

**The effects of conjugated linoleic acid (CLA) isomers  
on obesity-related hypertension:  
Insight into possible mechanisms involving adipocyte function**

BY

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## ABSTRACT

Enlargement of adipocytes in obesity leads to alteration in adipokine production and these changes are linked to the development of obesity-related cardiovascular diseases. Adipokines specifically associated with obesity-related hypertension include angiotensinogen and adiponectin. Conjugated linoleic acid (CLA) has been reported to reduce blood pressure in obese insulin-resistant rats, but its mechanism of action has not been identified. The objective of this study was to determine whether CLA's ability to improve obesity-related hypertension involves reducing adipocyte size and altering adipokine production. *Fa/fa* Zucker rats (6 or 16 week old) were fed diets containing CLA isomers for 8 weeks. The *trans(t)10,cis(c)12*-CLA isomer reduced adipocyte size in both younger and older rats. Despite beneficial changes in cell size of rats fed the t10,c12-CLA isomer, there were no changes in the renin-angiotensin system or pro-inflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6 or the anti-inflammatory cytokine IL-10. In contrast, the t10,c12-CLA isomer increased adiponectin levels both in the circulation and in adipose tissue. This was associated with increased phosphorylation of endothelial nitric oxide synthase (eNOS) in adipose tissue and aorta. Direct treatment of CLA isomers in cultured endothelial cells did not increase eNOS phosphorylation but increases were observed with adiponectin treatment. *In vivo*, infusion with adiponectin increased eNOS phosphorylation in adipose of *fa/fa* Zucker rats in parallel with improvements in blood pressure. Similarly, when circulating levels of adiponectin increased in rats fed the t10,c12-CLA isomer diet, blood pressure was also

attenuated. In younger rats, both the t10-c12 and c9,t11-CLA isomers were significantly different from the control group at week 8, however, only the t10,c12-CLA isomer was comparable to the commonly used anti-hypertensive medication captopril. In conclusion, the beneficial effects of the t10,c12-CLA isomer on blood pressure may in part be due to its ability to reduce the number of large adipocytes *in vivo*, thus increasing the production of adiponectin which subsequently activates vascular eNOS.

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## ABBREVIATIONS

AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AngII	Angiotensin II
ACE	Angiotensin converting enzyme
BMI	Body mass index
c	<i>Cis</i>
CLA	Conjugated linoleic acid
eNOS	Endothelial nitric oxide synthase
ELISA	Enzyme-linked immunosorbent assay
HAECs	Human aortic endothelial cells
I- $\kappa$ B $\alpha$	Inhibitor protein kappa-B alpha
IL	Interleukin
L-NAME	L-Nitro-arginine methyl ester
MAPK	Mitogen-activated protein kinase
NO	Nitric oxide
NF- $\kappa$ B	Nuclear factor kappa-B
OLETF	Otsuka Long Evans Tokushima Fatty
RAS	Renin-angiotensin system
rp	Ribosomal protein
SHHF	Spontaneously hypertensive heart failure
SHR	Spontaneously hypertensive rats

t	<i>Trans</i>
TNF	tumor necrosis factor
ZDF	Zucker diabetic fatty

# Chapter 1: Overall Introduction

## 1.1 Introduction

Conjugated linoleic acid (CLA) is a term used to describe a mixture of positional and geometric isomers of linoleic acid. CLA has been shown to reduce blood pressure in obese insulin-resistant rats [1, 2]. There is evidence to suggest that obesity-related hypertension may be due to activation of the renin-angiotensin system (RAS) in the adipose tissue, resulting in exaggerated production of the vasoconstrictor angiotensin II (AngII) and, consequently, an increase in blood pressure [3]. On the other hand, secretion of the adipokine adiponectin from adipose tissue is reduced in obesity and decreased serum levels of adiponectin may indirectly contribute to obesity-related hypertension via lower production of nitric oxide (NO), a vasodilator responsible for decreasing blood pressure [4]. In addition, cell size influences secretion of key molecules secreted by adipose and a shift towards a pro-inflammatory state is observed in obese individuals [5]. Thus, the high prevalence of hypertension observed in obesity may be due in part to regulatory mediators produced in adipose tissue and further investigation of the mechanism by which CLA affects hypertension is warranted. The main focus of this project was to investigate the effects of CLA isomers on blood pressure regulation in relation to adipocyte size, and consequent alterations in adipokine secretion in a model of obesity-related hypertension. Both *in vivo* and *in vitro* studies were employed to examine the effects of CLA isomers on key proteins involved in blood pressure regulation. The role of adiponectin on blood

pressure regulation was also investigated by infusion of recombinant adiponectin into obese hypertensive rats. The research in this thesis provides us insight into the cellular mechanisms responsible for the actions of CLA on blood pressure modulation in obesity. The following section will review the literature building up to the current study.

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## Chapter 2: Literature Review

### 2.1 Obesity-related hypertension

It is estimated that 27% of Canadians have high blood pressure, with only 36% receiving treatment, and of these only 17% actually being able to control their blood pressure [1]. Even more alarming is data from the Canadian Heart Health Survey, which indicate that just over half of hypertensive individuals are actually aware that they have high blood pressure [2]. Undetected hypertension is common because hypertension is usually asymptomatic until serious events such as stroke, myocardial infarction or renal dysfunction are observed. Hypertension can be used to describe elevations in mean arterial pressure, diastolic pressure and/or systolic pressure. A diastolic pressure  $\geq 90$  mmHg or systolic pressure  $\geq 140$  mmHg are used to define hypertension [3]. High blood pressure before the age of 35 is associated with atherosclerosis about 20 years later [4], and the incidence of coronary heart disease or stroke is doubled with every 20 mmHg increase in systolic pressure [5]. Many factors contribute to increased blood pressure such as such as heredity, sex, age, alcohol intake, blood lipids, blood sugar and a sedentary or inactive lifestyle, however, the Framingham study demonstrated that 65-78% of hypertension is attributed to obesity [6, 7]. In fact, 80% of individuals with hypertension are overweight/obese and 45% are obese [8]. Most individuals (95%) with hypertension also have one or more risk factors for cardiovascular disease such as dyslipidemia, diabetes and/or obesity.

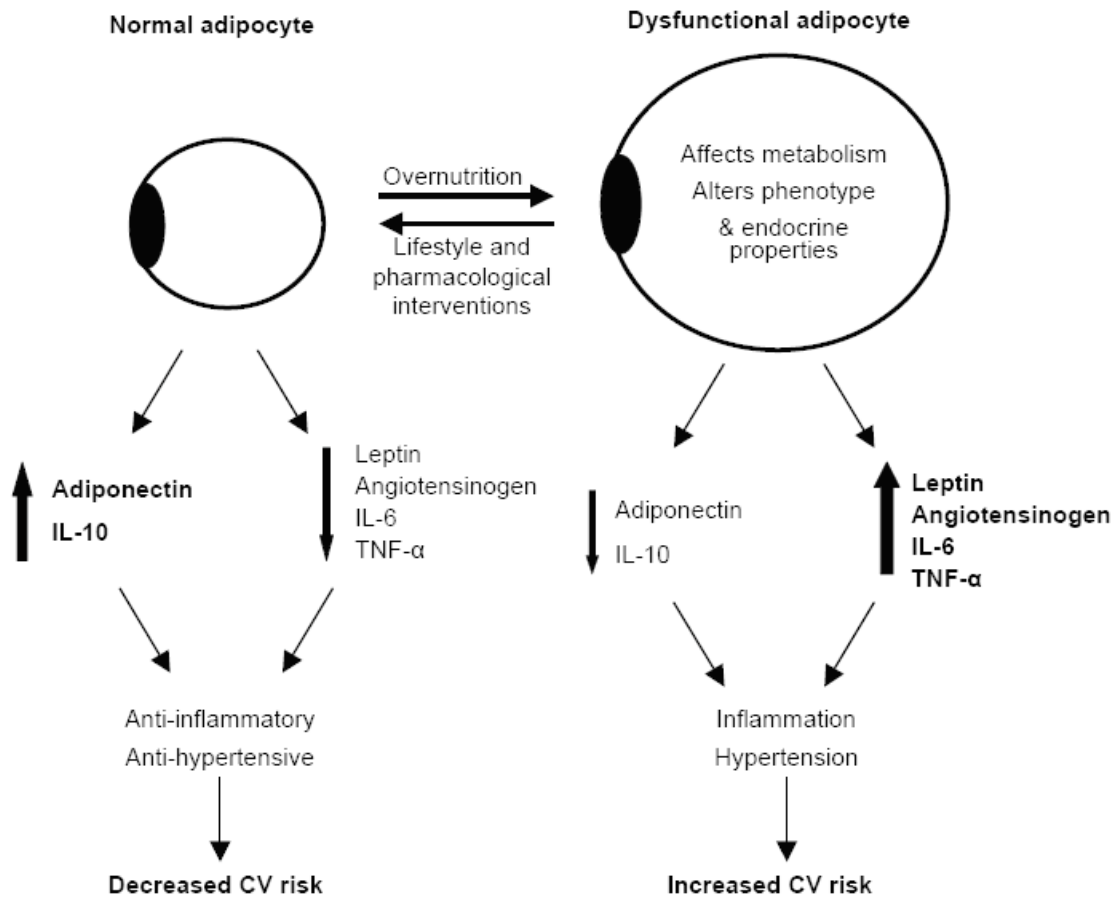
Rates of obesity in Canada have increased substantially during the past 15 years [9, 10], with almost 53% of Canadians being overweight and 18% obese [11]. In the United States, 68% of the population is overweight/obese and 34% are obese [12]. The high rates of obesity are estimated to contribute at least \$1.8 billion to health care costs in Canada [13]. This increase in obesity is associated with damaging effects on human health. Increases in Body Mass Index (BMI) are correlated with an increased risk of cardiovascular disease and mortality in both men and women [14]. BMI is a measure of weight for height that indicates weight status in adults [15]. A BMI below 18.5 kg/m<sup>2</sup> is considered underweight, between 18.5-24.9 kg/m<sup>2</sup> is normal, 25.0-29.9 kg/m<sup>2</sup> is overweight, and 30.0 kg/m<sup>2</sup> and above is considered obese. In those with a BMI  $\geq$  30 kg/m<sup>2</sup>, blood pressure is increased 38-42% in men and women [16]. Additionally, increases in blood pressure are greatest with abdominal obesity [17, 18]. Obesity is also defined as having a waist circumference  $\geq$ 40 inches (102 cm) in men and  $\geq$ 35 inches (88 cm) in women [15]. The distribution of body fat is a strong predictor of obesity-related risk factors and mortality with increases in visceral fat being associated with the metabolic complications of obesity and cardiovascular disease [17, 19]. Changes within the adipose tissue contribute to obesity-related complications.

## **2.2 Function of adipose tissue in obesity**

Apart from its major function as an energy storage depot, adipose tissue plays an important role in energy homeostasis and metabolism, and is involved in immune

and neuroendocrine functions [20]. Adipose tissue consists primarily of mature adipocytes surrounded by endothelial cell-lined vascular tissue as well as adipocyte precursors, macrophages and fibroblasts [21]. Mature adipocytes have the ability to synthesize and secrete bioactive molecules including various hormones and cytokines [22]. Many of these molecules have been shown to influence vascular function [20, 22, 23] and have been associated with an increased cardiovascular risk in obese individuals [24, 25].

Obesity occurs when adipose mass increases due to an overabundance of fatty acids. Storage of these fatty acids results initially in an enlargement of adipocyte size (hypertrophy), followed by an increase in adipocyte number (hyperplasia) [26-28]. These enlarged hypertrophic adipocytes are associated with changes in adipocyte metabolism and the release of a variety of bioactive molecules, termed adipokines, that may contribute the development of obesity-related complications (Figure 2.1) [23, 29, 30]. A study examining the size separated human adipocytes showed an increased secretion of more pro-inflammatory adipokines, such as leptin, interleukin (IL)-6 and IL-8, from the larger hypertrophic adipocytes, and reduced secretion of the anti-inflammatory IL-10 [29]. Increased secretion of pro-inflammatory adipokines may contribute to an increase in cardiovascular risk factors such as hypertension.



**Figure 2.1 Altered adipokine production in enlarged adipocytes contributing to obesity-related cardiovascular risk** (Adapted from DeClercq *et al.* 2008). CV= cardiovascular; IL= interleukin; TNF= tumor necrosis factor.

A strong relationship between obesity and hypertension has been established, however, the mechanism behind this relation remains poorly understood. The enlargement of adipocytes observed in obesity may contribute to obesity-related hypertension by altering the activity of the RAS as well as the production and secretion of adipokines implicated in blood pressure regulation [23, 31, 32]. The next sections will provide an overview of adipocyte-derived factors that have been linked to blood pressure.

## 2.3 Adipocyte-derived factors in blood pressure regulation

Research has just begun to explore the role of adipokines as regulators of blood pressure. The following sections will focus on the role of these molecules in obesity-related hypertension. For instance, leptin gained a lot of attention when it was discovered over 15 years ago. Normally, leptin decreases appetite and increases energy expenditure [33], however, a deficiency in leptin or disturbance of its signaling can contribute to the development of obesity [34]. Obese individuals have increased levels of circulating leptin [35, 36] due to leptin resistance, which further contributes to weight gain and predisposes individuals to the development of metabolic complications [37-39]. With respect to blood pressure regulation, leptin infusion in non-obese animal models increased blood pressure [40-42], however, this does not address complications due to the current obesity epidemic. In addition, obese *db/db* mice, which have a leptin receptor mutation, have elevated blood pressure [43, 44], suggesting that leptin signaling may not be involved in obesity-related hypertension. More research is needed to determine the exact role of leptin in obesity-related hypertension.

Resistin is an adipokine produced in murine adipocytes and to a lesser extent in human adipocytes (mostly from macrophages within the adipose) [45, 46].

Expression of the resistin gene is increased in obese rodents [47] and humans [48], however, its role in blood pressure regulation is unclear. Circulating resistin

positively correlated with mean blood pressure in type 2 diabetics but not nondiabetic hypertensive patients [49]. Nevertheless resistin has been shown to alter cell adhesion molecules and markers of inflammation, suggesting a role in vascular tissues [50, 51].

Adipose tissue produces a variety of factors that could contribute to the regulation of blood pressure in obesity, however, some adipokines have been better studied than others. Leptin and resistin both require more research involving obese models, knockout models and well controlled infusion studies to gain a better understanding of their potential role in obesity-related hypertension. On the other hand, research on specific adipokines in relation to obesity-related hypertension should also include angiotensinogen and adiponectin; the rest of the literature review will focus on these two molecules.

### **2.3.1 Renin-Angiotensin System (RAS)**

The RAS, which consists of components that are derived from the liver, kidney and vascular endothelium, is an important regulator of systemic blood pressure and is involved in altered cardiovascular pathophysiology [52]. Angiotensin (Ang) II is the main effector of the classical RAS [53]. Ang II is the product of a series of proteolytic cleavage events in which angiotensinogen is cleaved by renin to form Ang I and is further acted upon by angiotensin converting enzyme (ACE) which is responsible for the conversion of Ang I to Ang II [53]. Ang II binds to its receptors, angiotensin type-1 (AT<sub>1</sub>) and angiotensin type-2 (AT<sub>2</sub>), in various tissues to

modulate cardiovascular homeostasis [53]. Research on the actions of the AT<sub>2</sub> receptor are less clear than the AT<sub>1</sub> receptor, but growing evidence suggest the AT<sub>2</sub> receptor may mediate vasodilation, proliferation, differentiation, and apoptosis [54]. Most of the cardiovascular actions of Ang II are mediated through the AT<sub>1</sub> receptor, which is involved in vasoconstriction leading to an increase in systemic blood pressure and vascular inflammation [55, 56]. Ang II stimulates the release of pro-inflammatory cytokines, presumably via activation of nuclear factor-κB (NF-κB) [57]. NF-κB is a transcription factor shown to play a major role in the pathogenesis of hypertension in spontaneously hypertensive rats (SHR) [58]. Rodriguez-Iturbe *et al.* [58] demonstrated that early suppression of NF-κB activation completely abrogates hypertension.

More recently, the role of ACE2 in further converting Ang II to Ang-(1-7) has gained much attention [59, 60]. Activation of the Ang-(1-7) Mas receptor can produce nitric oxide and vasodilation [61-63]. Additionally, hypertension is ameliorated in Sprague-Dawley rats infused with Ang-(1-7) when maintained on a high-fructose diet [64]. Thus, the conversion of Ang II to Ang-(1-7) via ACE2 has been viewed as the counter-regulatory arm as it opposes ACE/Ang II/AT<sub>1</sub> receptor pathway [59, 60]. This second arm may prove to be an important target for cardiovascular therapies in the future but at this point the ACE2/Ang-(1-7) pathway requires further investigation to clarify its regulation and mechanism of action.

### 2.3.2 Local adipose RAS

Over the last several years the scientific community has gained an understanding that traditional RAS elements (eg. angiotensinogen, renin, ACE, AT<sub>1</sub> and AT<sub>2</sub>) are expressed in adipose tissue due to the presence of a tissue-localized RAS [53, 65]. A key study by Massiera *et al.* [66] showed that angiotensinogen is released locally from adipose tissue and it is released into circulation. This group developed a model that over-expressed angiotensinogen in adipose tissue on both a wild type background as well as in angiotensinogen-deficient mice [66]. Angiotensinogen could be detected in serum of mice over-expressing angiotensinogen in adipose on an angiotensinogen-deficient background and were normotensive, suggesting that adipose-derived angiotensinogen could contribute to systemic levels [66]. Interestingly, mice over-expressing angiotensinogen in adipose on a wild type background were hypertensive and displayed elevated levels of circulating angiotensinogen [66]. Together, the findings by Massiera *et al.* [66] demonstrate that adipose-derived angiotensinogen contributes to circulating levels of angiotensinogen and the development of hypertension [66]. Boustany *et al.* [67] demonstrated an increase in activity of adipose RAS in obesity-related hypertension. Their study showed that diet-induced obesity in Sprague-Dawley rats resulted in hypertension and increased angiotensinogen mRNA in adipose tissue [67]. Both Sprague-Dawley and obese Zucker rats display higher AT<sub>1</sub> receptor density in epididymal



adipocytes compared to retroperitoneal, mesenteric, and inter-scapular adipocytes [68, 69].

Furthermore, the local RAS may also be involved in regulating adipose growth. In angiotensinogen-deficient mice, body weight and adipose mass are lower than in wild type, and when angiotensinogen is over-expressed in adipose tissue, body and adipose mass increased [70]. Similarly, when angiotensinogen expression is limited to adipose tissue, body and adipose weight were normalized compared to angiotensinogen-deficient mice [70]. More recently, administration of the angiotensin type 1 receptor antagonist, valsartan, to obese KK-Ay mice reduced adipocyte size as well as enhanced expression of adiponectin and lowered expression of the pro-inflammatory cytokine tumor necrosis factor (TNF)- $\alpha$  [71].

Consistent with this link between the adipose RAS and obesity-related hypertension in animal models, human data have shown that BMI positively correlates with plasma angiotensinogen concentrations and blood pressure [72]. Klett *et al.* [73] suggest that angiotensinogen is important for long-term regulation of RAS. Additionally, angiotensinogen levels are higher in visceral adipose deposits compared to subcutaneous depots [74, 75]. Weight loss of 5% reduced systolic blood pressure and plasma levels of angiotensinogen and correlated with adipose angiotensinogen mRNA levels [76]. Thus, the high prevalence of hypertension observed in obesity may be due in part to regulatory mediators

produced in adipose tissue. As just discussed, a clear association has been made between obesity-related hypertension and adipose derived regulatory mediators of the RAS, but there is also a potential for other adipose-derived mediators such as adiponectin to be involved in blood pressure regulation.

