There was no overall treatment effect on feed intake however there was a time effect ($P <0.01$; data not shown) with intake increasing up to d 15 then decreasing slightly and leveling off. There was a treatment x time interaction as CLA fed mice had reduced ($P <0.01$) feed intake compared to controls between d 1 and d 5 and increased ($P <0.05$) feed intake compared to controls between d 11 and d 15 (Figure 4.0). There was no effect of diet on body weight (data not shown) and there was little or no effect of time on body weights (Figure 4.1).

Dietary CLA had no effect on pectoral, gastrocnemius, soleus or pooled muscle weights on either a gram or percent of body weight basis (Table 4.2; Figure 4.2). There were small changes in pectoral, gastrocnemius and pooled muscle weights over time ($P <0.01$, $P =0.03$ and $P <0.01$, respectively); however, there was no change between d 0

and d 30. Pooled muscle weight as a percent of body weight decreased between d 20 and d 30 (P <0.01), and there was no effect of time on soleus muscle weight.

On a percent of BW basis, dietary CLA significantly decreased (P <0.01) the perirenal fat depot weight on d 2 (23.3%) and perirenal and subcutaneous fat weights on d 4 (51.1 and 24.5%, respectively), 20 (67.3 and 65.7%, respectively) and 30 (72.5 and 85.8%, respectively) and epidydimal fat on d 20 (76.6%) and 30 (97.7%; Table 4.2). There was a significant effect of time (P <0.01) and an interaction effect between CLA and time (P <0.01) for all three fat depots as the fat lowering effect of CLA was more pronounced over time.

CLA increased liver weight (P <0.01) on a gram and percent of body weight basis on d 20 (81.3 and 86.8%, respectively) and 30 (108.2 and 119.9%, respectively). There was a significant effect of time (P <0.01) on liver weight and an interaction effect (P <0.01) between CLA and time as the effect of CLA on liver weight was more dramatic over time. CLA increased (P =0.04) heart weight on a gram basis on d 2 (18.8%) and on a percent of body weight basis (P =0.02) on d 2 (22.6%), 4 (17.4%) and 30 (19.7%). There was no effect of dietary CLA or day on spleen and kidney weights (Table 4.3).

There was no effect of time or dietary CLA on protein content (Figures 4.5 and 4.6, respectively), m-calpain content (Figures 4.7 and 4.8, respectively) or total calpain activity (Figures 4.9 and 5.0, respectively) of the gastrocnemius muscle. There was no effect of CLA on atrogin-1 gene expression; however expression of atrogin-1 did increase (87.6%; P <0.01) between d 0 and d 30 of experiment (Figure 5.1).

TABLE 4.2: Muscle and tissue weights of aged mice treated with control or CLA supplemented diet

		Day of Experiment														
		0	1		2		4		20		30			P-value		
Tissue			Cont	CLA	Cont	CLA	Cont	CLA	Cont	CLA	Cont	CLA	SEM	Day	CLA	DxC
Pectoral[1]	(mg)	210[a]	220	240	240	250	270[a]	220[b]	240	240	250	250	0.01	0.05	0.96	<0.1
	%	0.77	0.80	0.87	0.80	0.85	0.96	0.90	0.86	0.87	0.87	0.94	0.05	<0.01	0.43	0.51
Gastroc[2]	(mg)	130	130	120	140	140	140	120	140	130	130	130	0.01	0.05	0.53	0.15
	%	0.47	0.46	0.45	0.49	0.48	0.51	0.50	0.49	0.48	0.47	0.47	0.02	0.03	0.97	0.84
Soleus[3]	(g)	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.007	0.008	0.008	0.008	<0.01	0.08	0.42	0.36
	%	0.029	0.029	0.027	0.028	0.029	0.030	0.031	0.026	0.030	0.029	0.030	<0.01	0.72	0.27	0.78
Pooled[4]	(mg)	600	660	650	700	710	600	560	630	670	400	470	0.03	<.001	0.16	0.37
	%	2.24	2.38	2.41	2.38	2.42	2.14	2.22	2.24	2.43	1.42	1.73	0.10	<0.01	0.02	0.65
Epid[5]	(mg)	320	340	340	520	410	390	290	460[a]	100[b]	380[a]	10[b]	0.05	<0.01	<0.01	<0.01
	%	1.18	1.22	1.26	1.76	1.38	1.35	1.09	1.63[a]	0.38[b]	1.33[a]	0.03[b]	0.16	<0.01	<0.01	<0.01
SQ[6]	(mg)	270	269	292	342	296	280[a]	194[b]	290[a]	98[b]	303[a]	44[b]	0.03	<0.01	<0.01	<0.01
	%	0.97	0.97	1.07	1.15	1.00	0.98[a]	0.74[b]	1.02[a]	0.35[b]	1.06[a]	0.15[b]	0.08	<0.01	<0.01	<0.01
Perirenal[7]	(mg)	110	110	130	180[a]	140[b]	140[a]	60[b]	150[a]	50[b]	200[a]	50[b]	0.02	<0.01	<0.01	<0.01
	%	0.41	0.38	0.48	0.60[a]	0.46[b]	0.47[a]	0.23[b]	0.52[a]	0.17[b]	0.69[a]	0.19[b]	0.05	<0.01	<0.01	<0.01

Different superscripts within day indicate significant differences
[1]pectoral muscles; average of right and left
[2]gastrocnemius muscles; average of right and left
[3]soleus muscles; average of right and left
[4]pooled hind limb muscles; average of right and left
[5]epididymal adipose depot; pooled right and left
[6]subcutaneous adipose depot; pooled right and left
[7]perirenal adipose depot pooled right and left

TABLE 4.3: Organ weights of aged mice treated with control or CLA supplemented diet

		Day of Experiment															
		0	1		2		4		20		30			P-value		
Organ			Cont	CLA	Cont	CLA	Cont	CLA	Cont	CLA	Cont	CLA	SEM	Day	CLA	DxC
Liver	(g)	1.45	1.73	1.52	1.55	1.78	1.49	1.39	1.44[a]	2.61[b]	1.48[a]	3.23[b]	0.19	<0.01	<0.01	<0.01
	%	5.40	6.25	5.59	5.26	6.06	5.29	5.63	5.06[a]	9.45[b]	5.22[a]	11.48[b]	0.53	<0.01	<0.01	<0.01
Spleen	(g)	0.09	0.09	0.08	0.13	0.09	0.09	0.12	0.11	0.09	0.11	0.09	0.02	0.69	0.43	0.49
	%	0.35	0.31	0.30	0.45	0.31	0.33	0.49	0.39	0.34	0.39	0.31	0.07	0.75	0.56	0.33
Heart	(g)	0.19	0.18	0.18	0.16[a]	0.19[b]	0.19	0.20	0.19	0.21	0.19	0.21	0.01	0.03	0.04	0.43
	%	0.72	0.64	0.67	0.53[a]	0.65[b]	0.69[a]	0.81[b]	0.69	0.75	0.66[a]	0.79[b]	0.08	<0.01	0.02	0.13
Kidneys	(g)	0.41	0.42	0.40	0.44	0.44	0.41	0.38	0.43	0.40	0.41	0.40	0.02	0.16	0.87	0.04
	%	1.53	1.50	1.47	1.49	1.47	1.45	1.51	1.53	1.44	1.46	1.46	0.05	0.89	0.49	0.25