### **2.3.3 Adiponectin**

Another adipokine that is altered in obesity is adiponectin [77]. Adiponectin was originally identified in the 1990s by 4 independent research groups and termed ACRP30 (adipocyte complement-related protein of 30kDa), ADIPOQ, apM1 (adipose most abundant gene transcript 1) or GBP28 (gelatin-binding protein) [78-81]. Adiponectin is now the most widely accepted term. Adiponectin is produced and secreted from adipocytes [81] as a 30-kDa monomer composed of a globular C-terminal and a collagenous N-terminal domain [82]. Adiponectin can form low (trimer), medium (hexamer), and high (12-18 mer) molecular weight oligomers [83]. The low molecular weight trimer (LMW) is formed by non-covalent interactions of the collagenous domain, whereas disulfide linkages between trimers are involved in the formation of the medium molecular weight (MMW) and high molecular weight (HMW) oligomers [82, 84].

Adiponectin is normally found in the circulation at about 2-20  $\mu\text{g/ml}$  [85], however, levels of adiponectin are negatively associated with BMI and visceral adiposity [86-88]. As well, the amount of adiponectin secreted by the adipose tissue is decreased with increased fat accumulation, particularly in visceral

adipose depots [89]. For example, adiponectin secreted by omental adipocytes was significantly reduced compared to adipocytes from subcutaneous adipocytes of obese women, while secretion was not affected in lean individuals [89]. In addition, adiponectin secretion from omental adipocytes was negatively correlated with total body fat mass, visceral adipose area, and visceral adipocyte diameter [89]. A strong inverse association has been made between obesity and levels of adiponectin, which may prove to be a key protein in obesity-related diseases.

More recent research has demonstrated an association between hypoadiponectinemia and hypertension [86, 89-93]. Ohashi *et al.* [93] has shown that adiponectin-knockout (KO) mice maintained on a high-salt diet had elevated systolic blood pressure compared to wild-type mice. Additionally, adenoviral delivery of adiponectin lowered blood pressure in adiponectin-KO mice fed a high-salt diet [93]. This study also demonstrated a reduction in mRNA levels of endothelial nitric oxide synthase (eNOS) in the aorta of adiponectin-KO mice, suggesting a possible mechanism by which adiponectin exerts its anti-hypertensive effects.

### **2.3.4 Endothelial nitric oxide synthase (eNOS) and adiponectin**

eNOS is the enzyme responsible for catalyzing the oxidation of L-arginine to produce NO and L-citrulline [94]. Endothelial-derived NO is a potent vasoactive molecule that has a significant role in regulating blood pressure and vascular

homeostasis. Enzyme activity of eNOS is altered through phosphorylation at multiple sites. More specifically, the rate of NO production is diminished when eNOS threonine (Thr)-497 becomes phosphorylated whereas dephosphorylation of this site activates eNOS [95]. In contrast, eNOS function is increased when the serine (Ser)-1177 site is phosphorylated, while a mutation at this site reduces the release of NO [95]. The regulation of multi-site eNOS phosphorylation involves numerous kinases and phosphatases. There is increasing evidence to suggest that AMP-activated protein kinase (AMPK) stimulates eNOS activity by phosphorylating eNOS at Ser-1177 [96-98]. Deng *et al.* [96] have demonstrated improved endothelium-dependent vasodilatory response and increased eNOS (Ser-1177) activity in rat aortic rings incubated with globular adiponectin. Additionally, this group also showed enhanced phosphorylation of AMPK (Thr-172) in cultured endothelial cells treated with globular adiponectin [96]. AMPK activity can be altered by many known chemical inhibitors and activators such as Compound C and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), respectively [99]. The ability of adiponectin to phosphorylate eNOS is abrogated with the AMPK inhibitor, Compound C, suggesting that AMPK plays a key role in adiponectin-induced eNOS phosphorylation [96]. Previously discussed are possible adipose-derived factors and potential mechanisms that may contribute to the growing problem of obesity-related hypertension. Prevention and treatment strategies for obesity-related hypertension that target adipose-derived factors require further investigation.

## **2.4 Hypertension Prevention & Treatment**

Lowering blood pressure has many benefits for cardiovascular health. More specifically, it has been shown that antihypertensive therapy can reduce the incidence of stroke by 35-40%, myocardial infarction by 20-25%, and heart failure by over 50% [100-102]. These notable benefits of antihypertensive therapy emphasize the importance of therapeutic research.

### **2.4.1 Lifestyle management of high blood pressure**

Several steps can be taken to help prevent or control high blood pressure. The first step is adopting a healthy lifestyle, which includes engaging in physical activity, maintaining a healthy weight, moderate alcohol consumption, following the Dietary Approaches to Stop Hypertension (DASH) diet, undertaking stress management and avoiding tobacco smoke [103]. It is recommended that individuals should build up to 30-60 minutes of moderate intensity exercise 4-7 days per week. Maintenance of a healthy body weight is defined as a BMI between 18.5-24.9 kg/m<sup>2</sup>. Recommendations for alcohol include limiting intake to ≤ 2 drinks per day and no more than 14 and 9 drinks per week for men and women, respectively. Dietary recommendations are based on the DASH diet and emphasize fruit, vegetables, low-fat dairy products, fiber, whole grains and plant-based protein sources that are low in saturated fat and cholesterol. Additionally, dietary sodium intake is recommended to be less than 2300 mg per day. The target range for treatment is <140 mmHg systolic and <90 mmHg diastolic pressure for the general population. It is common for patients to require a

combination of therapies, both drug and lifestyle modifications, in order to achieve target blood pressure values.

#### **2.4.2 Pharmacological treatment of high blood pressure**

There are several different types of blood pressure medications including diuretics, beta-adrenergic receptor blockers, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor antagonists, calcium channel blockers, alpha- adrenergic receptor blockers, alpha-beta- adrenergic receptor blockers, nervous system inhibitors and vasodilators. Often two or more drugs in combination are required to achieve target blood pressure values [104]. The use of ACE inhibitors either alone or in combination has increased over the last several years, making them one of the most commonly used anti-hypertensive medications [104]. Interestingly, medications that target the RAS, such as ACE inhibitors and angiotensin receptor antagonists, also increase circulating adiponectin levels [105-108]. In addition to the drugs currently on the market, some nutraceuticals such as CLA are now being examined for their beneficial physiological effects. The remaining sections will cover some basic background information about CLA and its potential use as a nutraceutical in the treatment of high blood pressure.

## 2.5 Conjugated linoleic acid (CLA)

Functional foods and nutraceuticals are terms that refer to certain food-derived products that provide additional health benefits beyond their nutritional impact and may reduce the risk of chronic disease [109]. A functional food is similar to a conventional food, while a nutraceutical is isolated from a food and sold in dosage form; both contain active components, which occur naturally in the food [110].

In 1978, Dr. Pariza at the University of Wisconsin-Madison identified a particular extract from beef with anti-carcinogenic properties. In the 1980s, Dr. Pariza identified CLA as the active ingredient in the beef extract [111], after which time there was a surge of interest and research on the effects of CLA. Several additional physiological and pathological effects have subsequently been associated with CLA [112, 113].

CLA is a collective term used to describe a mixture of positional and geometric isomers of linoleic acid (C18:2 n-6), an 18-carbon polyunsaturated fatty acid. CLA contains a conjugated bond system, meaning it has single bonds alternating with two double bonds that are not methylene interrupted [112]. The two double bonds in CLA can occur in several positions along the carbon chain and each double bond can be found in either the *cis* (c) or *trans* (t) configuration.

CLA isomers are produced in the rumen during the microbial biohydrogenation of linoleic acid [112]. Biohydrogenation of dietary linoleic acid produces the c9,t11-CLA isomer or rumenic acid . Further hydrogenation results in t11-18:1 or vaccenic acid. Both rumenic acid and vaccenic acid can be absorbed, and taken up in tissues via the circulatory system. Once in tissues, vaccenic acid may then be converted back to CLA. The conversion back to CLA occurs via the enzyme, delta-9 ( $\Delta$ 9) desaturase [112]. The production of different CLA isomers is due the isomerase enzyme, which is responsible for the movement of the methylene-interrupted double bonds in linoleic acid to conjugated positions.

Dietary sources of CLA are found in ruminant meats (cow, sheep, goat), ruminant milk and other dairy products (yogurt, cheese, butter) [114]. The CLA content in fluid milk is 3.38 to 6.39 mg of CLA per gram of fat and ranges from 3.59 to 7.96 mg of CLA per gram of fat in cheese [115]. The c9,t11 CLA isomer comprises approximately 90% of the CLA found in foods [116]. Based on food intake records, the estimated intake of c9,t11-CLA isomer in the Canadian diet ranges from 15 to 174 mg per day [117]. CLA can also be chemically synthesized by alkali isomerization of high linoleic oil such as sunflower or safflower oils [112, 113]. These synthetic CLA oils usually contain a mixture of isomers, primarily c9,t11 and t10,c12-CLA isomers with minor amounts of other isomers. The major isomers being investigated for potential health benefits include c9,t11-CLA and t10,c12-CLA [112, 118].



### 2.5.1 CLA and adipokines

Many health benefits have been attributed to CLA [119-124], but this section of the literature review will focus on the effects of CLA isomers on adipose cell size and adipokines associated with obesity-related hypertension.

While investigations of the effect of CLA isomers on the RAS are limited, many studies have reported on alterations in adiponectin levels due to CLA. Most studies involving CLA and adiponectin have been conducted in animal models fed a mixture of CLA isomers. For example, 4-month old Wistar rats fed a high fat diet (15%, w/w) supplemented with either a 3% (w/w) or 1.5% (w/w) mixture of CLA isomers for 12 weeks had increased adiponectin mRNA levels in epididymal adipose tissue [125]. Likewise, in obese 6-week old *fa/fa* Zucker rats fed a 1.5% (w/w) mixture of CLA isomers for 8 weeks, adiponectin mRNA levels were increased in epididymal adipose tissue [126]. Interestingly these animals also had increased circulating adiponectin levels and reduced epididymal adipocyte size [126]. In contrast, 28-day old Wistar rats fed a saturated fat based diet (5.1% palm oil, w/w) supplemented with a mixture of CLA isomers (1.3%, w/w) for 8 weeks did not have altered serum adiponectin nor altered adipocyte size in either the inguinal or the retroperitoneal adipose tissues [127]. In mice, the effects of CLA are usually the opposite. ApoE-knockout mice fed an atherogenic diet supplemented with a mixture of CLA isomers (0.5%, w/w) for 12 weeks had reduced plasma adiponectin [128]. Similarly, adiponectin mRNA levels were

lower in the epididymal adipose tissue of 6-week old *ob/ob* mice fed a diet supplemented with a mixture of CLA isomers (1.5%, w/w) for 4 weeks [129].

In humans, a mixture of CLA isomers (3 g/day) in combination with omega-3 fatty acid supplement (3 g/day) for 12 weeks increased circulating adiponectin levels in young (average age=32 years) obese individuals (average BMI=32), however, there was no change in non-obese (average BMI=23) individuals or older (average age=57 years) obese individuals (average BMI=32) [130]. Obese women with type 2 diabetes mellitus taking a mixed isomer CLA supplement (6.4 g/day) for 16 weeks did not have changes in serum adiponectin levels [131]. Likewise, 5.5 g/day of a CLA mixture was unable to change adiponectin levels in women with a BMI <35 but who were otherwise healthy [132]. Taken together, the effects of a mixture of CLA isomers on adiponectin and cell size appear to vary between species and age, and may be dependent on level and type of fat in the background diet.

More recent studies investigating the effects of individual CLA isomers have provided further insight into the specific roles of each isomer. When apoE-knockout mice were fed an atherogenic diet supplemented with either 0.5% (w/w) c9,t11-CLA or 0.5% (w/w) t10,c12-CLA for 12 weeks, only the t10,c12-CLA isomer reduced plasma adiponectin [128]. In a mouse adipocyte cell line (3T3-L1), the t10,c12-CLA isomer alone or in combination with c9,t11-CLA reduced cellular [133, 134] and secreted adiponectin [134]. Serum adiponectin

concentrations were unchanged in Wistar rats fed a saturated fat-based diet (5.1% palm oil) supplemented with either 0.6% (w/w) c9,t11-CLA or 0.6% (w/w) t10,c12-CLA [127]. The c9,t11-CLA isomer did, however, result in larger adipocytes in both the inguinal and retroperitoneal adipose tissues [127]. Interestingly, obese Zucker rats fed an atherogenic diet (2% cholesterol) supplemented with either 1% (w/w) c9,t11-CLA or 1% (w/w) t10,c12-CLA for 5 weeks had increased serum adiponectin concentrations [135]. Again, the effects of CLA are different between species, and individual isomers appear to have different effects not only between species but within species with differing health status.

### **2.5.2 CLA and blood pressure**

There is growing evidence to suggest that CLA has the ability to attenuate blood pressure [136-139]. In SHR, a mixture of CLA isomers at 1-1.5% (w/w) of the diet reduced systolic blood pressure and abdominal adipose tissue after 5-8 weeks [138, 140]. Additionally, significant changes in serum adipokines such as increased adiponectin [138], and reduced resistin and TNF- $\alpha$  [140] were reported. In healthy males, a mixture of CLA isomers (4.7 g/day) over 5 weeks did not elicit a change in blood pressure [141]. Even in obese or overweight humans that are otherwise healthy, a mixture of CLA isomers up to 4 g/day for up to 12 weeks does not alter blood pressure [142, 143]. However, obese or overweight humans with an underlying condition such as rheumatoid arthritis (common inflammatory disease) [144] or hypertension [145] appear more

responsive to CLA treatment. Zhao *et al.* (2009) have shown that CLA supplementation (4.5 g/day) for 8 weeks enhanced the blood pressure lowering effects of ramipril, an ACE inhibitor, in obese hypertensive patients [145]. This treatment was also effective in increasing serum adiponectin and reducing leptin and angiotensinogen levels [145].

Alibin *et al.* [139] have also nicely demonstrated this same concept of CLA being more effective in disease conditions in rats. Sprague Dawley rats given 0.4% (w/w) CLA mixture for 8 weeks showed no change in blood pressure, however, Spontaneously Hypertensive Heart Failure (SHHF) rats had reduced blood pressure compared to both Sprague Dawley and SHHF controls [139].

Additionally, Nagao *et al.* [137] have shown that after 8 weeks of feeding 1% (w/w) dietary CLA mixture to Zucker Diabetic Fatty (ZDF) rats, blood pressure is approximately 10% lower than control animals. With the onset of obesity, blood pressure increased in the control group but this change was prevented in the group receiving the CLA diet. This study also demonstrated an inverse association between plasma adiponectin and blood pressure [137]. An additional study by Nagao *et al.* [136] showed that feeding 0.5% (w/w) t10,c12-CLA for 3 weeks resulted in significantly lower systolic blood pressure in Otsuka Long Evans Tokushima Fatty (OLETF) rats compared with those fed linoleic acid or c9,t11-CLA. Interestingly, angiotensinogen mRNA levels were reduced in adipose tissue by t10,c12-CLA isomer [136]. These results suggest that adipose tissue is a key regulator of blood pressure and that the beneficial effects of

t10,c12-CLA isomer may be due to the reduced secretion of hypertension-promoting adipokines in OLETF rats. However, unanswered questions remain about the effectiveness of CLA compared to known antihypertensive drugs, usefulness as a treatment agent in older animals with established hypertension, the direct effect of CLA isomers on adipocyte function (production and secretion of adipokines), and whether adipokines altered by CLA isomers can directly affect blood pressure in an obese hypertensive model.

## **2.6 Animal models for obesity-related hypertension studies**

The incidence of obesity and hypertension is rising, along with an increased number of individuals displaying characteristics of the metabolic syndrome, a clustering of risk factors including hypertension, abdominal obesity, impaired fasting glucose, dyslipidemia and insulin resistance [146]. Some models that can be used to study obesity-related hypertension include diet-induced obese Sprague Dawley rats, SHR, or *fa/fa* Zucker rats. Diet-induced obese SHR develop hypertension and insulin resistance after 12 weeks of feeding an energy dense diet [147]. Diet-induced obesity can also be induced in Sprague Dawley rats by high-fat or high-energy diets [148], however, it is important to note that some rats are resistant to obesity and it takes a long feeding period (e.g. 12 weeks) for obesity and its associated complications to develop. Also, the pathology and development of co-morbidities has not been well studied in diet-induced obese models. The *fa/fa* Zucker rat is leptin receptor deficient and as a result displays hyperphagia, impaired oral glucose tolerance and

hyperinsulinemia, dyslipidemia, insulin resistance, hypertension and obesity [149-152]. With respect to obesity, *fa/fa* Zucker rats are not visually different at 2-4 weeks of age but by 6 weeks there is a dramatic increase in body weight [153]. After 4 weeks of age, fat pads are heavier in *fa/fa* Zucker rats compared to their lean littermates and by 6 weeks of age the distribution of adipocyte size is substantially different, with significant increases in average adipocyte size [153]. Furthermore, liver angiotensinogen mRNA levels decrease with age, but in adipose there is an increase in angiotensinogen expression from day 21 [154]. Also, at 28 days there is an increase in cellular and secreted angiotensinogen in obese rats [154]. At 9-14 weeks of age *fa/fa* Zucker rats have increased mean arterial pressure [155, 156] and at 8-12 weeks endothelial contractile response to phenylephrine is attenuated in the aorta [157]. The *fa/fa* Zucker rats are responsive to commonly used hypertensive medication such as ACE inhibitors. Studies in obese Zucker rats treated with captopril demonstrated that captopril can prevent elevations in mean arterial pressure [158] and decrease systolic blood pressure from 157 to 136 mmHg [159]. Therefore, captopril is an established antihypertensive drug in various rat models and a response to this drug indicates the presence of hypertension is linked, at least in part, to an imbalance in the systemic RAS. Overall, the *fa/fa* Zucker rat has been well characterized with respect to the onset of obesity, adipose hypertrophy, the development of hypertension, and responsiveness to anti-hypertensive treatment, which makes the *fa/fa* Zucker rat a useful model for investigating the effects of dietary compounds on obesity-related hypertension.

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## Chapter 3: Rationale, Hypotheses, and Objectives

### 3.1 Rationale

Alterations in the normal functioning of adipose tissue have been linked to the growing rates of obesity-related cardiovascular disease. Obesity results in more large hypertrophic cells within the adipose tissue, and this alters adipocyte metabolism and the secretion of a variety of bioactive molecules that may contribute to obesity-related health problems such as hypertension, metabolic syndrome and an increased risk of cardiovascular disease [1-4]. The development of obesity-related hypertension may be due to hypertrophic adipocytes producing and secreting more pro-inflammatory adipokines such as leptin, TNF- $\alpha$ , IL-6 and angiotensinogen, and less adiponectin [3, 5-7]. In obesity there is increased activation of the local adipose RAS, which is involved in regulating blood pressure and adipose growth [8, 9]. By activating NF- $\kappa$ B [10], AngII also promotes the release of pro-inflammatory cytokines, thus further contributing to obesity-related cardiovascular risk [11]. Unlike most other adipokines that are elevated with obesity, adiponectin secretion is reduced in obesity [12] and low levels are associated with the development of obesity-related hypertension [13], possibly by contributing to a decrease in eNOS activity [13]. Despite the clear link between adiponectin and hypertension, the direct effects of adiponectin treatment on high blood pressure and underlying mechanisms remain to be determined.