Different superscripts indicate significant differences within day

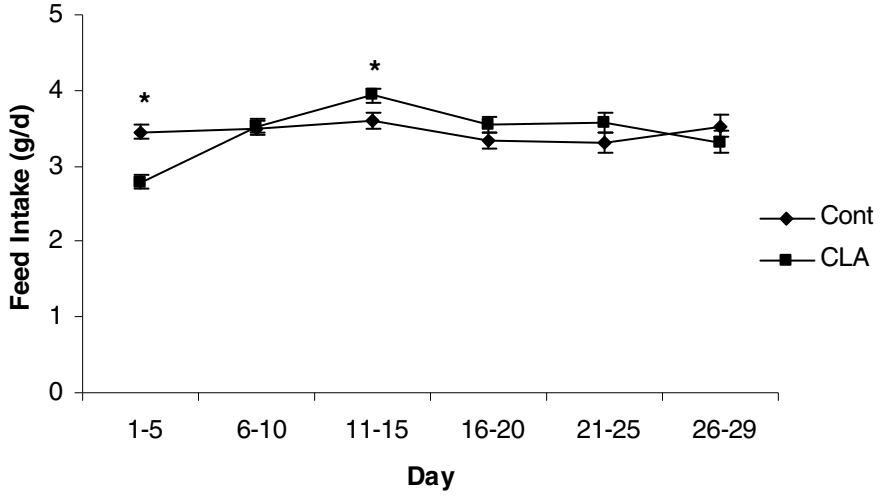

FIGURE 4.0: Effect of diet on feed intake in control and CLA fed aged male mice

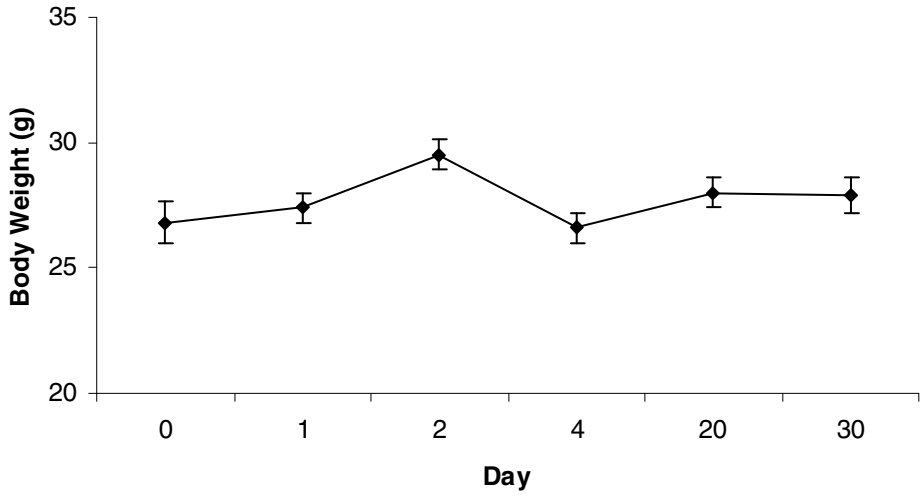

FIGURE 4.1: Effect of day on body weight in control and CLA fed aged male mice

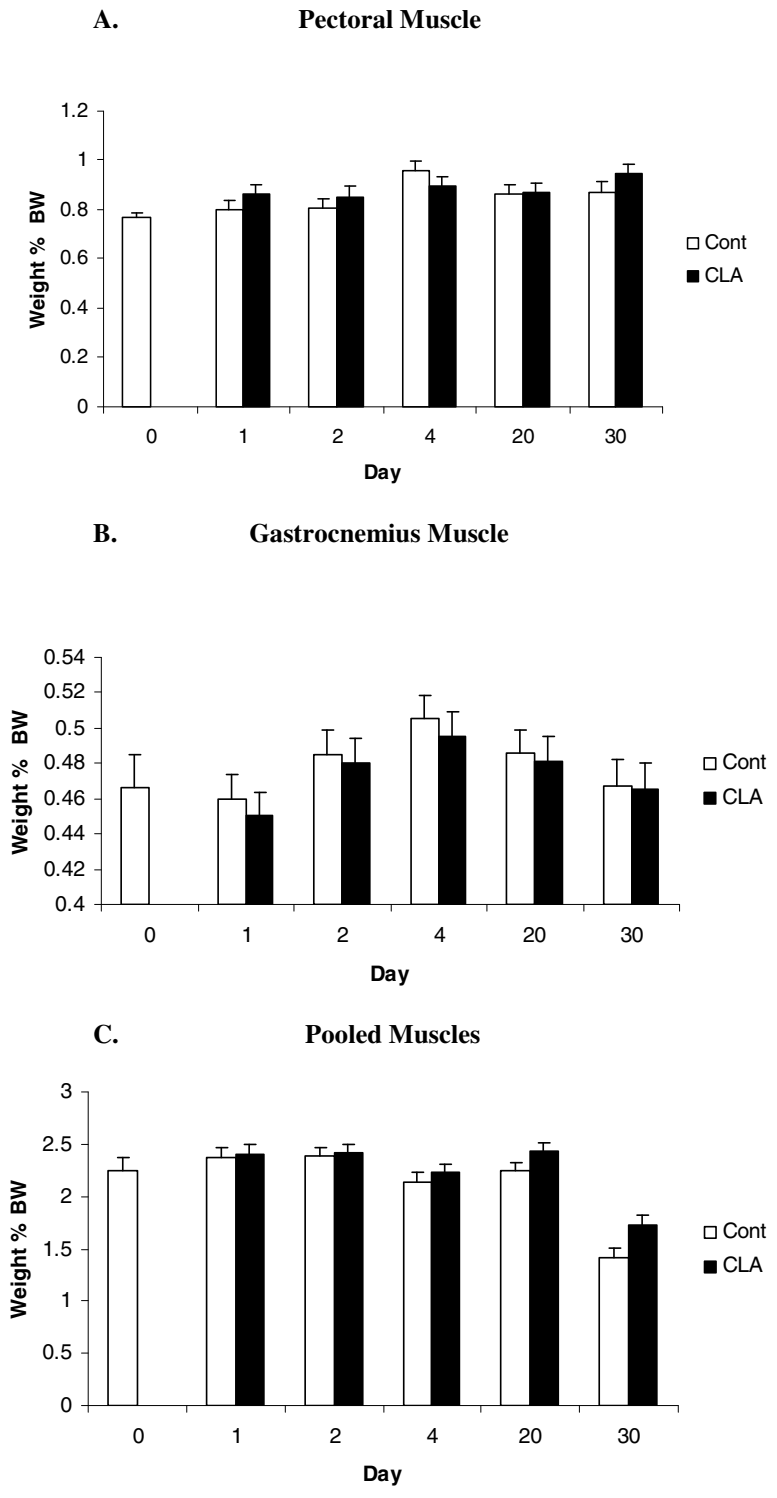

FIGURE 4.2: Effect of dietary CLA on the pectoral (A), gastrocnemius (B) and pooled hind limb (C) muscle weights (as a % of BW) in aged male mice