Many studies have demonstrated the ability of CLA to reduce cardiovascular risk factors, however, there is limited research investigating the mechanisms. Beneficial effects of CLA on blood pressure in young growing rats have been reported [14-16], however, the effects of CLA isomers in older animals with established hypertension have not been examined. Furthermore, since feeding a mixture of CLA isomers has been shown to both reduce adipocyte size and alter adipokine status [17], it seems likely that CLA's ability to improve blood pressure may be due to its ability to reduce adipocyte size and favorably modify the production of adipokines involved in regulating blood pressure. However, research comparing the effects of specific CLA isomers and the mechanisms responsible for CLA's attenuation of obesity-related hypertension is lacking. Therefore, further investigation is required to understand the mechanism by which CLA isomers affect hypertension, with specific emphasis on the role of adipocyte size and adipokine secretion from adipose tissue. Investigation into the direct effect of CLA isomers on adipokine production and secretion in both normal and dysfunctional adipocytes is also needed. In addition, there are no studies comparing the anti-hypertensive effects of CLA to that of an established ACE inhibitor such as captopril, nor is there evidence that adipokines such as adiponectin have a direct influence on blood pressure. Therefore, we propose to investigate the mechanisms by which CLA isomers improve blood pressure in obesity by examining the role of bioactive molecules produced by the adipose tissue.



## 3.2 Hypotheses

The t10,c12-CLA isomer will improve blood pressure of *fa/fa* Zucker rats by reducing adipocyte size and improving adipocyte function, which will be associated with positive changes in blood pressure regulating molecules such as angiotensinogen and adiponectin.

Direct adiponectin treatment will attenuate elevated blood pressure of *fa/fa* Zucker rats by activating eNOS in adipose tissue.

## 3.3 Objectives

1. Determine if direct supplementation with CLA isomers prevents the increase in adipocyte size and pro-inflammatory status associated with the development of obesity in growing *fa/fa* Zucker rats.
  - a. Evaluate the effects of CLA isomers on adipocyte size in growing *fa/fa* Zucker rats.
  - b. Determine the effects of CLA isomers on levels of angiotensinogen, angiotensin receptor type 1, IL-6, TNF- $\alpha$ , and IL-10 mRNA and protein in adipose tissue of growing *fa/fa* Zucker rats.
  - c. Determine the effects of CLA isomers on phosphorylation of NF- $\kappa$ B and its promoter binding activity in adipose tissue of growing *fa/fa* Zucker rats.

2. Determine if dietary supplementation with CLA isomers could treat hypertension and reduce adipocyte size in obese *fa/fa* Zucker rats with established hypertension and directly improve the adipocyte profile of adipokines involved in blood pressure regulation (angiotensinogen and adiponectin).
  - a. Examine the effects of CLA isomers on blood pressure in obese hypertensive *fa/fa* Zucker rats.
  - b. Evaluate the effects of CLA isomers on adipocyte size in obese hypertensive *fa/fa* Zucker rats.
  - c. Determine the effects of direct treatment with CLA isomers on cellular levels of angiotensinogen and adiponectin in both large and small primary adipocytes isolated from obese *fa/fa* Zucker rats.
  - d. Determine the effects of direct treatment with CLA isomers on secreted levels of AngII and adiponectin in both large and small primary adipocytes isolated from obese *fa/fa* Zucker rats.
  
3. Determine the effect of CLA isomers on the development of obesity-related hypertension in *fa/fa* Zucker rats in relation to adiponectin secretion and eNOS activation.
  - a. Examine the effects of CLA isomers on blood pressure in growing *fa/fa* Zucker rats.
  - b. Determine the effects of CLA isomers on plasma and adipose levels of adiponectin in growing *fa/fa* Zucker rats.

- c. Determine the effects of direct treatment with CLA isomers on protein levels of eNOS and phosphorylated eNOS in adipose and aorta of growing *fa/fa* Zucker rats.
- d. Determine the effects of direct treatment with CLA isomers on protein levels of phosphorylated eNOS and AMPK in cultured human aortic endothelial cells.
- e. Examine the effects of direct adiponectin treatment (acute and chronic) on blood pressure in obese *fa/fa* Zucker rats.
- f. Determine the effects of direct adiponectin treatment on protein levels of phosphorylated eNOS and AMPK in adipose and aorta of obese *fa/fa* Zucker rats.

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**Chapter 4: Effects Of Conjugated Linoleic Acid Isomers  
On Adipocyte Size And The Local Adipose Renin-  
Angiotensin System In *fa/fa* Zucker Rats.**

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## 4.1 Abstract

In obesity, increased activity of the local renin-angiotensin system (RAS) and enlarged adipocytes with altered adipokine production are linked to the development of obesity-related health problems and cardiovascular disease. Mixtures of conjugated linoleic acid (CLA) isomers have been shown to reduce adipocyte size and alter the production of adipokines. The objective of this study was to investigate the effects of feeding individual CLA isomers on adipocyte size and adipokines associated with the local adipose RAS. *Fa/fa* Zucker rats received either (i) control, (ii) *cis(c)9,trans(t)11*-CLA, or (iii) *t10,c12*-CLA diet for 8 weeks. The *t10,c12*-CLA isomer reduced adipocyte size in epididymal adipose tissue. RT-PCR and Western blot analysis showed that neither CLA isomer altered mRNA or protein levels of angiotensinogen or AngII receptors in adipose tissue. Likewise, mRNA and protein levels of the pro-inflammatory cytokines TNF- $\alpha$  and IL-6 or the anti-inflammatory cytokine IL-10 were unchanged in adipose tissue. Similarly, neither CLA isomer had any effect on phosphorylation or DNA binding of NF- $\kappa$ B. Our results suggest that although the *t10,c12*-CLA isomer had beneficial effects on reducing adipocyte size, this did not translate into changes in the local adipose RAS or associated adipokines.



## 4.2 Introduction

It is well known now that adipose tissue not only stores triglycerides for energy but also functions as an endocrine organ participating in energy homeostasis, neuroendocrine control and immune functions [1, 2]. Increases in adipose tissue, such as those observed in obesity, are associated with enlargement (hypertrophy) of adipocytes and subsequent changes in the production of bioactive proteins or adipokines to favor a more pro-inflammatory profile [3-5]. These changes are associated with obesity-related health problems, the development of metabolic syndrome and an increased risk of cardiovascular disease [6-8]. Some of the pro-inflammatory adipokines involved in pathogenesis of these diseases include angiotensinogen, tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6.

All components of the renin-angiotensin system (RAS), including angiotensin II (AngII) and its precursor angiotensinogen, are present in adipose tissue and this local RAS has been implicated in the regulation of adipose growth as well as the regulation of blood pressure [9, 10]. In support of this argument, mice that selectively over-express angiotensinogen in adipose tissue exhibit an increase in circulating angiotensinogen and are hypertensive [9]. Upon binding to its receptor, AngII also stimulates pro-inflammatory cytokines through the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) [11], which further contributes to the pathogenesis of cardiovascular complications [12].

Due to the large increases in obesity-related cardiovascular complications, the need for preventative strategies is at an all time high. The use of dietary compounds to prevent or treat obesity-related cardiovascular risk factors may be an attractive idea for some. Conjugated linoleic acid (CLA) is a fatty acid derived from ruminant animals that is being examined for its potential health benefits [13]. Our laboratory has shown that feeding a mixture of CLA isomers to obese *fa/fa* Zucker rats favorably alters levels of serum adipokines such as leptin and adiponectin, and at the same time reduces adipocyte size [14]. At this time, however, it remains unclear which CLA isomer is responsible for reducing adipocyte size in an obese model and if other adipose-derived proteins may be affected. Research from other groups suggest that the t10,c12-CLA isomer may be responsible for the cardiovascular benefits, such as improving blood pressure by reducing adipose angiotensinogen mRNA [15]. This study was therefore designed to investigate the effects of individual CLA isomers on adipocyte size and to examine the relationship with the local adipose RAS in obesity.

## 4.3 Methods

### ***Animals & Diet***

During an 8-week experimental period, 6-week old male *fa/fa* Zucker rats (Harlan, Indianapolis, IN) received diets based on the AIN-93 formula differing in the amounts of CLA isomers (Appendix A). There were 3 groups (i) control diet (ii) 0.4% (w/w) *cis(c)9,trans(t)11*-CLA diet, and (iii) 0.4% (w/w) *t10,c12*-CLA diet. At the end of the feeding period, rats were euthanized by CO<sub>2</sub> asphyxiation and epididymal adipose tissue was dissected, frozen in liquid nitrogen or Cryogel embedding medium and subsequently stored at –80°C until analyzed.

Epididymal adipose tissue was examined because abdominal obesity in humans is primarily to adipocyte hypertrophy [16] and in the *fa/fa* Zucker rat adipose growth occurs mostly by hypertrophy in epididymal adipose as compared to hyperplasia in the peri-renal adipose depot [17]. The University of Manitoba Protocol Management and Review Committee approved the animal protocol which was in agreement with the Canadian Council on Animal Care Guidelines [18].

### ***Morphometry***

Ten micrometer thick adipose sections were mounted on slides and fixed in formaldehyde. Adipocyte cell size was quantified with Image J software as previously described [14].

### ***RT-PCR Analysis***

Total RNA was isolated from epididymal adipose tissue using TRIzol reagent. The concentration of RNA and the purity of the samples were assessed spectrophotometrically. RNA was digested with DNase, reverse transcribed into cDNA and amplified with an Access RT-PCR system kit (Promega, Madison, WI). Primer sequences for detection of angiotensinogen, AngII type 1a receptor, AngII type 1b receptor, TNF- $\alpha$ , IL-6, IL-10 and ribosomal protein L32 (rpL32) mRNA can be found in Table 1. Since high levels of pro-inflammatory mediators have been linked to obesity and CLA is considered effective against obesity [13], we also examined the anti-inflammatory mediator IL-10. cDNA products were run on an agarose gel, visualized with Vista Green Nucleic Acid Stain and relative intensity of the bands was quantified by densitometry. Results are expressed as arbitrary units after normalization to rpL32 expression.

### ***Protein Extraction & Western Blotting***

The bicinchoninic acid assay (Pierce) was used to determine total protein in samples isolated from adipose tissue using a mortar and pestle and 3 $\times$  sodium dodecyl sulfate sample buffer. Adipose samples were analyzed by Western blotting as described previously [19] with antibodies (diluted 1:1000) for angiotensinogen (Fitzgerald), AngII type 2 receptor (Santa Cruz), TNF- $\alpha$  (Cell Signaling), IL-6 (Biosource), IL-10 (Biosource), phosphorylated NF- $\kappa$ B p65 (Ser536) (Cell Signaling) and total mitogen-activated protein kinase (MAPK) (Cell Signaling). The latter was used to account for variability in sample loading.

Autoradiography and scanning densitometry were used to capture and quantify band intensities.

### ***NF- $\kappa$ B Activation***

Nuclear extracts from epididymal adipose tissue samples were prepared using a Nuclear Extract Kit according to the manufacturer's instructions (Active Motif). NF- $\kappa$ B binding activity was determined using a TransAM NF- $\kappa$ B Transcription Factor ELISA Kit according to manufacturer's instructions (Active Motif).

### ***Statistical Analysis***

Data were analyzed with Statistical Analysis Software (SAS 6.04; SAS Institute, Cary, NC) to examine the effect of diet on cell size, mRNA and protein levels by a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences were considered significant at  $P < 0.05$  and all results are expressed as mean  $\pm$  standard error.

## 4.4 Results

### ***Growth performance and feed intake***

There was no significant difference in body weight gained (control, 433±10 g; c9,t11-CLA, 452±9 g; t10,c12-CLA, 453±8 g) or epididymal adipose mass (data not shown) among groups. Likewise, daily food intake was similar among groups (control, 27.2±0.5 g/day; c9,t11-CLA, 28.8±0.6 g/day; t10,c12-CLA, 28.9±0.4 g/day).

### ***Adipocyte Size***

Previously we reported that a mixture of CLA isomers reduced adipocyte size [14], however, the contribution of the individual CLA isomers was not investigated. Therefore, the current study was designed to examine the effect of individual CLA isomers on adipocyte size. The group receiving the t10,c12-CLA isomer had 60% smaller adipocytes compared to the control and c9,t11-CLA groups (Figure 4.1A and 4.1B). The distribution pattern of cell size shows that 80% of adipocytes in the t10,c12-CLA isomer group were <2000  $\mu\text{m}^2$ , with less than 2% of cells >5000  $\mu\text{m}^2$ , whereas the control group and the c9,t11-CLA group had a 53-63% of cells in the 2000-5000  $\mu\text{m}^2$  range and more than 25% of the cells were in excess of 5000  $\mu\text{m}^2$  (Figure 4.1C).

### ***Adipokines in adipose tissue***

It is suggested that adipocyte size alters the production of adipokines and since the t10,c12-CLA isomer reduced adipocyte size we decided to explore adipokine

status in the adipose tissue. Unexpectedly, the AngII precursor angiotensinogen was not changed at the gene (Figure 4.2A) or protein level (Figure 4.3A) in the adipose tissue of the rats fed CLA isomers. There was also no change in AngII receptors (Figure 4.2B, 4.2C and 4.3B). Similarly, TNF- $\alpha$ , IL-6 and IL-10 mRNA were unchanged with CLA isomers (Figure 4.2D, 4.2E and 4.2F). Likewise, protein levels of TNF- $\alpha$ , IL-6 and IL-10 were similar among all groups (Figure 4.3C, 4.3D and 4.3E).

### ***NF- $\kappa$ B Activation***

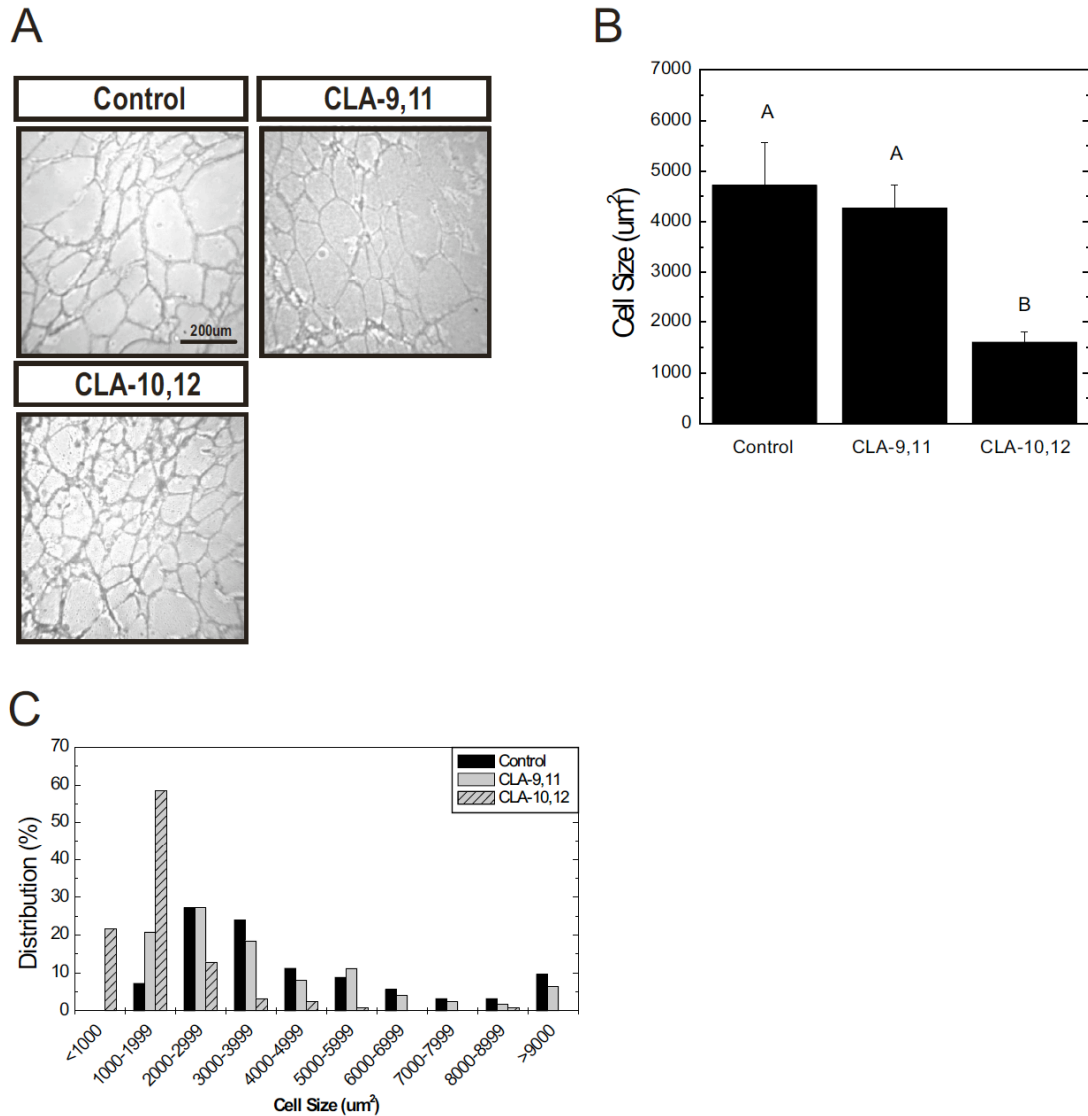
Since NF- $\kappa$ B regulates many genes involved in inflammation, we next examined the levels of phosphorylated NF- $\kappa$ B and its promoter binding activity in epididymal adipose tissue. Neither CLA isomer affected either NF- $\kappa$ B phosphorylation (Figure 4.4A) or DNA binding (Figure 4.4B). This result was not unexpected given that levels of the inflammatory adipokines examined were unchanged by the treatments.

**Table 4.1 Primer Sequences for RT-PCR**

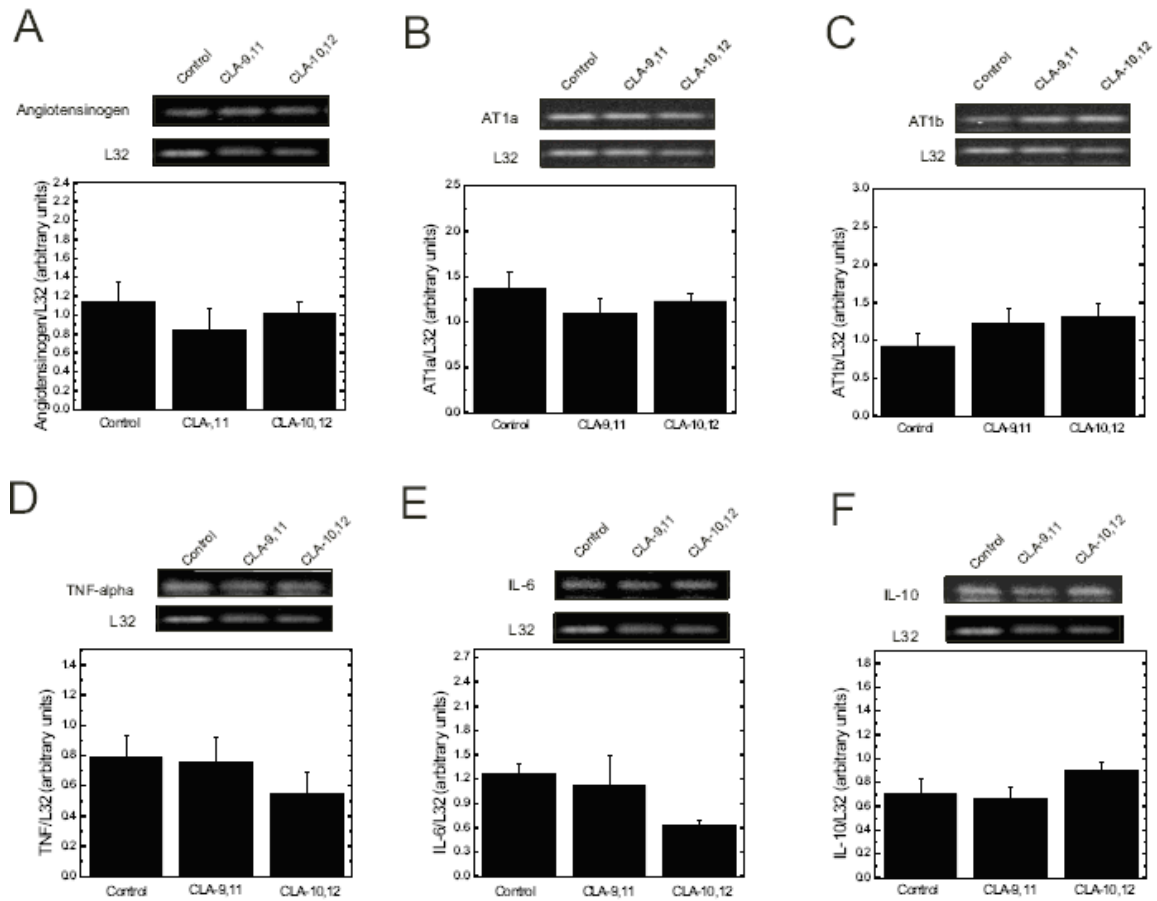
Gene name	Primer sequence
Angiotensinogen	F: 5'-CAC GGA CAG CAC CCT ATT TT -3' R: 5'-GCT CTT CTC CAC CCA GAA CT-3'
TNF- $\alpha$	F: 5'-CTC TGA CAG GGC AGG ATT TT-3' R: 5'-TGA CCT CCC ATC TCC TTT TG-3'
IL-6	F: 5'-CCC AAC TTC CAA TGC TCT CCT AAT G-3' R: 5'-GCA CAC TAG GTT TGC CGA GTA GAC C-3'
IL-10	F: 5'-GGC TCA GCA CTG CTA TGT TGC C-3' R: 5'-AGC ATG TGG GTC TGG CTG ACT G -3'
rpL32	F: 5'-TAA GCG AAA CTG GCG GAA AC-3' R: 5'-GCT CGT CTT TCT ACG ATG GCT T-3'
AngII type 1a receptor	F: 5'-CTC AAG CCT GTC TAC GAA AAT GAG-3' R: 5'-TAG ATC CTG AGG CAG GGT GGA T-3'
AngII type 1b receptor	F: 5'-CTT TCC TAC CGC CCT TCA GAT A-3' R: 5'-TGA GTG CTT TCT CTG CTT CAA C-3'

F = forward primer; R = reverse primer

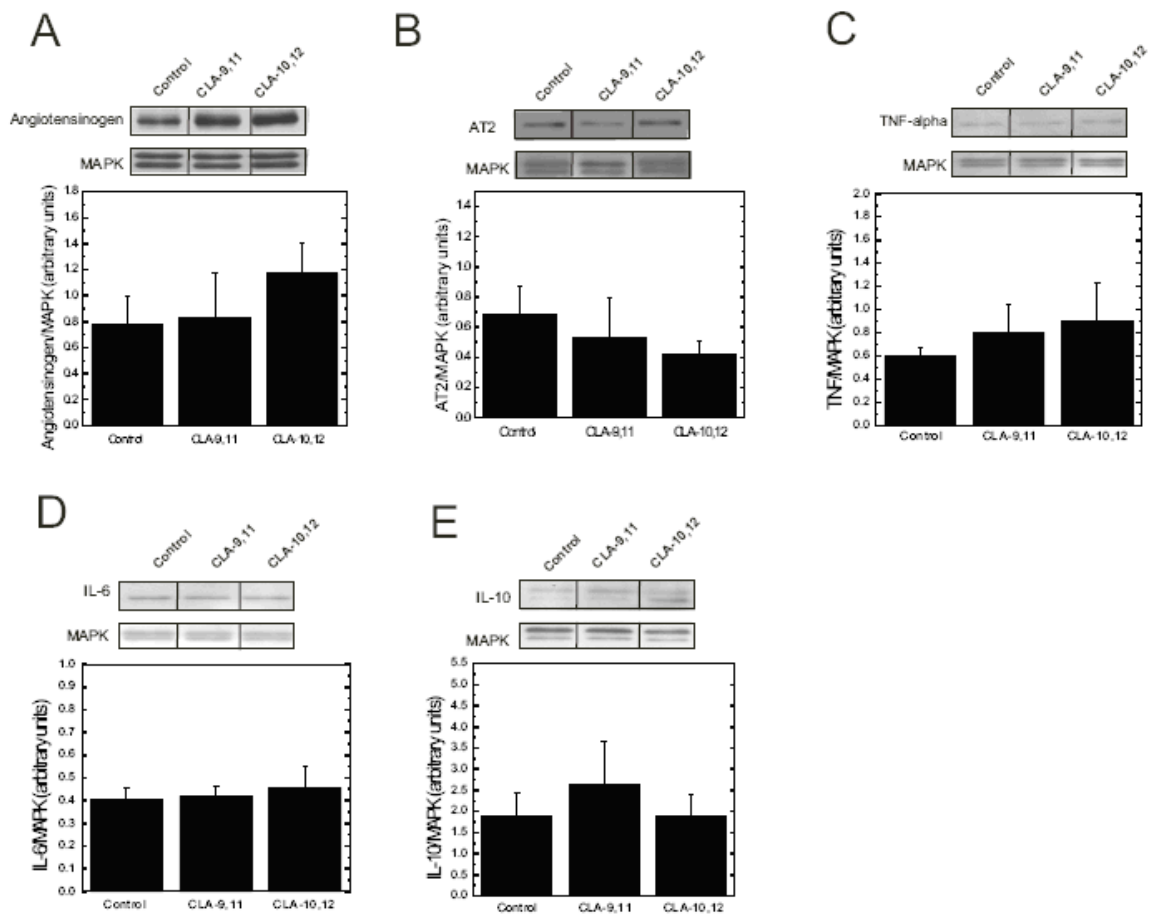




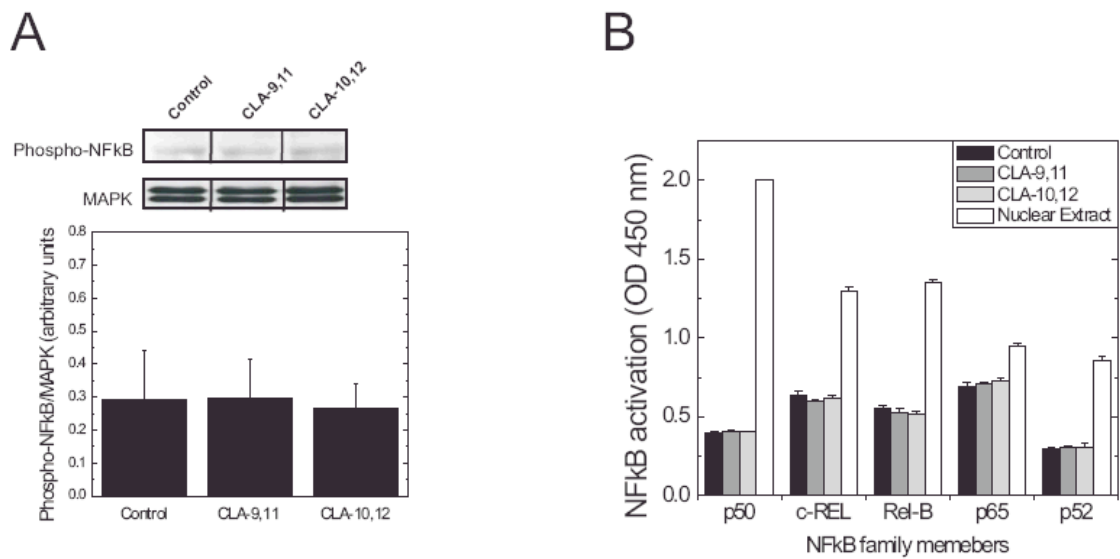
**Figure 4.1 Morphometry of epididymal adipose tissue from 14-week old *fa/fa* Zucker rats fed CLA isomers.** Adipocyte size (A) was quantified for each treatment group and area means (B) are reported in  $\mu\text{m}^2$  for the overall means  $\pm$  SEM, ( $n=5/\text{group}$ ). Area means with different letters are significant different ( $P \leq 0.05$ ) as determined by Duncan's multiple range test. The distribution of adipocyte size (C) in each treatment group is also shown. Control = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA.



**Figure 4.2** Epididymal adipose tissue mRNA levels of angiotensinogen (A), AngII type 1a receptor (B), AngII type 1b receptor (C), TNF- $\alpha$  (D), IL-6 (E) and IL-10 (F) in *fa/fa* Zucker rats ( $n=6/\text{group}$ ). mRNA was analyzed using RT-PCR. The relative intensity of the bands was quantified by densitometry and normalized to that of ribosomal protein L32. Representative gels are shown for each plot. Data are presented as means  $\pm$  SEM. No significant differences ( $P>0.05$ ) were detected by ANOVA. Control = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; AT1a = AngII type 1a receptor; AT1b = AngII type 1b receptor; L32 = ribosomal protein L32.



**Figure 4.3** Epididymal adipose tissue protein levels of angiotensinogen (A), AngII type2 receptor (B), TNF- $\alpha$  (C), IL-6 (D) and IL-10 (E) in *fa/fa* Zucker rats ( $n=4-6$ /group). Protein levels were measured using Western blot analysis. The relative intensity of the protein bands was quantified by densitometry and normalized to that of MAPK. Representative Western blots are shown for each plot. Data are presented as means  $\pm$  SEM. No significant differences ( $P>0.05$ ) were detected by ANOVA. Control = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; AT2 = AngII type 2 receptor.



**Figure 4.4 Epididymal adipose tissue phosphorylated NF- $\kappa$ B p65 (Ser536) levels (A) and binding activity (B) in *fa/fa* Zucker rats.** Protein levels were measured using Western blot analysis (n=6/group). Relative intensity of the protein bands was quantified by densitometry and normalized to that of MAPK. A representative Western blot is shown. Activation of NF- $\kappa$ B was measured with TransAM ELISA kits (Active Motif). Data are presented as means  $\pm$  SEM. No significant differences ( $P>0.05$ ) were detected by ANOVA. Control = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; Nuclear extract = positive control.

## 4.5 Discussion

The current study has demonstrated that feeding t10,c12-CLA to *fa/fa* Zucker rats reduces epididymal adipocyte size. Earlier work examining the effects of CLA mixtures on adipocyte size revealed that other types of fat in the diet may influence the effects of CLA [14, 20, 21]. For example, rats consuming diets based on a soybean oil background and supplemented with a mixture of CLA isomers have reduced cell size [14, 20], whereas the effects of CLA are lost when the background consists of saturated palm oil [21]. In the same study that used palm oil, isomer specific effects were also examined and the c9,t11-CLA isomer increased adipocyte size in the inguinal and retroperitoneal depots of Wistar rats [21]. The increase observed with the c9,t11-CLA isomer was abolished when a mixture of CLA isomers was provided [21], suggesting the t10,c12-CLA may have beneficial effects for reducing cell size. In agreement with this hypothesis, our study demonstrated that the t10,c12-CLA isomer is responsible for reducing cell size. Additionally, since earlier work in *fa/fa* Zucker rats fed a mixture of CLA isomers demonstrated that reduced adipocyte size was associated with altered adipokines, we wanted to test if the beneficial changes associated with adipocyte size observed with the t10,c12-CLA isomer also produce changes in local adipose RAS and cellular adipokine status.

Evidence suggests that obesity-related complications may be due in part to the activation of a local RAS in adipose tissue [9, 22, 23], however, in the current study, no significant differences were detected in adipose angiotensinogen levels

or AngII receptors of CLA fed rats. In contrast, OLETF rats fed the t10,c12-CLA isomer had suppressed angiotensinogen gene expression in peri-renal adipose tissue [15]. Furthermore, Hainault *et al.* [24] showed angiotensinogen levels were greater in inguinal adipocytes isolated from obese compared to lean Zucker rats. Perhaps assessment of angiotensinogen mRNA or protein levels in different adipose depots or different models may be responsible for the varying results. Epididymal adipose tissue was investigated in this study because alterations in adipokine levels in epididymal adipose tissue have been noted in this animal model [14]. We measured angiotensinogen protein levels in epididymal adipose tissue as well as peri-renal adipose tissue (Appendix B), however, we were unable to detect any dietary effects on angiotensinogen levels in either depot. Additionally, it may be that a reduction in fat mass is required in rats to achieve changes in angiotensinogen mRNA [15, 25].

Despite observing changes in adipocyte size, we did not note any changes in adipokine status in the epididymal adipose tissue. Similarly, Wendel *et al.* [26] did not detect any changes in epididymal adipose mRNA levels of TNF- $\alpha$  or IL-6 in *ob/ob* mice fed a mixture of CLA isomers (1.5%, w/w) for 4 weeks. Another study in Sprague Dawley rats examined exercise as a means to reduce cell size and inflammatory cytokines, however, results showed that maintenance of a small adipocyte size by exercise did not alter IL-6 or TNF- $\alpha$  mRNA compared to sedentary rats with increased adipocyte size [27]. Thus, lifestyle interventions such as dietary components and exercise aimed at altering adipocyte size to

improve inflammatory adipokine status require more investigation before conclusions can be drawn.

Cell culture studies suggest that CLA isomers may be able to modulate NF- $\kappa$ B activity [28-30], thus we examined phosphorylation and binding activity of NF- $\kappa$ B in adipose tissue. Our results in obese rats show no effect of either CLA isomer on NF- $\kappa$ B phosphorylation or binding activity, consistent with our findings on inflammatory molecules in the adipose tissue. In contrast, direct treatment of isolated human adipocytes with t10,c12-CLA isomer increased TNF- $\alpha$  and IL-6 mRNA and was associated with increased I- $\kappa$ B $\alpha$  phosphorylation and NF- $\kappa$ B binding activity [28]. Likewise, increased NF- $\kappa$ B binding activity was demonstrated in human umbilical vein endothelial cells treated with either the c9,t11-CLA or t10,c12-CLA isomer [29]. In a mouse macrophage cell line (RAW264.7), however, a mixture of CLA isomers reduced I- $\kappa$ B $\alpha$  phosphorylation and NF- $\kappa$ B binding activity [30]. These conflicting results emphasize that a great deal more work is necessary to determine if CLA isomers have depot and species-specific effects, as well as determine if therapeutic strategies that reduce cell size can directly alter adipokine production and secretion.

In conclusion, this study demonstrates that t10,c12-CLA is the isomer responsible for the reduction in adipocyte size that was observed in an earlier study using a mixture of CLA isomers in *fa/fa* Zucker rats [14]. The reduction in adipocyte size, however, did not alter components the local adipose RAS such

as angiotensinogen and AngII receptors, or associated adipokines such as TNF- $\alpha$ , IL-6 and IL-10. Therefore, further work is needed to determine if there are any functional changes in the adipocyte as a result of reduced cell size via t10,c12-CLA isomer treatment.



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**Chapter 5: Isomer-specific Effects Of Conjugated  
Linoleic acid (CLA) On Blood Pressure, Adipocyte Size  
And Function.**

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## 5.1 Abstract

Obesity-related hypertension may be caused by activation of the local adipose renin-angiotensin system, resulting in exaggerated production of the vasoconstrictor angiotensin II. Additionally, secretion of adiponectin from adipose tissue, which prevents endothelial dysfunction, is altered in obesity. Consumption of conjugated linoleic acid (CLA) has been shown to modulate cytokine release from adipocytes and positively influence blood pressure in younger rats, but its physiological actions in older models with established hypertension and isomer specific effects on adipocyte size remain to be determined. Therefore, we investigated the effects of CLA isomers on adipocyte dysfunction in relation to blood pressure and adipokine production by hypertrophic adipocytes in older fa/fa Zucker rats with established hypertension. Fa/fa Zucker rats were fed cis(c)9,trans(t)11-CLA or t10,c12-CLA isomers for 8 weeks and compared with lean and obese rats fed the control diet. Blood pressure and adipocyte size were subsequently measured. Collagenase-isolated adipocytes were size separated and angiotensinogen and adiponectin protein levels quantified by Western blotting. The t10,c12-CLA group had reduced blood pressure, fewer large adipocytes, and increased serum adiponectin. Angiotensinogen was present at higher levels in the large adipocytes, while the converse was observed for adiponectin. In conclusion, the beneficial effects of the t10,c12-CLA isomer on blood pressure and adipocyte size in vivo results from its ability to reduce the number of large adipocytes, which is associated with altered the levels of vasoactive molecules secreted from adipose tissue.

## 5.2 Introduction

The rising incidence of obesity-related cardiovascular disease has been linked in part to changes that affect the normal function of adipose tissue. Normally, adipose tissue produces and secretes a range of bioactive molecules involved in the regulation of glucose and lipid metabolism, inflammation, blood pressure, coagulation and appetite control [1, 2]. In obesity, growth of adipose tissue is initially due to enlargement of adipocyte size (hypertrophy) due to the increased delivery of fatty acids to adipose, followed by an increase in adipocyte number (hyperplasia) [3]. Previous work has shown that enlarged adipocytes have altered expression and secretion of pro- and anti-inflammatory adipokines, with large adipocytes preferentially displaying a pro-inflammatory state [4]. Altered production and secretion of adipokines, such as adiponectin and angiotensinogen, from enlarged adipocytes may affect cellular processes associated with obesity-linked cardiovascular diseases such as hypertension [5-7].

In obesity, increased adipose tissue renin-angiotensin system activity enhances production of angiotensin II (AngII), resulting in elevated blood pressure [8, 9]. Angiotensinogen-deficient mice have lower body and epididymal adipose weights, reduced adipocyte size as well as lower blood pressure compared to wild type mice [10]. In contrast, overexpression of adipose angiotensinogen results in significantly higher body weight and epididymal adipose tissue mass, increased adipocyte size and elevated blood pressure [10]. On the other hand,



secretion of the adipokine adiponectin is reduced with increased adipose accumulation, specifically in omental adipose [11, 12]. But weight loss of 5-10% can increase circulating adiponectin concentrations and result in smaller adipocytes [13]. Ohashi *et al.* [14] have shown that hypoadiponectinemia is also associated with the development of obesity-related hypertension in a mouse model, however, the mechanism of action remains unclear.

The potential of nutraceuticals to prevent or treat obesity-related diseases has gained much attention over the last several years. Consequently, the use of food components to alter adipocyte size and function is an emerging area of research. Conjugated linoleic acid (CLA) is a naturally occurring fatty acid found in ruminant animals and is a mixture of positional and geometric isomers of linoleic acid (C18:2 n-6). CLA has beneficial effects on blood pressure in younger rats (6 weeks of age) prone to hypertension, possibly via a reduction in angiotensinogen mRNA in adipose tissue due to the actions of the *trans*(t)10,*cis*(c)12-CLA isomer [15-17]. Although benefits have been reported in younger rats prone to hypertension, there are several gaps in our understanding of the effects of CLA on older animals (greater than 14 weeks of age) with established hypertension, isomer-specific responses, and effects on adipocyte size. Previous work from our laboratory has shown that feeding 6 week old *fa/fa* Zucker rats a mixture of CLA isomers has no effect on total body weight, but it does result in reduced epididymal adipocyte size [18]. Treatment with the CLA mixture also increased serum adiponectin and adiponectin mRNA levels in adipose tissue of younger

rats [18]. Given that the physiological effects of changing adipocyte size have not been adequately explored, we elected to investigate the effects of CLA isomers on adipocyte dysfunction in relation to blood pressure and adipokine production by large and small adipocytes in older *fa/fa* Zucker rats with established hypertension.