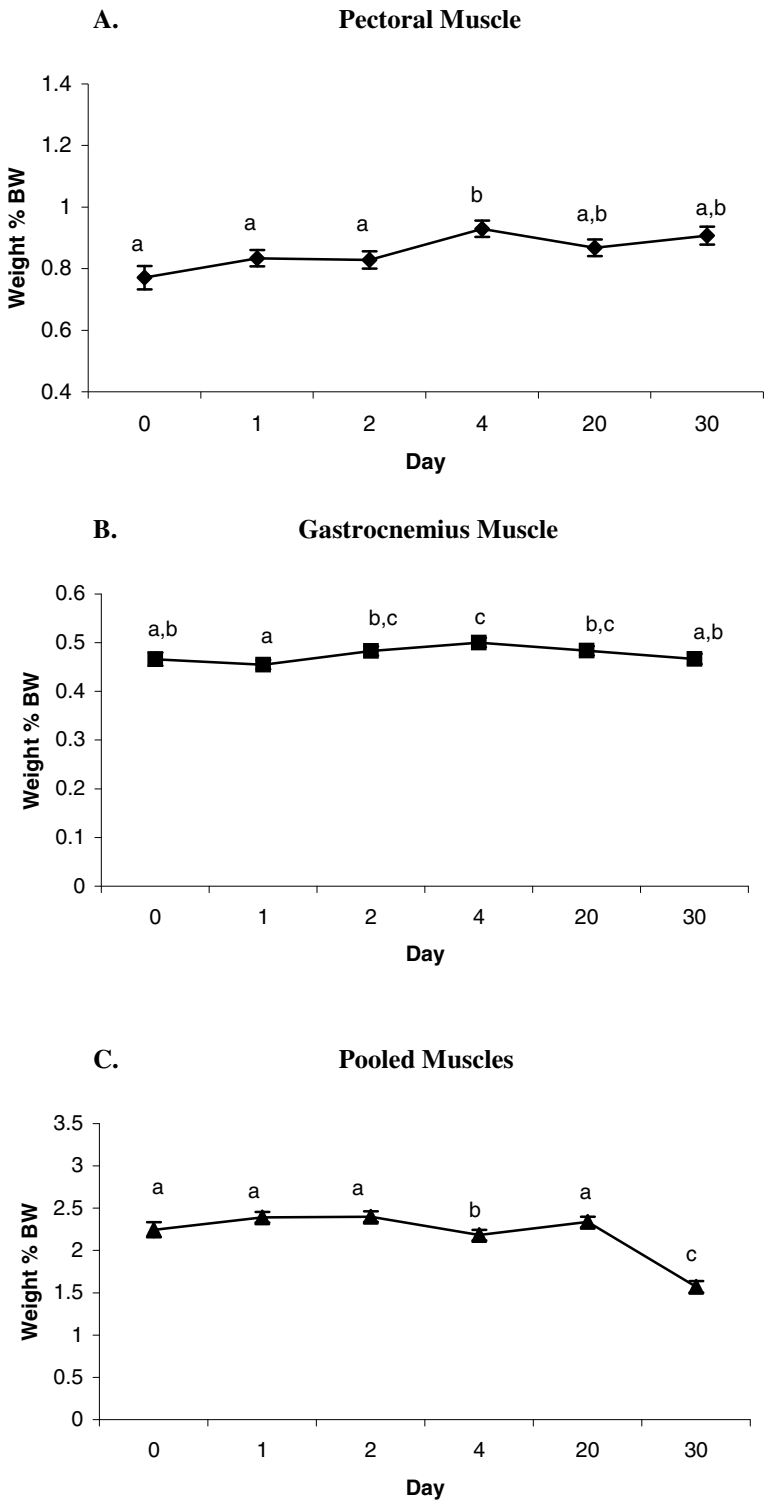

FIGURE 4.3: Effect of time on pectoral (A), gastrocnemius (B) and pooled hind limb (C) muscle weights (as a % of BW) in aged male mice

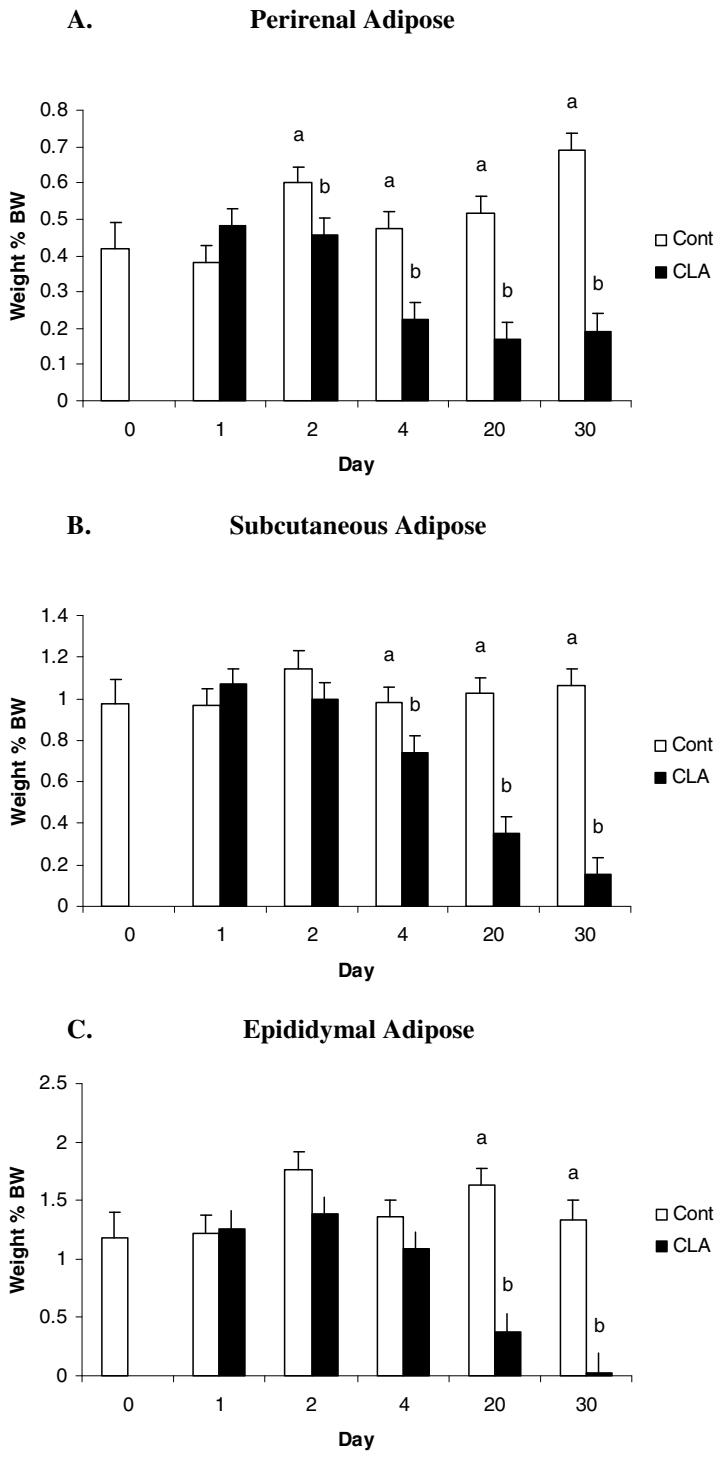

FIGURE 4.4: Effect of dietary CLA on perirenal (A), subcutaneous (B) and epidydimal (C) adipose tissue weight (as a % of BW) in aged male mice