## **5.3 Methods**

### ***Animals and diet***

The protocol used for these experiments was approved by the University of Manitoba Protocol Management and Review Committee. Fifteen-week old male *fa/fa* Zucker rats (n=35) and their lean littermates (n=10) (Harlan, Indianapolis, IN) were housed individually. The rats were randomly assigned to 1 of 3 dietary groups (n=10/group): (i) obese control, (ii) 0.4% (w/w) c9,t11-CLA, (iii) 0.4% (w/w) t10,c12-CLA, and (iv) lean rats fed the same diet as the obese control group. The diet formulation can be found in Appendix A. At the end of the 8-week feeding period, the rats were euthanized by CO<sub>2</sub> asphyxiation followed by decapitation. Blood samples were collected from the trunk of the animals, centrifuged at 3000g for 15 minutes at 4°C and subsequently stored at –80°C until analyzed for serum adiponectin using a rat-specific total adiponectin EIA kit (Alpco, Salem, NH).

### ***Blood pressure measurements***

The indirect tail cuff method (IITC Life Sciences blood pressure monitoring system with the manual amplifier, Woodland Hills, CA) was used to measure systolic blood pressure in the conscious state at baseline and at the end of the study. Three measurements were taken at each time point and the average was calculated to determine mean systolic pressure.

### ***Morphometry***

Epididymal adipose tissue samples (n=5/group) frozen in Cryogel were sectioned (10  $\mu\text{m}$  thick), mounted on Super Frost plus slides and fixed in formaldehyde. Images were captured with a BH2-RFCA microscope (Olympus, Center Valley, CA) using a Q-Imaging camera and Q-Capture Pro 6.0 software (Surrey, BC). Adipocyte size was quantified with Image J software 1.42 (Rasband, WS, ImageJ, US National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/download.html>) as previously described [18]. A continuous block of 25 cells was measured in every field to determine average cell size and size distribution for each treatment group.

### ***Isolation and size separation of adipocyte***

Adipocytes were isolated following previously published procedures [19] with slight modifications. Briefly, freshly dissected epididymal adipose tissue (10 g) from sixteen week old *fa/fa* Zucker rats (n=5) was rinsed with phosphate buffered saline (PBS), minced with scissors, and then placed into a 37°C solution of Minimum Essential Medium (Invitrogen, Carlsbad, CA) containing 1.05 mg/ml collagenase Type A (Roche, Mississauga, ON), 4% bovine albumin (Invitrogen), and 1 ml/100 ml Antibiotic/Antimycotic (Invitrogen) for 60 minutes. The digested samples were then filtered through a 250  $\mu\text{m}$  nitex screen (Dynamic Aqua-Supply, Surrey, BC) and washed 3 times with fresh media. The flow-through was collected in 50 ml conical tubes that were gently inverted 3 times by hand and cells that resurface were collected and subsequently filtered through a 70  $\mu\text{m}$

nitex screen. To collect large adipocytes, the mesh was inverted over a new conical tube and washed with fresh media to capture the large adipocytes. Dense cells that did not surface after 30 seconds were filtered through a 50  $\mu\text{m}$  nitex screen and the cells that passed through were considered small adipocytes. Both small and large adipocyte populations were cultured and treated with CLA isomers as described in the figure legend.

### ***Biochemical Assays***

Adiponectin concentrations in the cell media were determined using a ELISA kit for total mouse/rat adiponectin (Otsuka Pharmaceutical, Montreal QC).

Angiotensin II concentrations were analyzed using an angiotensin II EIA kit (SPIbio, Montigny le Bretonneux, France), which exhibited some cross-reactivity with both angiotensin I (4%) and angiotensin III (36%). Absorbance readings for both assays were obtained with a microplate reader (SpectraMax) using SOFTmax Pro software.

### ***Western Blot Analysis***

Cell lysates were analyzed by Western blotting as previously described [20] for angiotensinogen (1:1000; Fitzgerald, Concord, MA) and adiponectin (1:1000; Calbiochem, Nottingham, UK). Membranes were stained for total protein with Ponceau S (Sigma, Oakville, ON) to confirm protein loading [20]. Data are expressed as arbitrary units after normalizing to the intensity control (same sample on each gel) and loading control (Ponceau S).

### ***Statistical Analysis***

Data were analyzed with SAS (SAS 6.04; SAS Institute, Cary, NC). In the dietary study, a one-way ANOVA was used to determine the effect of diet on organ weights and cell size whereas the effects of diet and duration of feeding on blood pressure were analyzed as repeated measures 2-way ANOVA with main effects of time and diet. In the primary adipocyte experiments, differences in size between the two cell populations were analyzed by Student's t-test and a 2-way ANOVA was used to determine the effect of cell size and treatment on adipokine production and secretion. Following ANOVA, differences among groups were determined by Duncan's multiple range test. A *P*-value of  $\leq 0.05$  was considered statistically significant and all results are reported as means  $\pm$  SEM.

## 5.4 Results

### ***Body Mass and Blood Pressure***

At the end of the 8-week feeding period, the feed intake (data not shown), the body weight (Figure 5.1A) and adipose weight (Figure 5.1B) of the obese *fa/fa* Zucker rats were similar among the three groups, but were higher than that of the lean animals. In parallel with weight, all obese animals had a systolic pressure at baseline almost 30 mmHg higher than the lean (obese:  $162 \pm 4$  mmHg; lean:  $132 \pm 3$  mmHg). Furthermore, unlike the lean animals in which blood pressure remained constant, systolic pressure increased in the *fa/fa* control group by more than 10 mmHg over the 8 week period of the study (Fig 5.1C), resulting in a 40 mmHg difference with lean at week 8 (obese:  $175 \pm 5$  mmHg; lean:  $127 \pm 2$  mmHg). Similar results were obtained with the c9,t11-CLA group. In contrast, supplementation with the t10,c12-CLA isomer prevented the increase in systolic blood pressure observed in the obese group at week 8 ( $159 \pm 5$  mmHg vs  $175 \pm 5$  mmHg, respectively) (Figure 5.1C). Even though there was a 3% reduction in blood pressure in the t10,c12-CLA group from week 0 to week 8 ( $163 \pm 4$  vs  $159 \pm 5$  mmHg, respectively), the values of the t10,c12-CLA group still remained significantly higher than those of the lean animals at week 8 ( $159 \pm 5$  mmHg vs  $127 \pm 2$  mmHg, respectively) (Figure 5.1C). Conversely, serum adiponectin was significantly higher in the t10,c12-CLA group ( $18.3 \pm 1.0$   $\mu\text{g/ml}$ ) compared to the obese and lean groups ( $15.7 \pm 0.7$  and  $9.3 \pm 0.7$   $\mu\text{g/ml}$ , respectively) (Figure 5.1D).

### ***Adipocyte Size***

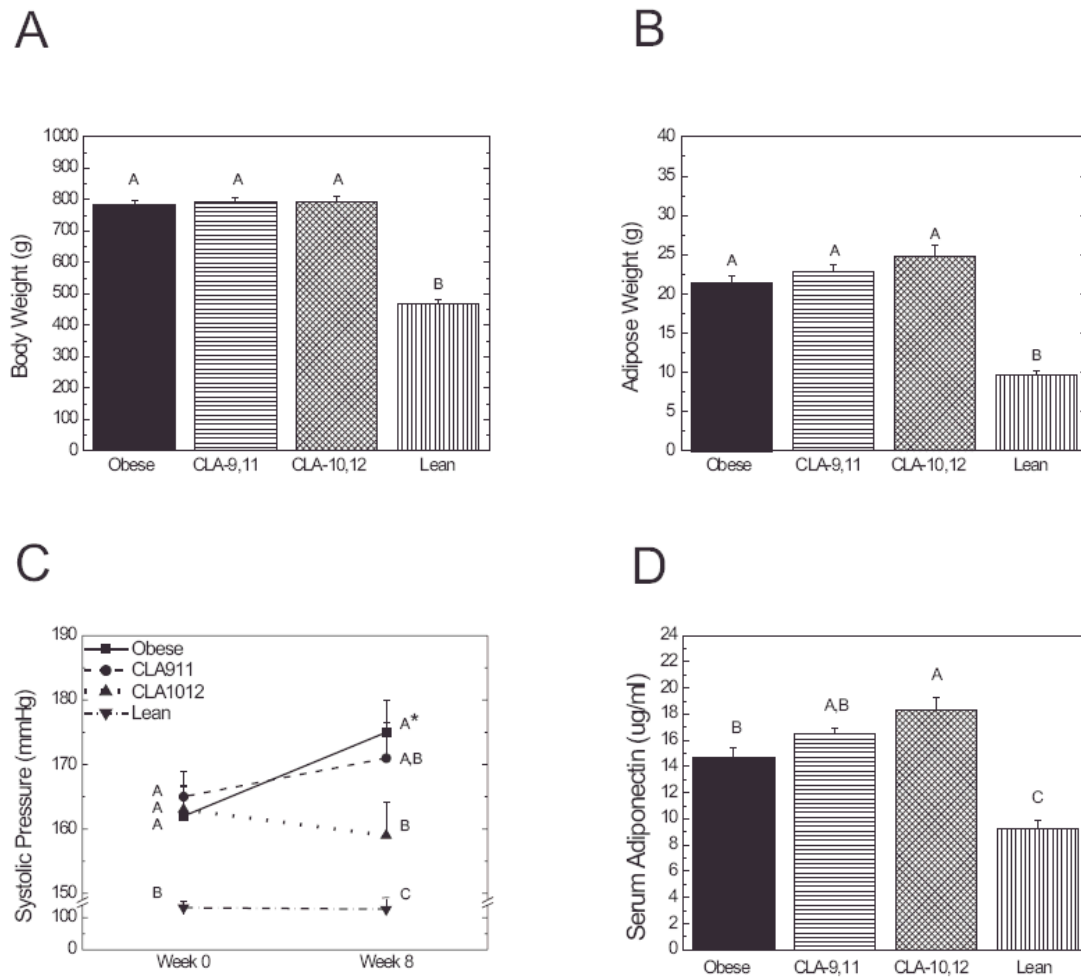
The obese control group had significantly larger cells in comparison to the lean control group (Figure 5.2A). Adipocyte area was similar in both the c9,t11-CLA isomer group and the obese control group (Figure 5.2A). In contrast, the adipocytes of the group receiving the t10,c12-CLA isomer were similar in size relative to those of the lean group. The adipocytes of these two groups (t10,c12-CLA and lean) were 50% smaller than in the obese control and c9,t11-CLA groups (Figure 5.2A). The distribution pattern of adipocyte size shows lean rats and obese rats receiving the t10,c12-CLA isomer had no cells  $>5000 \mu\text{m}^2$ , whereas the obese control group and the obese rats receiving the c9,t11-CLA isomer had some cells in excess of  $6000 \mu\text{m}^2$  (Figure 5.2B). It appears the adipocytes from animals receiving the t10,12-CLA isomer may function more like adipocytes from non-obese animals.

### ***Cytokine Production by Large and Small Adipocytes***

To test whether the effects of CLA were direct, adipocytes isolated from epididymal adipose of *fa/fa* Zucker rats were size-separated and then treated with CLA isomers. The technique employed to separate adipocytes according to size achieved a 10-fold difference in the mean cell area between the large and small populations (Figure 5.3A and 5.3B). This significant distinction in cell size enabled us to investigate the specific cytokines secreted by each cell population within the context of blood pressure regulation. Small adipocytes expressed significantly higher levels of adiponectin than large adipocytes (Figure 5.4A). In

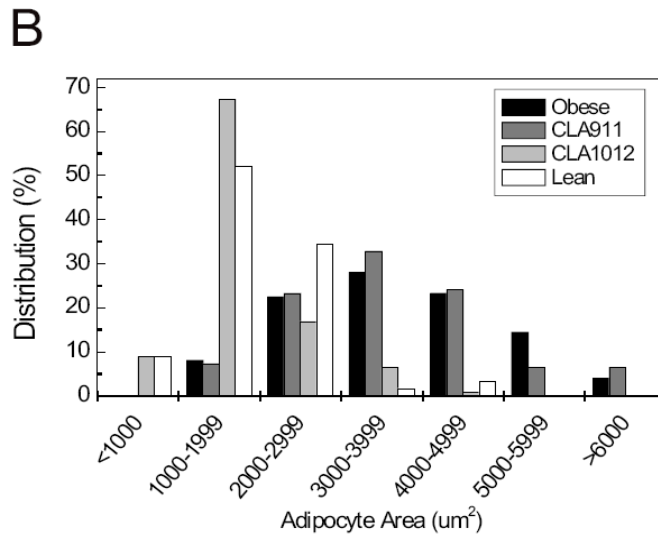
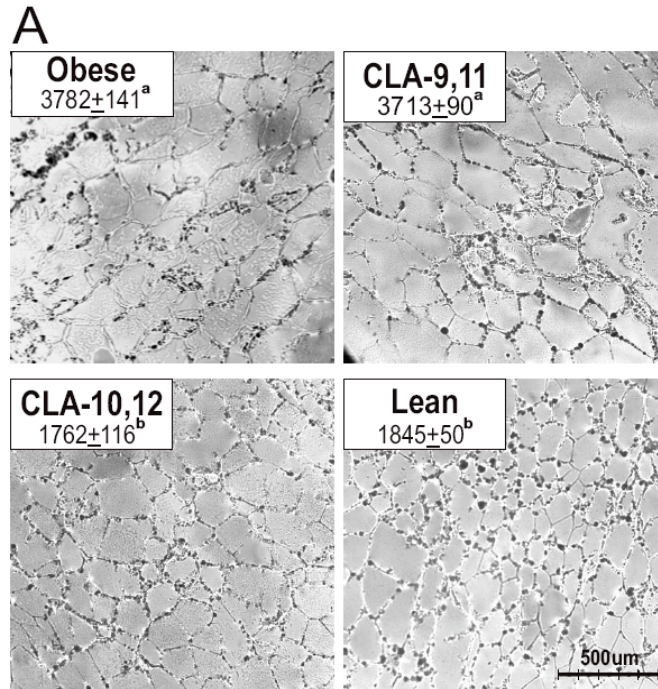


contrast, small adipocytes had significantly lower levels of angiotensinogen than large adipocytes (Figure 5.4B). CLA isomers did not significantly influence levels of adiponectin or angiotensinogen in large adipocytes (Figure 5.4A and 5.4B, respectively). However, in small adipocytes, both CLA isomers increased adiponectin levels (Figure 5.4A). Differences in the cytokine content of the cell medium were also observed for the large and small adipocytes (Figure 5.4C and 5.4D). While treatment with the CLA isomers did not influence adiponectin secretion by either the small or large cells (Figure 5.4C), the c9,t11-CLA isomer reduced AngII in the cell medium of large adipocytes compared to the linoleic acid control (Figure 5.4D).



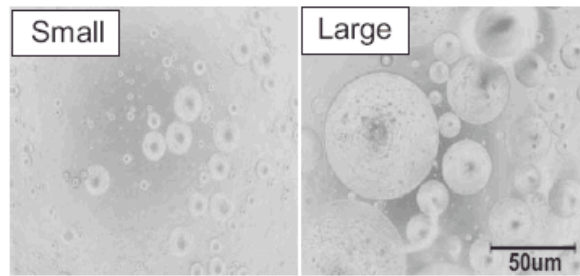
**Figure 5.1 Physiological parameters of *fa/fa* Zucker rats fed CLA isomers for 8 weeks.**

Body weight (A), epididymal adipose tissue weight (B), systolic blood pressure (C) and total serum adiponectin (D). Data are presented as means  $\pm$ SEM. Different letters denote significant differences ( $P \leq 0.05$ ) at the same time point as determined by Duncan's multiple range test and an asterisk (\*) denotes a significant change in systolic blood pressure from week 0. Obese = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; Lean = lean Zucker rats fed 0% CLA.

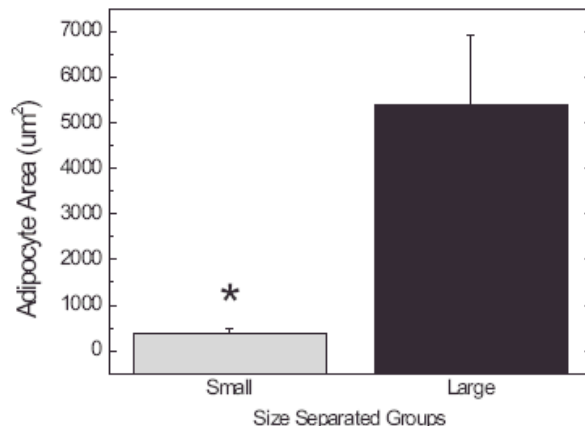


**Figure 5.2 Morphometry of epididymal adipose tissue from 24-week old *fa/fa* Zucker rats fed CLA isomers.** Adipocyte size (A) was quantified for each group and cell area is reported in µm<sup>2</sup> for the overall means ± SEM; different letters indicate significant differences ( $P \leq 0.05$ ) as determined by Duncan's multiple range test. The distribution of adipocyte size (B) in each treatment group is also shown. Obese = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; Lean = lean Zucker rats fed 0% CLA.

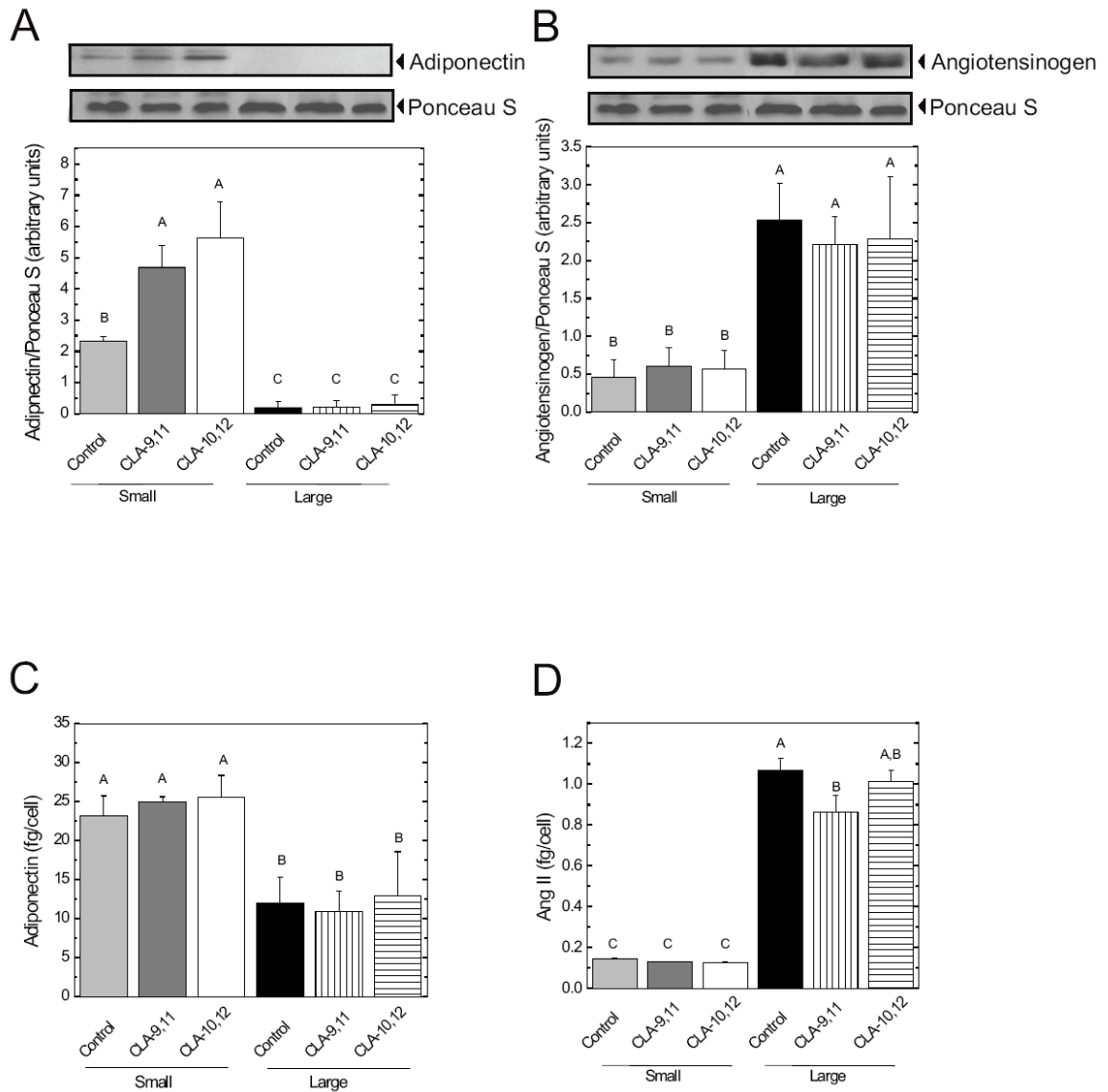
A



B



**Figure 5.3 Large and small adipocyte populations from *fa/fa* Zucker rats.** Representative images of small and large adipocytes obtained by size separation (A). The area in  $\mu\text{m}^2$  of these cell populations was quantified and the data are presented as means  $\pm$ SEM (B). \* denotes a significant difference as determined by Student's t-test ( $P \leq 0.005$ ).



**Figure 5.4 Adipokines produced by large and small adipocytes from *fa/fa* Zucker rats.** Both small and large adipocytes were plated on 6 well plates and treated with either (i) 60  $\mu$ M linoleic acid, (ii) 60  $\mu$ M c9,t11-CLA, or (iii) 60  $\mu$ M t10,c12-CLA for 96 hours. Western blotting was subsequently used to analyze adiponectin (A) and angiotensinogen (B) levels in cells and enzyme immunoassays were used to analyze total adiponectin (C) and angiotensin II (D) in the culture media. Protein levels expressed as arbitrary units relative to Ponceau staining for total protein. Data are presented as means  $\pm$ SEM. Different letters denote significant differences ( $P \leq 0.05$ ) as determined by Duncan's multiple range test. Ang II = angiotensin I

## 5.5 Discussion

Due to the increased incidence of obesity in our society there is great demand for the development of novel lifestyle interventions and/or new pharmacological targets that can reduce the incidence of obesity-related diseases. Hypertension exemplifies a condition for which there is excellent epidemiological evidence showing a positive relationship with obesity and that leads to serious health consequences [21, 22]. The current study was therefore designed to examine the utility of dietary intervention with CLA in the treatment of established obesity-related hypertension. Our investigation demonstrates for the first time that inclusion of the t10,c12-CLA isomer in the diet attenuates systolic blood pressure, increases serum adiponectin and leads to a decrease in adipocyte size in older *fa/fa* Zucker rats that already have hypertension. Furthermore, adipocyte size reverts with t10,c12-CLA feeding to that of lean rats. These positive changes in blood pressure, adipocyte size and adiponectin occurred without a reduction in body weight thus highlighting the importance of adipose function. Lastly, synthesis and secretion of adipokines involved in blood pressure regulation are distinctly different in small and large adipocytes, with higher levels of adiponectin in small adipocytes compared to large adipocytes, and the converse with angiotensinogen.

This study shows that the t10,c12-CLA isomer provides beneficial effects on systolic blood pressure in an older animal model that already has hypertension. This adds to previous reports showing that CLA isomers may be beneficial for attenuating systolic blood pressure in growing rats during the development of hypertension [15-17].

Interestingly, Zhao *et al.* [23] recently published a study in obese hypertensive men and women showing that CLA supplementation (4.5 g/day) enhanced the blood pressure lowering effect of ramipril, an angiotensin converting enzyme (ACE) inhibitor. Since a mixture of CLA isomers (5.5 g/day) can be safely consumed by healthy human males (BMI < 30 kg/m<sup>2</sup>) with normal blood pressure without causing hypotension [24], the blood pressure lowering effects of CLA appear to be therapeutic, producing desirable results in disease conditions such as obesity-related hypertension.

Serum adiponectin concentrations were investigated in this study as previous research has demonstrated a negative correlation between blood pressure and serum adiponectin concentrations [25-29]. Furthermore, blood pressure treatments that block the renin-angiotensin system also increase adiponectin levels [30, 31]. As expected, feeding the t10,c12-CLA isomer increased serum adiponectin concentrations in *fa/fa* Zucker rats. Our data are in agreement with previous work showing a mixture of CLA isomers increases serum adiponectin concentrations in disease models such as *fa/fa* Zucker rats, Zucker diabetic fatty rats and spontaneously hypertensive rats [16-18]. These observations have recently been extended to humans by Zhao *et al.* [23] who showed that the combination of CLA and ramipril led to a significant increase in circulating adiponectin levels in obese hypertensive humans.

We also demonstrated that the t10,c12-CLA isomer is responsible for reducing epididymal adipocyte size in *fa/fa* Zucker rats without decreasing body or adipose weight. Most of the previous work investigating CLA and adipocyte size was conducted

in growing animals using a mixture of CLA isomers [18, 32, 33]. Data from these studies show an increase in the proportion of small adipocytes in growing female Sprague-Dawley or male *fa/fa* Zucker rats fed CLA mixtures with soybean oil in the background diet. Interestingly, this effect is lost in male Wistar rats when saturated palm oil is used in the background diet [33]. A single study has looked at individual isomers of CLA and adipocyte size; it found the c9,t11-CLA isomer increased adipocyte size in the inguinal and retroperitoneal depots of Wistar rats, but there was no effect when the isomers were combined [33]. These data suggest that the t10,c12-CLA isomer may be preventing the negative actions of the c9,t11-CLA isomer.

The shift in adipocyte size observed with CLA treatment brings up the question: does the change in size actually affect adipocyte function, particularly the ability to produce and secrete various molecules? Size separated adipocytes allowed us to study the treatment of different cell populations *in vitro* and we observed detectable differences in adipokines from cells of the same animal. Large adipocytes expressed more angiotensinogen, resulting in higher amounts of angiotensin II in the medium. These data add to previous work by Hainault *et al.* [34] showing both secreted and cellular angiotensinogen levels are greater in obese compared lean rat adipocytes. Likewise, our study showed reduced adiponectin expression and secretion in large adipocytes compared to small adipocytes. However, until the current study, research examining the effect of CLA treatment on angiotensinogen and adiponectin production in adipocytes was lacking. Although CLA isomers did not influence angiotensin II secretion by small adipocytes, the c9,t11-CLA isomer reduced angiotensin II secretion by large adipocytes.



These results suggest CLA isomers may independently contribute to the blood pressure effects of CLA. We also demonstrated that CLA isomers increase levels of adiponectin in small adipocytes from *fa/fa* Zucker rats without affecting secretion of adiponectin. Current available literature on the ability of CLA to alter adiponectin production and secretion has been mostly carried out in 3T3-L1 mouse adipocytes [35-37] showing that the c9,t11-CLA isomer increases adipocyte adiponectin levels [37] as well as adiponectin secretion [36], whereas the t10,c12-CLA-isomer decreases cellular and secreted levels of adiponectin [35]. In primary epididymal adipocytes from Wistar rats, a mixture of CLA isomers (up to 200  $\mu$ M) decreased the secretion of adiponectin [38]. We did not note any differences in adiponectin secretion by CLA isomers in the current study, however, a comparison to other studies is difficult since cell size was not considered.

In conclusion, our study is the first to show that the t10,c12-CLA isomer displays beneficial effects on systolic blood pressure in adult hypertensive *fa/fa* Zucker rats. We also showed beneficial effects of the t10,c12-CLA isomer on adipocyte size, and distinct differences in cellular and secretion levels of adipokines involved in blood pressure regulation from small and large adipocytes. However, treatment of isolated adipocytes with individual CLA isomers did not mimic the effects on adipokines seen *in vivo*, thus suggesting that cross-talk between the various cells types present in adipose tissue may play an important role in regulating the production and release of adipokines *in vivo*. Further research investigating individual as well as combined CLA isomers in different obese hypertensive models looking at both total and size separated adipocytes

is warranted to better understand the effects of CLA isomers on adipocyte function in the content of adipokine production and secretion. On the other hand, this study clearly establishes the existence of a link between CLA's ability to improve blood pressure, increase adiponectin and reduce adipocyte size, however, the causal relationships among these benefits requires further exploration.

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**Chapter 6: Conjugated Linoleic Acid Improves Blood  
Pressure By Increasing Adiponectin And Endothelial Nitric  
Oxide Synthase Activity**

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## 6.1 Abstract

Conjugated linoleic acid has been reported to reduce blood pressure in obese insulin-resistant rats, but its mechanism of action has not been identified. The objective of this study was to determine whether conjugated linoleic acid isomers can reduce obesity-related hypertension in the *fa/fa* Zucker rat and to determine the mechanism of action, focusing on adiponectin production and endothelial nitric oxide synthase activation. Obese *fa/fa* Zucker rats were randomly assigned to: 1) *cis9,trans11*-conjugated linoleic acid, 2) *trans10,cis12*-conjugated linoleic acid, 3) control, or 4) captopril. After 8 weeks, systolic blood pressure was increased 30% in control obese rats. This increase was attenuated 11-13% in the *trans10,cis12*-conjugated linoleic acid isomer and captopril groups. The *trans10,cis12*-conjugated linoleic acid isomer concurrently elevated adiponectin levels in both plasma and adipose tissue and increased phosphorylated endothelial nitric oxide synthase in adipose tissue as well as the aorta. Although a direct effect of conjugated linoleic acid was not observed in cultured endothelial cells, direct adiponectin treatment increased phosphorylation of endothelial nitric oxide synthase. Endothelial nitric oxide synthase phosphorylation was also increased in adipose of *fa/fa* Zucker rats infused with adiponectin in parallel with improvements in blood pressure. Our results suggest that the *trans10,cis12*-conjugated linoleic acid isomer attenuates development of obesity-related hypertension, at least in part, by stimulating adiponectin production, which subsequently activates vascular endothelial nitric oxide synthase.

## 6.2 Introduction

Hypertension makes a significant contribution to the morbidity and mortality associated with cardiovascular disease, and obesity is a major contributor to hypertension [1].

Although the relationship between obesity and hypertension is well established, the reason for this association remains poorly understood, but it is likely linked to endothelial dysfunction [2]. The increase in adipose tissue that leads to obesity initially results from an enlargement of adipocytes and is associated not only with changes in adipocyte metabolism, but also their endocrine properties [3]. The resultant alteration in secretion of a variety of bioactive molecules may alter gene expression and cell signaling in various tissues, including the vasculature, and thus contribute to obesity-related hypertension [3].

The amount of adiponectin secreted by the adipose tissue decreases as fat accumulates [4], and an association between low plasma adiponectin levels and increased risk of cardiovascular disease is now recognized [5]. Adiponectin is a 30 kDa protein secreted from the adipose tissue that exhibits anti-atherogenic and anti-diabetic properties [6]. While it is typically found in the circulation at concentrations between 0.5-30  $\mu\text{g/ml}$  [7], plasma levels are lower in obesity. Recently, hypoadiponectinemia has been associated with the development of obesity-related hypertension, specifically by altering expression of endothelial nitric oxide synthase (eNOS) [8], the enzyme responsible for the production of the vasodilatory molecule nitric oxide (NO).

Adiponectin can also increase NO production through phosphorylation of eNOS via AMP-activated protein kinase (AMPK) [9, 10]. Although a clear link between adiponectin

and hypertension has been made, the direct effects of adiponectin treatment on obesity-related hypertension remain to be determined.

Nonpharmacological interventions with dietary compounds are receiving increased attention as a novel approach to treat obesity-related hypertension. Conjugated linoleic acid (CLA) is a term used to describe a mixture of positional and geometric isomers of linoleic acid, an 18-carbon polyunsaturated fatty acid. Nagao *et al.* [11] have shown that feeding the *trans*(t)10,*cis*(c)12-CLA isomer results in significantly lower systolic blood pressure in Otsuka Long Evans Tokushima Fatty (OLETF) rats compared with those fed linoleic acid or c9,t11-CLA. Current work from our laboratory has shown that feeding *fa/fa* Zucker rats a mixture of CLA isomers reduces the levels of pro-inflammatory agents that are elevated in obesity, reduces adipocyte hypertrophy and increases serum adiponectin [12]. Given these multiple actions of CLA, this study was designed to examine the relationship between adiponectin and eNOS activity in the blood pressure lowering actions of CLA in an obese rat model. We also investigated the effects of adiponectin treatment on blood pressure of *fa/fa* Zucker rats and the contribution of eNOS to this process.

## 6.3 Methods

### ***CLA feeding intervention***

The animal protocol was approved by the University of Manitoba Protocol Management and Review Committee. Thirty-six male, 5-week old, *fa/fa* Zucker rats (Harlan) were acclimatized for 5-8 days and then randomly assigned to 1 of 4 groups for 8 weeks: 1) 0.4% *c9,t11*-CLA, 2) 0.4% *t10,c12*-CLA, 3) control, 4) captopril. The control and CLA diets were based on the AIN-93 formulation (Appendix A). Captopril was added to the drinking water at 50 mg/kg/day [13]. Systolic blood pressure was measured in the conscious state by the indirect tail cuff method using the IITC Life Sciences system. Research shows that the tail-cuff method provides comparable blood pressure results to telemetry measurements [14, 15] and current research continues to use this method for measuring blood pressure in rats [16]. At the end of the study, tissues were collected for additional analysis as described below.

### ***Cell culture***

Human aortic endothelial cells (HAECs; Clonetics) were expanded in growth medium (EBM-2) and passaged when 70% confluent. Experiments were performed with cells at passage 5. HAECs were treated for 1 hour and then lysed with 2× sodium dodecyl sulfate sample buffer. Cell lysates were subsequently analyzed by Western blotting.

### ***Acute adiponectin injection***

Detailed methods for cloning and purification of adiponectin can be found in the Supplemental material (page 134) and Appendix A. Sixteen-week old *fa/fa* Zucker rats

(n=6) were implanted with telemetry transponders (C50-PXT; Data Sciences International) in the femoral artery and allowed 10 days to recover. Each rat was given a bolus injection, via the tail vein, of saline and adiponectin (50 µg), separated by a period of 48 hours to ensure responses had normalized. Blood pressure was measured for 120 minutes following the injections. Finally, the rats were given the nitric oxide synthase inhibitor (L-NAME) in their drinking water (80 mg/kg/day) for 24 hours at which time adiponectin was injected and blood pressure monitored for 120 minutes. In the acute study, blood pressure readings were taken by telemetry because measurements needed to be taken continuously over a 2-hour period.

### ***Chronic adiponectin infusion***

Osmotic pumps (model 2001; ALZET) were implanted subcutaneously into 16-week old *fa/fa* Zucker rats (n=6/group) to infuse either saline or adiponectin (50 µg/day). Blood pressure was measured every 48 hours by the tail cuff method (CODA-6, Kent Scientific). On day 7 of infusion, rats were euthanized, plasma samples were collected for detection of recombinant adiponectin (Figure S6.1, Supplemental material) and tissues were collected for post-mortem analysis by Western blotting.

### ***Plasma and tissue collection***

Rats were euthanized by CO<sub>2</sub> asphyxiation at the end of the treatment period and trunk blood was collected, centrifuged and stored at -80°C. The aorta and epididymal adipose tissue were removed, weighed, frozen in liquid nitrogen or embedding medium

(Cryo-Gel; Instrumedics) and stored at  $-80^{\circ}\text{C}$ . Adipocytes were isolated from the epididymal adipose tissue according to Jernas *et al.* [17].

### ***Measurement of plasma adiponectin***

Total plasma adiponectin levels were measured using a commercial rat ELISA kit (Alpco Diagnostics) following the manufacturer's guidelines (n=5/group).

### ***Western blotting***

Protein was extracted from frozen aorta and adipose tissue samples and subjected to Western blotting [12] with antibodies (diluted 1:1000) to adiponectin (Calbiochem), and phosphorylated eNOS (Ser1177), eNOS, phosphorylated AMPK- $\alpha$  (Thr172), and AMPK- $\alpha$  (Cell Signaling). Membranes were stripped and re-blotted for either mitogen-activated protein kinase (MAPK) (Cell Signaling) or  $\beta$ -tubulin (Abcam) to correct for variability in sample loading. Results are expressed as arbitrary units after being normalized to the intensity control (same sample on each gel) and loading control.

### ***Statistical analysis***

Differences were considered significant at  $P < 0.05$  and all results are expressed as mean  $\pm$  SEM. Data were analyzed by repeated measures two-way ANOVA for main effects of treatment and time (blood pressure *fa/fa* Zucker rats) or one-way ANOVA for treatment effects (parameters in *fa/fa* Zucker rats, HAECs, acute adiponectin infusion) followed by means testing with Duncan's multiple range test. Student's t-test was used

to determine treatment changes over time (0 vs 8 weeks) or effects of chronic adiponectin infusion (blood pressure, protein levels).



## 6.4 Results

### ***Physiological actions of CLA***

In our experiment, no differences in feed intake or body weight due to the dietary CLA supplementation or captopril treatment were observed (Table 6.1). Epididymal adipose tissue weight was also similar among all groups (Table 6.1). These data indicate that neither the CLA isomers nor captopril altered these parameters. Over the 8-week study, body weight increased approximately 3-fold in all groups. In parallel, systolic blood pressure increased more than 30% in obese rats fed the control diet (Figure 6.1A). This increase in blood pressure was significantly attenuated in all treatment groups, with t10,c12-CLA and captopril having the greatest effect (Figure 6.1A). At the end of the study, systolic blood pressure was 7%, 11%, and 13% lower in the c9,t11-CLA, t10,c12-CLA and captopril groups than the control group, respectively. These data are the first to show that c9,t11-CLA has the ability to affect blood pressure, albeit not as potently as t10,c12-CLA.

### ***Effect of CLA feeding on adiponectin and eNOS***

Circulating levels of total adiponectin were 1.7-fold higher in the group receiving the t10,12-CLA isomer compared to control (Figure 6.1B). In contrast, neither c9,t11-CLA nor captopril significantly affected plasma adiponectin concentrations. Treatment with the t10,c12-CLA isomer also resulted in a 2-fold increase in adiponectin levels in adipose tissue relative to the control group (Figure 6.1C). No effect was seen with either captopril or the c9,t11-CLA on adipose adiponectin levels. These results suggest the

increase in circulating adiponectin in the t10,c12-CLA group results from elevated production.

NO is produced in adipose tissue by eNOS and causes vasodilation of blood vessels, thus the potential contribution of eNOS activity was examined. Phosphorylation of eNOS was increased 5- to 13-fold in the adipose of the t10,c12-CLA isomer group compared to all other groups (Figure 6.2A), implicating eNOS as a target for the actions of t10,c12-CLA. There was no change in total eNOS levels in adipose with any treatment (Figure 6.2B). To identify the source of eNOS that is susceptible to CLA treatment, microscopy (please see Supplemental material) and Western blotting were used to examine eNOS levels in two abundant cell types present in adipose tissue. These data clearly show that eNOS is present in the endothelial cells and not in adipocytes (Figure S6.2, please see Supplemental material) suggesting that NO is produced by the blood vessels that perfuse adipose tissue rather than by the adipocytes themselves.

To explore the possibility that the change in eNOS phosphorylation seen in adipose tissue could represent a systemic effect of CLA, we next examined eNOS in the aorta. A 3- to 8-fold increase was observed in the levels of phosphorylated eNOS in the t10,c12-CLA isomer group compared to all other groups (Figure 6.2A). However, in contrast to the adipose tissue, eNOS was also increased 2- to 3-fold in the aorta of the rats receiving the t10,c12-CLA isomer compared to all other groups (Figure 6.2B). From these results, it is plausible that the physiological actions of t10,c12-CLA on blood

pressure are mediated via eNOS and that t10,c12-CLA specifically targets endothelial cells.