*****Different superscripts within day indicate significant differences (P<0.1)

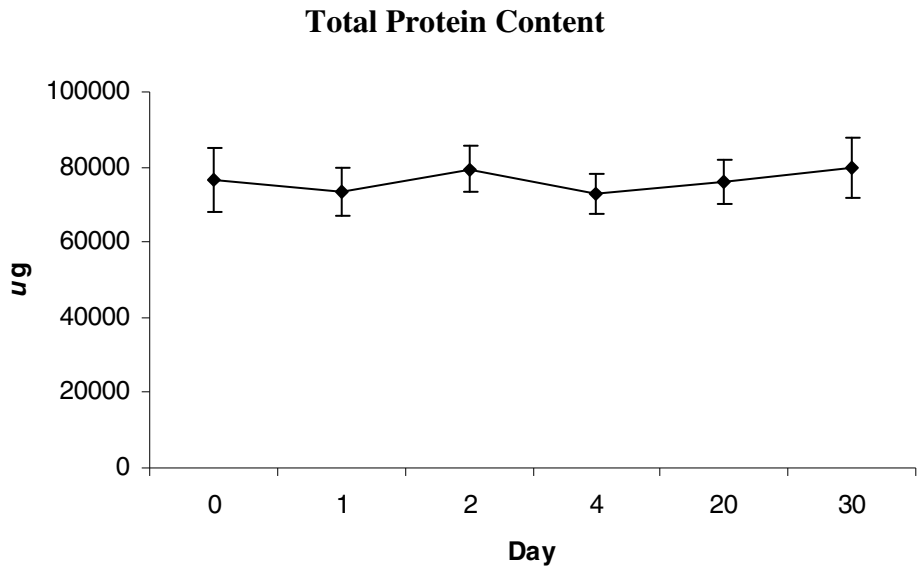

FIGURE 4.5: Effect of day on total protein content in gastrocnemius muscle of control and CLA fed aged male mice

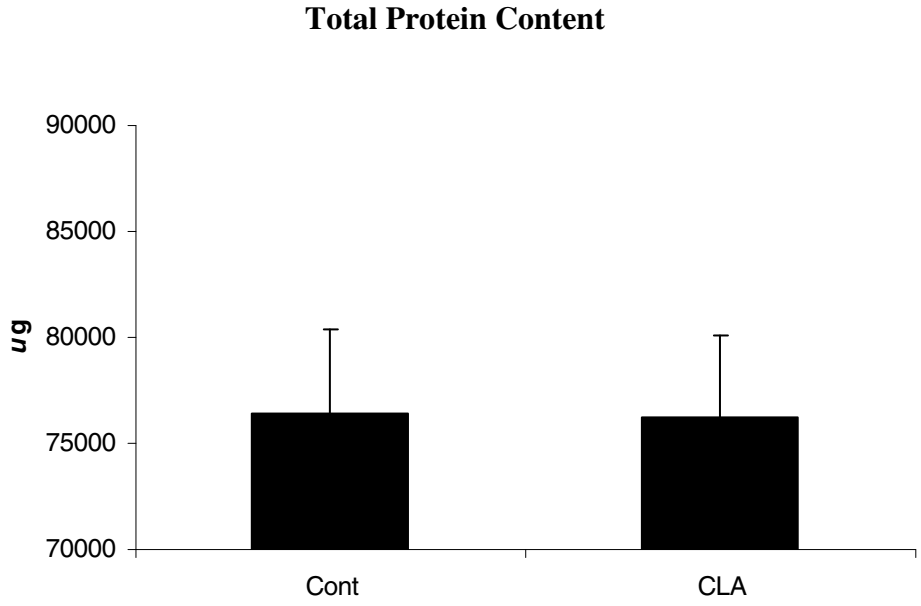

FIGURE 4.6: Effect of CLA on total protein content in gastrocnemius muscle of aged male mice

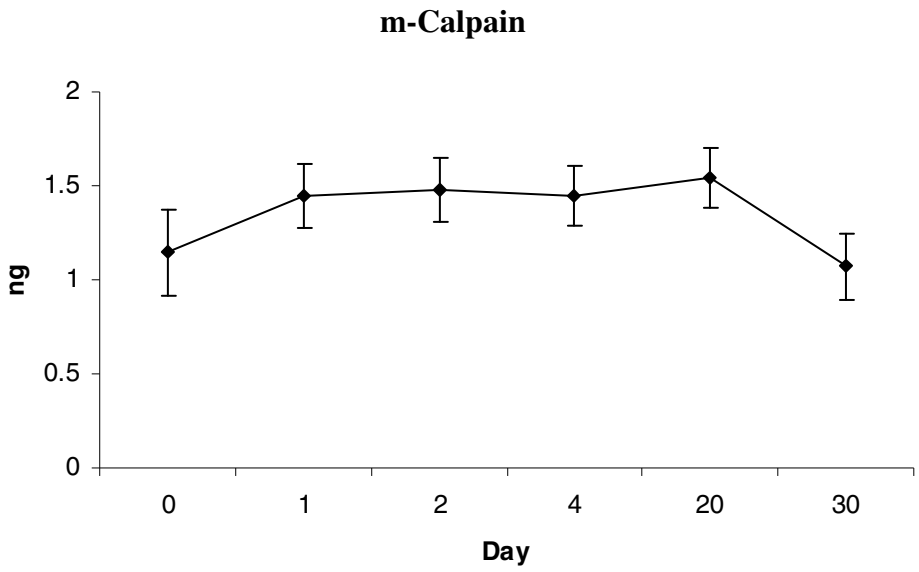

FIGURE 4.7: Effect of time on m-calpain amount in gastrocnemius muscle of control and CLA fed aged male mice

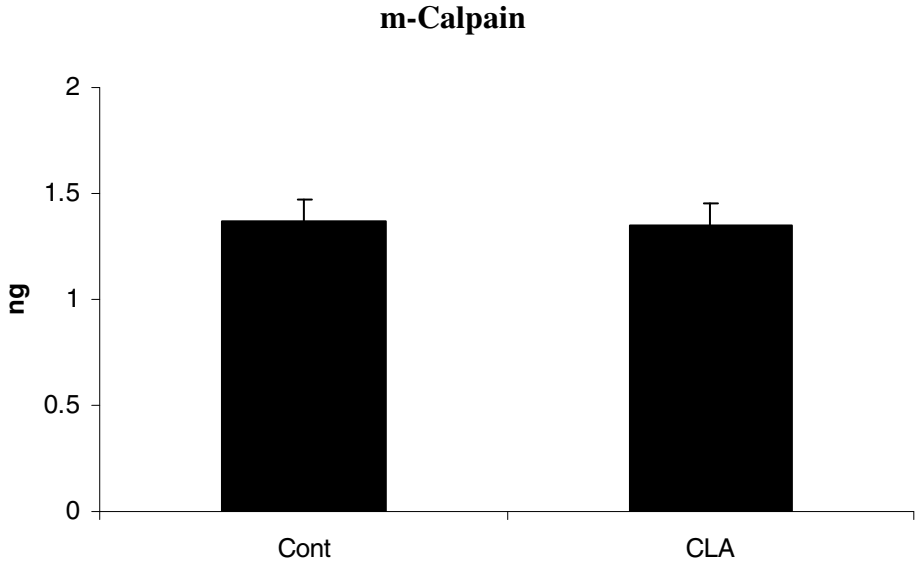

FIGURE 4.8: Effect of CLA on m-calpain amount in gastrocnemius muscle of aged male mice

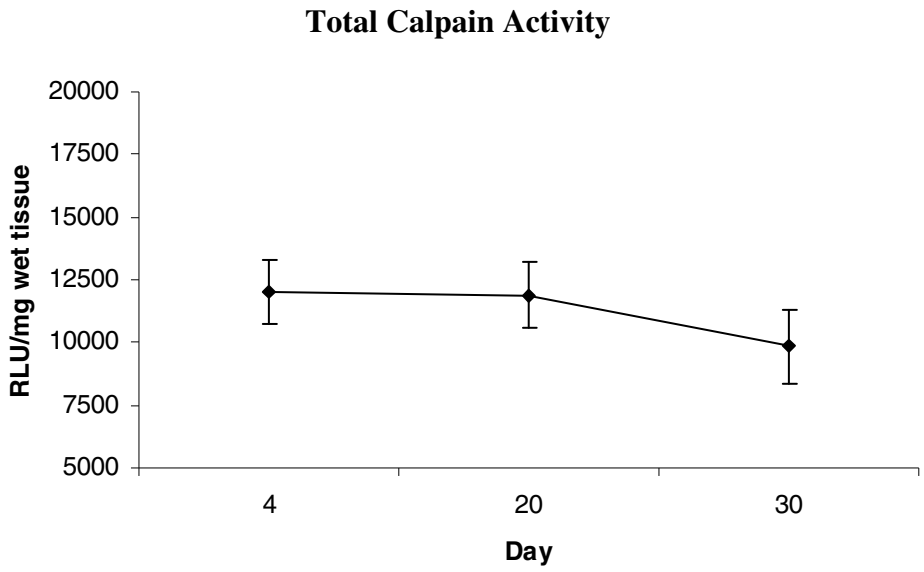

FIGURE 4.9: Effect of time on total calpain activity in gastrocnemius muscle of control and CLA fed aged male mice