### ***Endothelial cell response to CLA***

To determine whether CLA can promote NO production directly, HAECs were treated *in vitro* with CLA. No increase in phosphorylation of eNOS was detected with either CLA treatment (Figure 6.3A), indicating the effects of CLA on vascular tissue may be indirect and mediated, instead, through its ability to promote adiponectin secretion from adipocytes. To determine whether adiponectin can increase eNOS activity, endothelial cells were treated with adiponectin or the AMPK activator 5'-Aminoimidazole-4-carboxamide riboside (AICAR). Both adiponectin and AICAR strongly stimulated eNOS phosphorylation (Figure 6.3A). All treatments increased AMPK phosphorylation (Figure 6.3B). None of the treatments affected total eNOS or AMPK levels (Figure S6.3, please see Supplemental material).

### ***Adiponectin infusion and blood pressure***

Although improved blood pressure was associated with increased circulating adiponectin in obese rats treated with CLA isomers, it could not be resolved whether the increase in adiponectin has a direct effect on blood pressure. Therefore, we elected to test the effect of a known concentration of adiponectin on obesity-related hypertension directly. A bolus injection of adiponectin had no effect on systolic blood pressure compared to saline (Figure 6.4A). Furthermore, adiponectin did not reverse the blood pressure increase obtained by placing L-NAME in their drinking water for 24 hours

(Figure 6.4A). Diastolic blood pressure was also unchanged after adiponectin injection (Figure S6.4, please see Supplemental material). In contrast, when given adiponectin continuously for 7 days, systolic blood pressure was improved compared to saline infused rats (Figure 6.4B), although diastolic blood pressure did not change (Figure S6.4, please see Supplemental material). These data clearly establish that adiponectin is capable of modulating blood pressure, although exposure over several days is necessary for its actions.

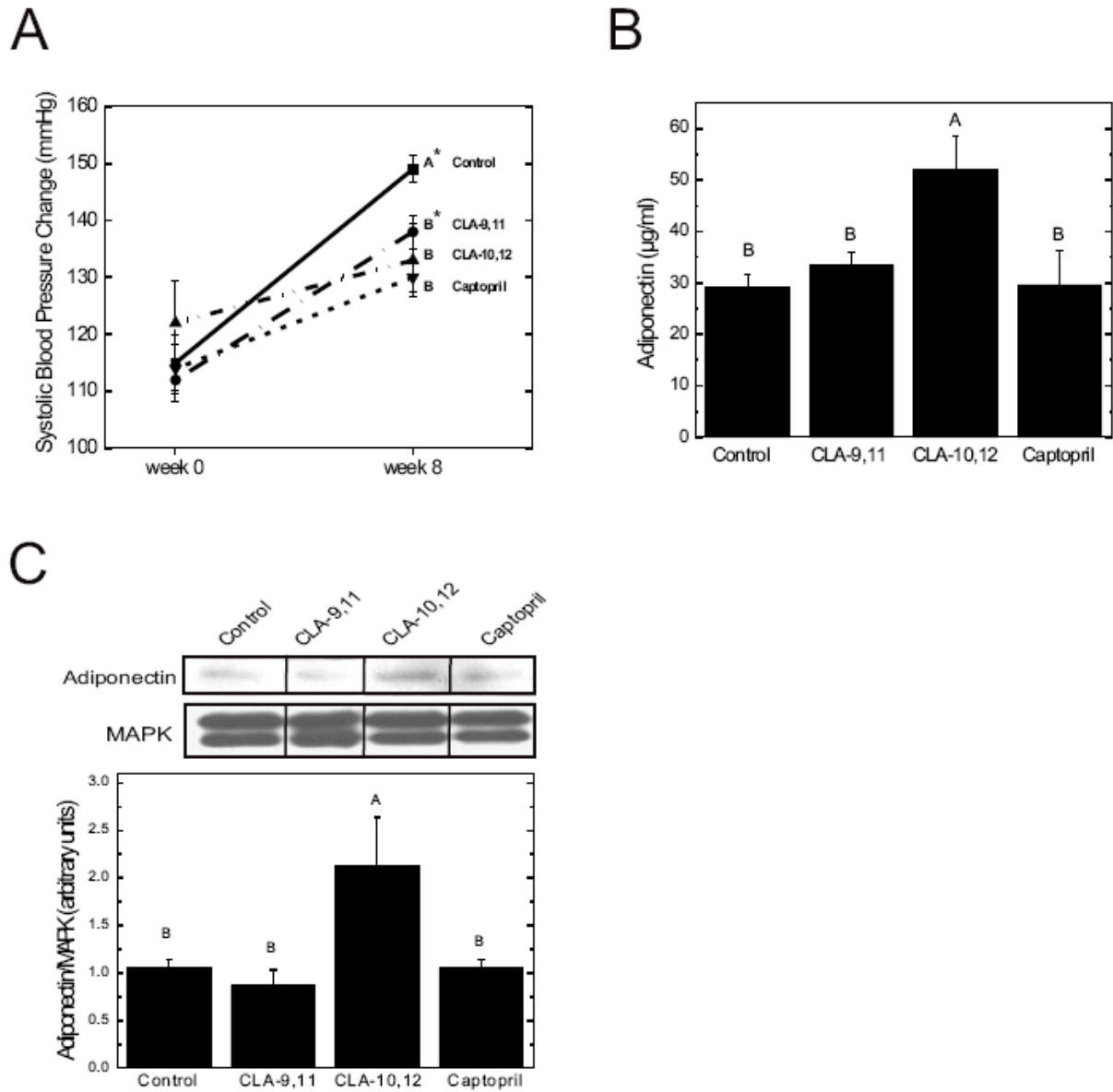
### ***Adiponectin infusion on aortic and adipose proteins***

In the aorta, levels of phosphorylated eNOS (Figure 6.5A) and AMPK (Figure 6.5B) were unchanged after the 7-day infusion. Similarly, no changes were observed in the levels of total eNOS or AMPK (Figure S6.5, please see Supplemental material). These data indicate aortic endothelial cells are not the target of adiponectin. Since adipose tissue also contains endothelial cells, we tested if eNOS activity was altered in adipose tissue after the 7-day infusion and found adiponectin infusion increased phosphorylated eNOS levels more than 5-fold compared to the saline infused group (Figure 6.5A), whereas phosphorylated AMPK was unaltered (Figure 6.5B). No changes were observed in the levels of total eNOS or AMPK in adipose tissue (Figure S6.5, please see Supplemental material). Based on these results, adiponectin appears to selectively target the microvascular endothelial cells that modulate blood pressure rather than macrovascular endothelial cells.

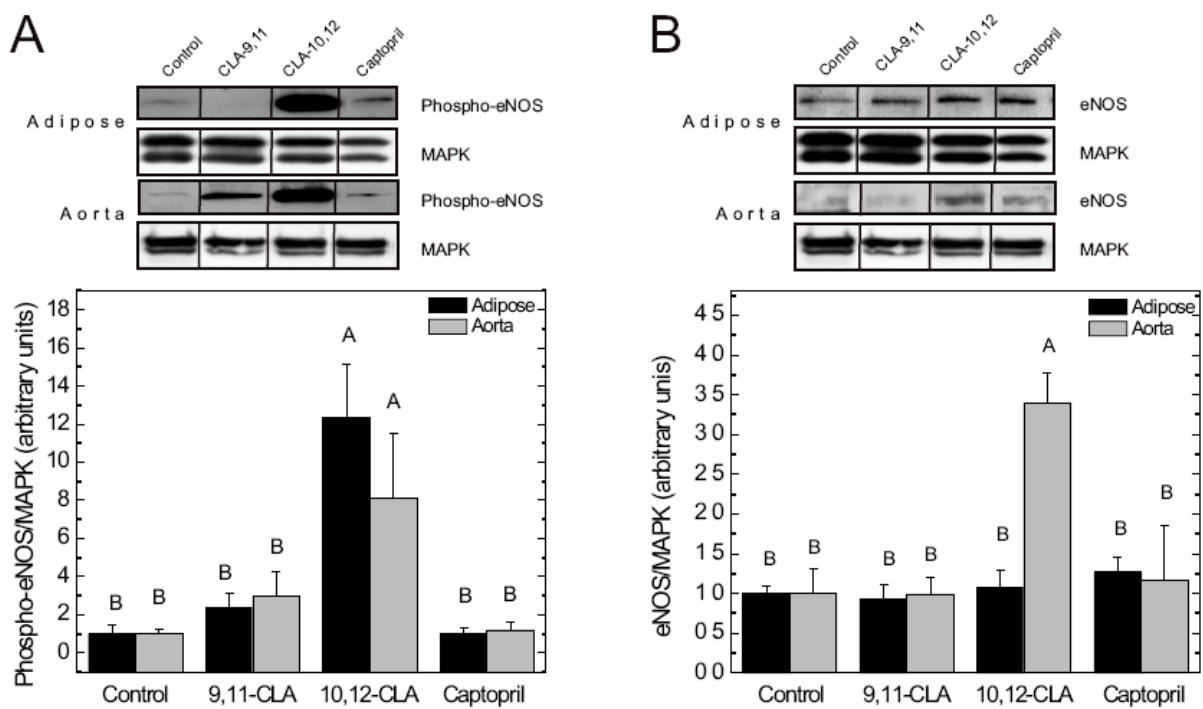
**Table 6.1** Characteristics of *fa/fa* Zucker rats fed CLA isomers.

	<b>Control</b>	<b>c9,t11-CLA</b>	<b>t10,c12-CLA</b>	<b>Captopril</b>
Total feed intake (g)	1524±29	1615±35	1619±24	1569±32
Initial body weight (g)	135±5	139±3	127±7	142±6
Final body weight (g)	569±9*	591±8*	580±11*	573±13*
Epididymal adipose (g)	17.1±1.1	18.7±1.8	19.7±0.7	19.3±1.0
Epididymal adipose (g/100 g body weight)	3.00±0.19	3.16±0.17	3.40±0.15	3.38±0.18

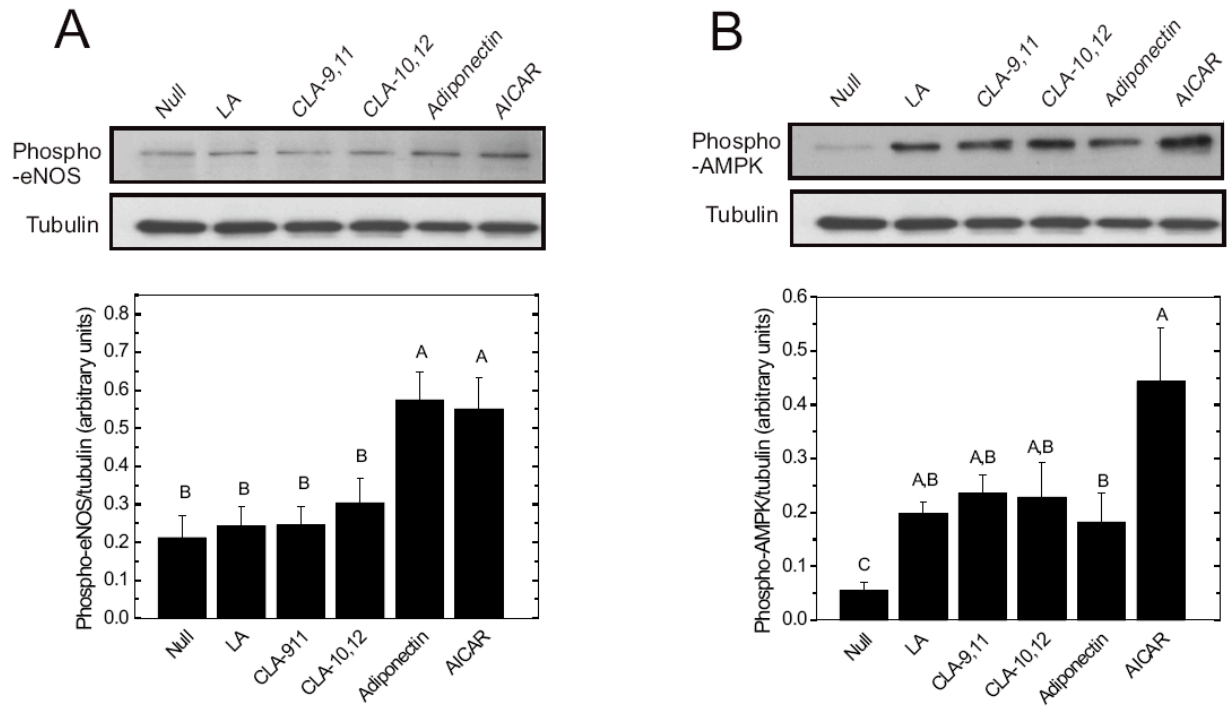
Asterisk (\*) denotes significant change ( $P \leq 0.05$ ) within a group from initial to final body weight. Other parameters are not significantly different.



**Figure 6.1** Systolic blood pressure (A), plasma adiponectin (B) and adipose tissue levels of adiponectin (C) in *fa/fa* Zucker rats fed CLA diets. Different letters denote significant differences ( $P \leq 0.05$ ) at the same time point and an asterisk (\*) denotes significant change in systolic blood pressure from week 0,  $n=5-12/\text{group}$ .



**Figure 6.2 Adipose tissue and aortic levels of phospho-eNOS (A) and eNOS (B) in *fa/fa* Zucker rats fed CLA diets.** Relative intensity of the bands was normalized to MAPK. For each tissue different letters denote significant differences ( $P \leq 0.05$ ),  $n=5/\text{group}$ .

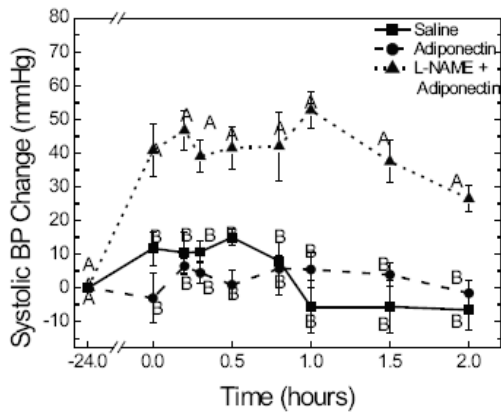


**Figure 6.3 Levels of phospho-eNOS (A), and phospho-AMPK (B) in HAECs treated with CLA**

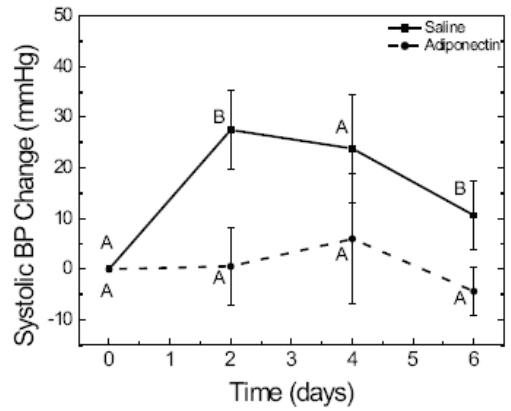
**isomers.** Relative intensity of the bands obtained by Western blot analysis of 6 independent experiments were normalized to  $\beta$ -tubulin. Cells were either untreated (null) or treated with 60  $\mu$ M linoleic acid (LA), 60  $\mu$ M individual CLA isomers (CLA-9,11 and CLA-10,12), 10  $\mu$ g/ml adiponectin or  $10^{-3}$  M 5'-aminoimidazole-4-carboxamide riboside (AICAR). Different letters denote significant differences ( $P \leq 0.05$ )



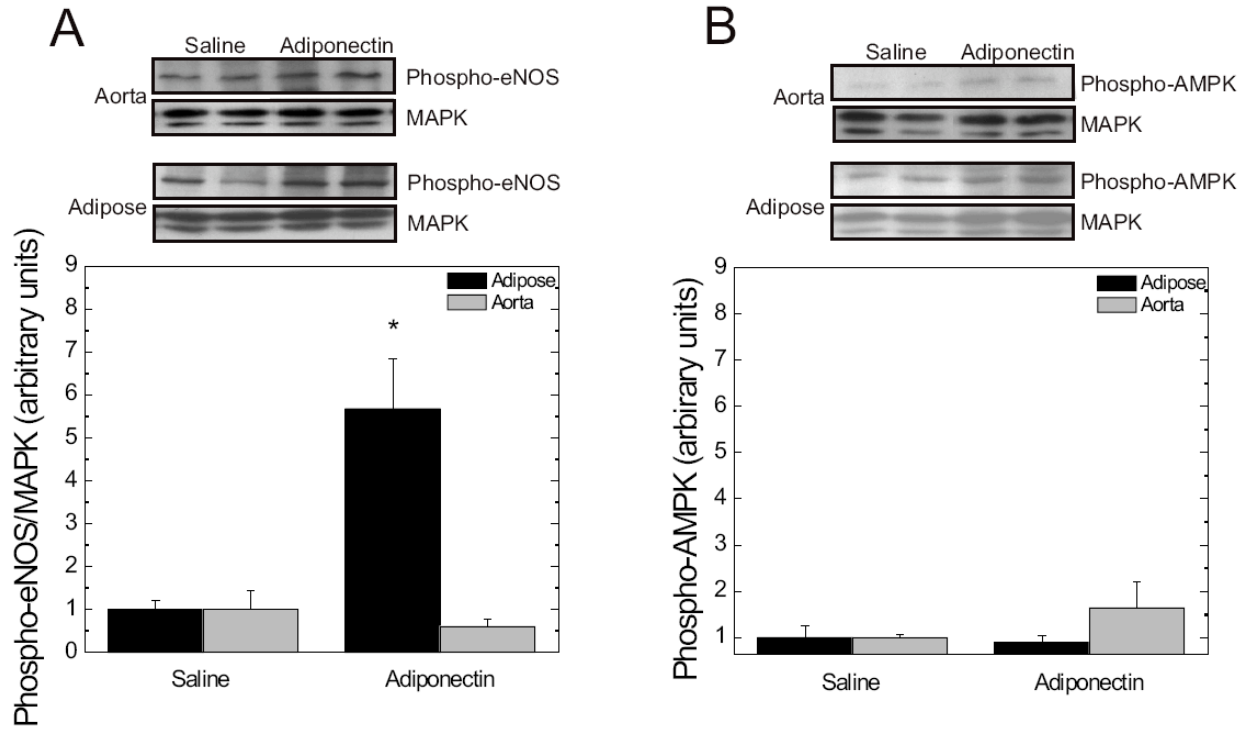
A



B



**Figure 6.4** Systolic blood pressure in *fa/fa* Zucker rats given adiponectin for 120 minutes (A) or 7 days (B). At the same time point, different letters denote significant differences ( $P \leq 0.05$ ),  $n=4-6$ /group.



**Figure 6.5 Levels of phospho-eNOS (A) and phospho-AMPK (B) in adipose and aorta of *fa/fa***

**Zucker rats treated with adiponectin for 7 days.** The relative intensity of the Western blot bands was normalized to MAPK. Asterisk (\*) denotes denote significant differences ( $P \leq 0.05$ ) from saline-treated,  $n=6/\text{group}$ .

## 6.5 Discussion

The current study has demonstrated that feeding t10,c12-CLA to *fa/fa* Zucker rats prevents the increase in blood pressure associated with obesity. We have also shown that the t10,c12-CLA treatment (0.4% w/w) was as efficacious as a standard anti-hypertensive drug (captopril; 50 mg/kg/day) in blocking the increase in blood pressure. Rats receiving the t10,c12-CLA isomer displayed increased levels of adiponectin in both plasma and adipose tissue. These changes were accompanied by increased levels of phosphorylated eNOS in both the adipose tissue and aorta, most likely in the endothelial cells of both tissues. However, a direct effect of CLA on eNOS phosphorylation in cultured endothelial cells was not observed, which suggests that the effect of CLA on blood pressure is indirectly mediated through adipose tissue-dependent production and secretion of adiponectin. In support of this conclusion, we are the first to show that infusion with a known amount of adiponectin into an obese rat improves blood pressure and increases eNOS phosphorylation in adipose tissue.