*RLU= relative luminescence units

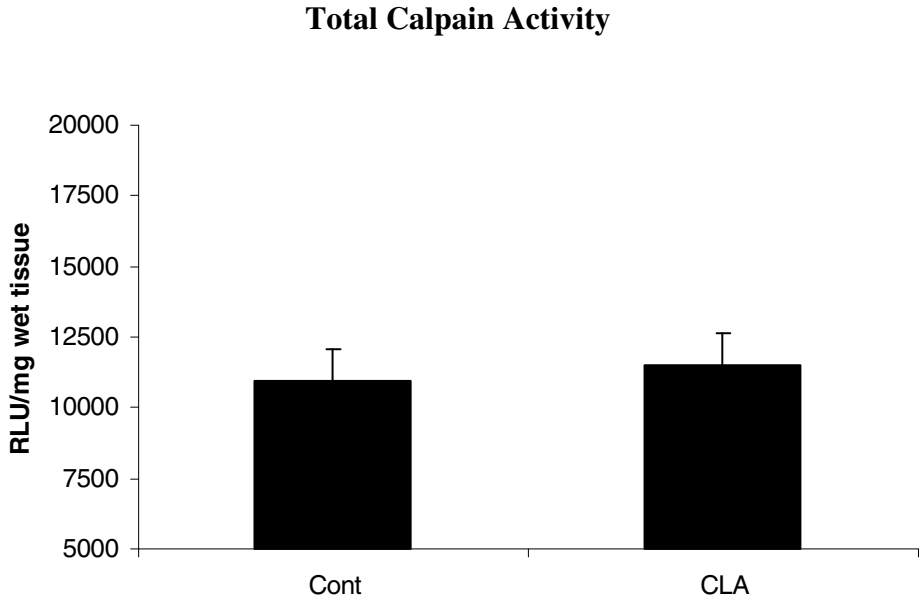

FIGURE 5.0: Effect of CLA on total calpain activity in gastrocnemius muscle of aged male mice

*RLU= relative luminescence units

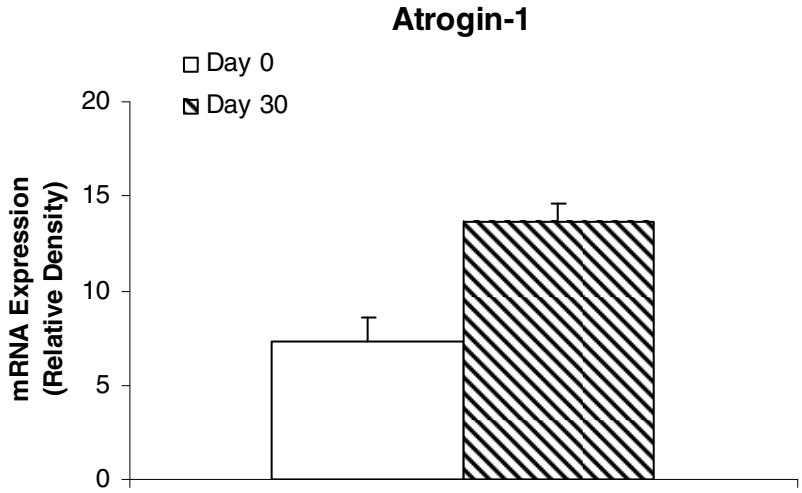

FIGURE 5.1: Change in atrogin-1 gene expression over time (d 0 vs. d 30) in soleus muscle of aged male mice

Discussion

As the life expectancy continues to increase (Newton, 2006), sarcopenia related problems will become a more significant health issue. Sarcopenia leads to increased frailty, a loss of functional independence and increased morbidity and mortality (Roubenoff, 2004) and the estimated healthcare cost attributed to sarcopenia is greater than $18 billion (Janssen et al., 2004). Unfortunately, neither an effective treatment nor an effective sarcopenia prevention plan exists. Therefore, continued research on the mechanisms responsible for sarcopenia and potential treatments is of interest.

Studies indicate that dietary CLA can prevent muscle loss associated during a disease state (i.e. cachexia; Yang and Cook, 2003; Graves et al., 2005) and our lab has recently demonstrated that CLA increases whole carcass protein mass in aged animals (Baker et al., 2005). To explore this further, we evaluated the effect of CLA on specific

muscle and adipose depot weights as well as parameters of muscle degradation (the calpain system and atrogin-1 gene expression) in aged mice.

In the current study we did not detect a time-induced decrease in pectoral, gastrocnemius, or soleus muscle weights and the decrease in pooled hind limb muscle weight between day 20 and 30 is likely do to variability in tissue dissection (Table 4.2; Figure 4.3). This lack of muscle atrophy is surprising as the mice were at 88% (23 mo) of the 50% survival rate curve (26 mo; Turturro et al., 1999). The lack of a decrease in specific muscle mass suggests these animals were not experiencing severe sarcopenia and therefore this specific model may have limited our ability to observe CLA-induced increases in muscle mass. Utilizing older animals or extending the length of the experiment may have allowed us to detect changes in muscle mass as hypothesized. Despite the lack of changes in muscle mass, there was a significant increase in soleus atrogin-1 expression between d 0 and d 30 (Figure 5.1). Atrogin-1 is an enzyme necessary for muscle protein degradation and its expression in increased during muscle atrophy (Lecker et al., 2004). However, atrogin-1 expression was not measured in the other muscles and this is important as changes in gene expression in different muscle types (fast vs. slow twitch and oxidative vs. glycolytic) are likely specific to each muscle and different muscles exhibit different rates of protein synthesis and degradation (Garlick et al., 1989).

Dietary CLA had no effect on muscle weights, gastrocnemius protein content, or soleus atrogin-1 expression. Although there are many inconsistencies within the literature, there is evidence suggesting that CLA increases lean tissue mass in growing

(Bhattacharya et al., 2005) and aging mice (Rahman et al., 2006), hamsters (Zabala et al., 2006), pigs (Meadus et al., 2002; Ostrowska et al., 2003) and cachectic animals (Yang and Cook, 2003; Graves et al., 2005). Animals in the aforementioned studies were either growing, and likely gaining muscle, or were experiencing muscle wasting. Animals in the current study were neither gaining nor loosing muscle mass (Figure 4.3) and the difference in rates of muscle protein synthesis and/or degradation compared to previous animal models may explain the unresponsiveness to CLA supplementation in this study. Additionally, differences in how muscle mass was measured, physiological differences between species and sex, and differences in the diets fed and length of feeding may also explain the inconsistency.

Sarcopenia is thought to be caused by age-associated increases in inflammatory cytokines, particularly TNF-α (Licastro et al., 2005). Dietary CLA decreases TNF-α gene expression *in vitro* (Yu et al., 2002) and *in vivo* (Roche et al., 2002) and reduces circulating TNF-α levels (Yang and Cook, 2003). Additionally, the beneficial effect of CLA on lean mass in middle aged mice was associated with decreased levels of circulating TNF-α (Rahman et al., 2006) which supports the hypothesis that CLA may improve sarcopenia by decreasing the catabolic effect of TNF-α on muscle.

To our knowledge, this is the first study to look specifically at the effect of CLA supplementation on parameters of the calpain system. Calpains are believed to play a key role in the degradation of myofibrillar proteins and calpain expression and activity increases in atrophic conditions (Bartolli and Richard, 2005). There was no effect of CLA on m-calpain protein levels or on total calpain activity (Figures 4.8 and 5.0,

respectively), and this is consistent with the literature (Meadus et al., 2002). However, because we did not detect changes in muscle mass, detecting changes in the calpain system may have been difficult.

Similar to previous trials (Tsuboyama-Kasaoka et al., 2000; Akahoshi et al., 2002; Nakanishi et al., 2004), dietary CLA markedly reduced subcutaneous, perirenal, and epidydimal fat mass (Table 4.2; Figure. 4.4). To our knowledge, this is the first experiment to elucidate temporal effects of CLA on specific adipose depots. The perirenal depot was the first tissue to respond to CLA (d 2) with subcutaneous responding shortly after (d 4) and the epidydimal fat depot did not respond until d 20. Despite responding slower (d 2 vs. d 20), the epididymal depot was more sensitive with regards to ultimate depletion (97.4 vs. 75.0 and 86.7% in epididymal, perirenal and subcutaneous, respectively).

There are many proposed mechanisms to explain the fat-lowering effect of CLA including increases in energy expenditure, adipocyte apoptosis, fatty acid oxidation, adipocyte lipolysis, decreased lipogenesis and fatty acid uptake, and decreased adipocyte differentiation (House et al., 2005). In control mice, adipose depot weights remained relatively constant over the course of the experiment (Figure. 4.4) suggesting rates of lipogenesis and lipolysis were similar. We are unable to determine whether CLA supplementation decreased adiposity by increasing adipocyte lipolysis and/or apoptosis or decreasing the rate of lipogenesis. Adipocyte apoptosis is induced by the *trans*-10, *cis*-12 isomer (Hargrave et al., 2002) and CLA's effect on lipolysis is controversial, as studies have demonstrated CLA to both increase (Chung et al., 2005) and have no effect

on lipolysis (Simon et al., 2005). CLA decreases adipocyte lipogenesis by decreasing the expression of key lipogenic enzymes including acetyl-CoA caboxylase (ACC), fatty acid synthase (FAS) and Δ^9-desaturase. Additionally, CLA decreases the expression of sterol regulatory element-binding protein-1 (SREBP-1) which regulates the expression of lipogenic enzymes (House et al., 2005).

Heterogeneity in the responsiveness of adipocytes to factors regulating lipolysis and apoptosis may explain the differences in adipose depot sensitivity to CLA. It is well established that adipocytes from different depots are dissimilar with respect to cell size, lipolytic, and lipogenic rates and this has been attributed to differences in insulin sensitivity, blood flow and lipoprotein lipase activity (Hartman, 1977; Hartman and Christ, 1978; Fried et al., 1982). Additionally, differences in CLA responsiveness may be due to differences in the incorporation of CLA isomers into the plasma membrane (Sanders et al., 2004; Baker et al., 2005).

CLA supplementation increased liver weight (Table 4.3) and this is consistent with the literature (Takahashi et al., 2003; Sanders et al., 2004; Baker et al., 2005; Kelley et al., 2006). CLA did not cause a significant increase in liver weight until d 20, indicating that the hepatomegaly associated with CLA feeding is probably a secondary effect of the CLA-induced changes in lipid metabolism. There was no effect of CLA on spleen or kidney weight (Table 4.3) and this agrees with previous reports (Sanders et al., 2004; Baker et al., 2005; Kelley et al., 2006). CLA increased heart weight (grams and percent of body weight; Table 4.3) which is inconsistent with previous studies (Sanders et al., 2004; Baker et al., 2005; Kelley et al., 2006); the reason is unknown but is of interest.

In conclusion, dietary CLA had no effect on muscle weights, gastrocnemius protein content, parameters of the calpain system or atrogin-1 gene expression, although the male 22 mo mice in this study may not have been a proper model of sarcopenia. CLA decreased adipose depot weights in a depot specific manner over time, and investigation into the metabolism of each depot may further the understanding of CLA's mechanism and the regulation of whole animal adiposity. Although we did not detect muscle wasting and therefore could not determine the effect of CLA on sarcopenia, recent findings in the literature warrant further investigation into the potential benefits of CLA on sarcopenia.

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