A positive relationship between obesity and hypertension has clearly been established [18], which explains the blood pressure increase observed with *fa/fa* Zucker rats in this report. Previous studies using obese rats have shown that improvements in blood pressure with t10,c12-CLA treatment are achieved simultaneously with reduced epididymal adipose tissue mass [11]. In contrast, the current study demonstrates that dietary treatment with t10c12-CLA

attenuates systolic blood pressure in *fa/fa* Zucker rats despite the fact that these rats had similar epididymal adipose mass to the untreated controls. One reason for the differing results may be the use of different rat models; the OLETF rat model responds rapidly to dietary CLA intervention, with changes in adipose weight and blood pressure evident after only 3 weeks of feeding [11]. Another reason that may have influenced the outcome on adipose mass may be the type of background oil used: corn oil by Nagao *et al.* [11] versus soy oil in the current study. This is also the first study to show attenuation of systolic blood pressure with the c9,t11-CLA isomer, which is naturally present in ruminant meats and dairy products, although it is not as effective as the t10,c12-CLA isomer. The mechanism for the c9,t11-CLA isomer requires further investigation as plasma and adipose adiponectin were unchanged.

In humans, only a few studies examining CLA and blood pressure have been reported. One study in healthy non-obese normotensive males showed no change in blood pressure after consuming CLA-enriched butter (predominantly c9,t11-CLA) [19], whereas a mixture of CLA isomers significantly enhanced the effect of ramipril on reducing blood pressure in obese men and women with hypertension [20].

Evidence from human and animal studies [8, 21, 22] has demonstrated a link between low levels of adiponectin and hypertension. We were able to show that adiponectin infusion in obesity can improve blood pressure. Furthermore,

beneficial changes in the blood pressure of rats receiving the t10,c12-CLA isomer occurred in conjunction with increases in adiponectin levels in both plasma and adipose tissue. In agreement with our findings, Nagao *et al.* [23] showed CLA isomers increase plasma adiponectin in Zucker Diabetic Fatty rats, and they attributed the changes in adiponectin to enhanced gene expression in white adipose tissue. Likewise, we previously showed increases in serum adiponectin and mRNA levels in epididymal adipose tissue of *fa/fa* Zucker rats when given a diet containing mixed CLA isomers [12]. However, direct treatment of large adipocytes with CLA does not increase adiponectin production (Chapter 5), emphasizing the importance of cross-talk between different adipose-derived cells in mediating the effects of CLA on adiponectin production.

Impaired endothelial function is evident in obesity [24] and low levels of circulating adiponectin are associated with impaired endothelium-dependent vasorelaxation [22, 25], suggesting that plasma adiponectin levels might be a useful indicator of endothelial dysfunction. In agreement with this concept, we demonstrated that adiponectin infusion improves blood pressure and increases eNOS phosphorylation in adipose tissue. Furthermore, direct treatment of HAECs with adiponectin increased both phosphorylated eNOS and AMPK. Similar observations were made by Hattori *et al.* [9] in human umbilical vein endothelial cells. Our study examined the effects of CLA isomers on HAECs and showed that neither isomer affected eNOS activity, however, in bovine aortic endothelial cells a mixture of CLA isomers increases eNOS mRNA levels under

asynchronous hemodynamics [26]. More research directed towards understanding the effects of CLA on endothelial cell function will be required to resolve these conflicting observations.

Obesity-related hypertension is prevalent in today's world, so understanding the mechanisms causing this condition will help to develop new therapeutic strategies for prevention and treatment. The current study adds to the body of literature by showing utilizing *in vivo* and *in vitro* approaches that the t10,c12-CLA isomer has beneficial effects on endothelial cell function that can manifest as lower blood pressure. Furthermore, we have demonstrated that direct treatment with adiponectin improves blood pressure in obese rats, thus establishing that altered cross-talk between adipocytes and endothelial cells within adipose tissue is a major underlying factor in the development of obesity-related blood pressure. Consequently, it may be concluded that improvements in adipocyte function through consumption of t10,c12-CLA isomer attenuates blood pressure in part by increasing adiponectin, which improves endothelial function by increasing the phosphorylation of eNOS. Additionally, our findings suggest the t10,c12-CLA isomer lowers blood pressure through a different mechanism than the c9,t11-CLA isomer and captopril. Further work will be required to elucidate the actions of CLA on blood pressure regulation and to uncover the details of the interactions between endothelial cells and adipocyte-derived molecules.

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## 6.7 Supplementary Material

### Expanded Methods

#### *Cloning, expression, and purification of recombinant adiponectin*

Adiponectin was amplified by PCR and sub-cloned into an expression vector. Rat adiponectin clones were purchased from Invitrogen and amplified using 5'ATTACTGCAACCGAAGGGCCAGG-3' as the sense primer and 5'GCGGATCCTCAGTTGGTATCATGGTAGAG-3' as the antisense primer. The adiponectin gene was sub-cloned into the expression vector (pET-45) using standard procedures. Briefly, the pET vector was digested with Pml1 and BamH1, then both the digested vector and insert were run on a 1% agarose gel, and a QIAquick Gel Extraction kit (Qiagen) was used to purify the products. The purified products were ligated with T4 DNA ligase then transformed in bacterial cells (DH5-alpha). Samples were mini-prepped using the standard kit protocol (Invitrogen Mini Prep Kit) and sent for DNA sequencing (University of Calgary) to verify.

The plasmid containing the adiponectin gene was then transformed into Rosetta Gami Cells (Novagen) and streaked out on agar plates. An isolated colony was selected from the plate and used to inoculate 5 ml Terrific broth (TB). The culture was incubated at 37°C until the OD 600 reached 0.6-1.0. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the culture at 1 mM at 37°C for 3

hours to induce expression. Cultures were centrifuged and pellets were subsequently stored at -80°C.

Frozen pellets were thawed on ice and cells were suspended in Bugbuster (Novagen) followed by centrifugation and collection of supernatant. Adiponectin in the supernatant was isolated by affinity chromatography. His-tagged full-length adiponectin was purified from the bacterial lysates using Ni-NTA resin (BioRad) columns. The samples were loaded onto the Ni-NTA columns, washed with binding buffer then eluted with imidazole. Purification of adiponectin was determined by running SDS-PAGE followed by Coomassie blue staining. The His-tag enabled detection of recombinant adiponectin in infused animals.

#### ***Plasma detection of recombinant adiponectin***

Nylon membranes were cut to appropriate size and placed in ddH<sub>2</sub>O. Once the membrane was fully hydrated, it was placed in a slot blot apparatus. Wells were washed with 500 µl of ddH<sub>2</sub>O, then plasma samples (50 µl) were loaded in to the wells of the slot blot apparatus and a vacuum was applied to pull the samples through the wells. Wells were washed with 500 µl of ddH<sub>2</sub>O, then the membrane was removed from the slot blot apparatus. Recombinant adiponectin in the plasma of infusion animals was detected with a His6 antibody (1:5000; Abcam) following our standard Western blot procedure.

### ***Immunofluorescence staining in epididymal adipose tissue***

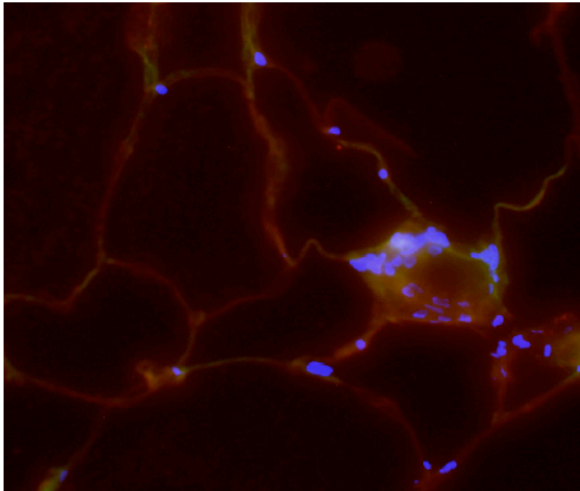
At room temperature, 10  $\mu\text{m}$  thick sections of adipose tissue were fixed in 4% paraformaldehyde for 10 minutes under UV-light then washed 2 times with PBS for 5 minutes each time. The slides were blocked in 3% BSA-PBS for 1 hour at room temperature followed by 3 more washes. The slides were incubated overnight at 4°C with primary antibodies for adiponectin (3  $\mu\text{g}/\text{ml}$ ; Abcam) and phosphorylated eNOS (1:100; Cell Signaling). The slides were washed 3 times with PBS for 10 minutes each time, then incubated with secondary anti-rabbit antibody coupled to an Alexa Fluor 488 tag (2.5  $\mu\text{l}/\text{ml}$ ; Molecular Probes) and anti-mouse antibody with a Cy3 tag (1:250; Jackson ImmunoResearch) for 1 hour at room temperature. The slides were washed 2 times with PBS for 10 minutes then incubated with the nuclear stain, Hoechst 33342 (5  $\mu\text{l}/\text{ml}$ ; Sigma), for 1 minute at room temperature. The slides were washed 2 more times for 10 minutes with PBS before cover-slips were applied with mounting medium. Images of immunostained adipose sections were captured using an BH2-RFCA Olympus fluorescence microscope, a Q-Imaging digital camera and Q-Capture Pro 6.0 software.

## Supplementary Figures

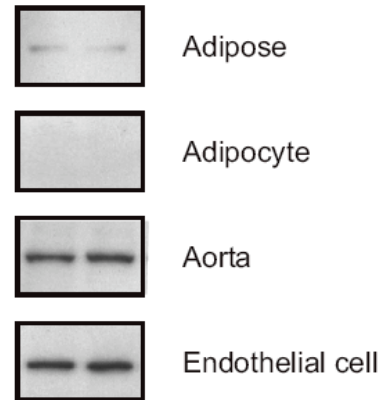


**Figure S6.1 Slot Blot analysis of plasma from saline and adiponectin-infused *fa/fa* Zucker rats at day 7.** Blot shows His-tagged adiponectin in the adiponectin group and not the saline-infused group.

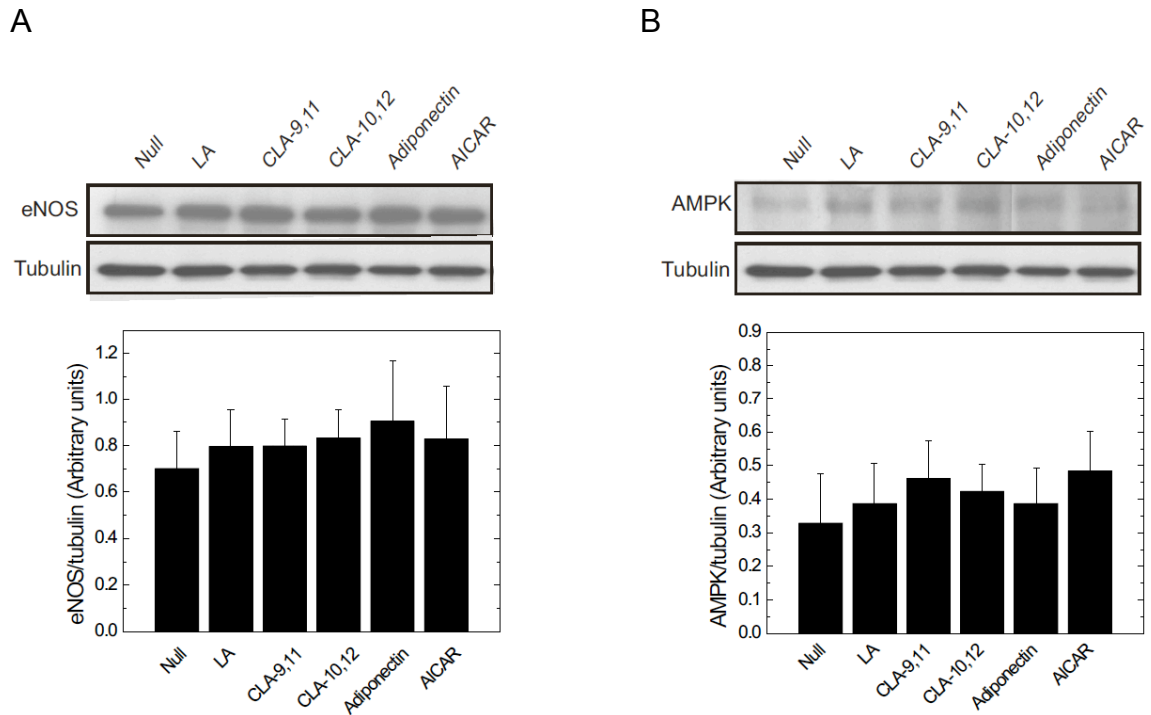
A



B



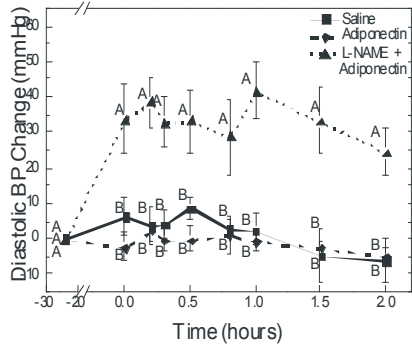
**Figure 6.2S** Representative fluorescent image of epididymal adipose tissue from *fa/fa* Zucker rats showing phospho-eNOS is associated with vascular structures and is absent in adipocytes (A) and comparative Western blot (B) to determine the presence of eNOS in different cell types and tissues including adipose tissue, adipocytes, aorta and human endothelial cells. Each section was stained with Hoechst 33342 (blue), adiponectin (red) and phospho-eNOS (green).



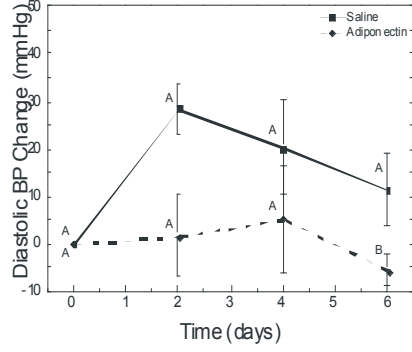
**Figure S6.3 Human aortic endothelial cell levels of eNOS (A) and AMPK (B).** Relative intensity of the bands was quantified by densitometry and normalized to  $\beta$ -tubulin. Representative Western blots are presented in each panel. Data are presented as means  $\pm$  SEM from 6 independent experiments. Cells were either untreated (null) or treated for 1 hour with 60  $\mu$ M linoleic acid (LA; Cayman), 60  $\mu$ M individual CLA isomers (CLA-9,11 and CLA-10,12; Cayman), 10  $\mu$ g/ml adiponectin (BioVendor) or 10 $\cdot$ 3M 5'-aminoimidazole-4-carboxamide riboside (AICAR; Calbiochem).



A

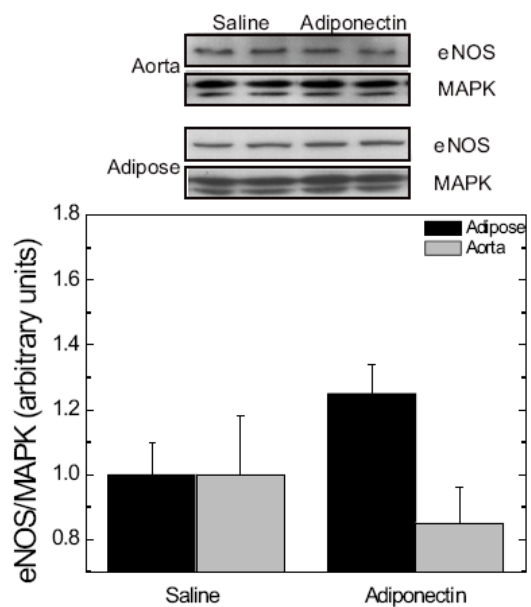


B

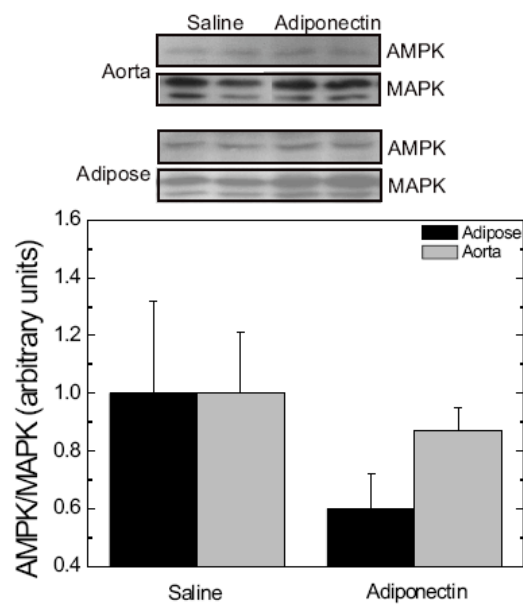


**Figure S6.4 Diastolic blood pressure in *fa/fa* Zucker rats given adiponectin for 120 minutes (A) and 7 days (B) adiponectin treatments.** Data are expressed as means  $\pm$  SEM. At the same time point, different letters denote significant differences ( $P \leq 0.05$ ).

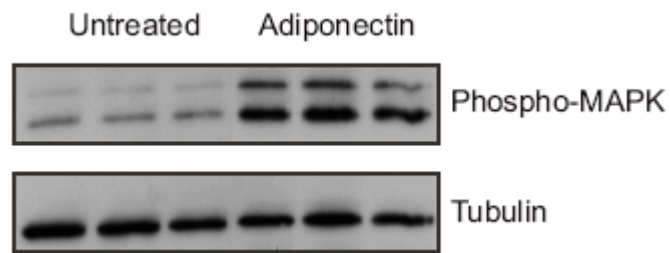
A



B



**Figure S6.5 Levels of eNOS (A) and AMPK (B) in epididymal adipose and aorta of *fa/fa* Zucker rats treated with adiponectin.** The relative intensity of the bands was quantified by densitometry and normalized to that of MAPK. Representative Western blots are presented in each panel. Data are presented as means  $\pm$  SEM. No significant differences were observed.



**Figure S6.6** Western blot of smooth muscle cell extracts prepared 1 hour after treatment with recombinant adiponectin (10  $\mu\text{g/ml}$ ). The increase in phosphorylated MAPK (Cell Signaling) indicates the adiponectin used in the infusion study is biologically active.

## Chapter 7: Conclusions

### 7.1 Discussion

The main findings of this study were: (1) the t10,c12-CLA isomer attenuated systolic blood pressure, increased serum adiponectin and reduced adipocyte size in both growing and old *fa/fa* Zucker rats, (2) the t10,c12-CLA isomer was as effective as a standard anti-hypertensive drug in blocking the increase in blood pressure associated with obesity, (3) there were distinct differences in adipokine status of small and large adipocytes from *fa/fa* Zucker rats, (4) the t10,c12-CLA isomer increased the levels of adiponectin and eNOS phosphorylation in adipose tissue, (5) t10,c12-CLA did not directly cause changes in eNOS phosphorylation but increased phosphorylated eNOS did occur with adiponectin treatment, and (6) direct infusion with adiponectin attenuated blood pressure and increased eNOS phosphorylation in adipose tissue of obese rats (Figure 7.1).

The current study was designed to evaluate the efficacy of dietary intervention with CLA isomers in preventing and treating obesity-related hypertension and to explore possible mechanisms for its action. This study is important because the increasing incidence of obesity-related complications such as hypertension are at an all time high in our society and contribute greatly to serious cardiovascular events [1-3]. Thus, the heightened prevalence of obesity-related complications increases the need for novel therapeutic interventions. A possible therapeutic target for obesity-related complications may be adipocyte size.

Review of the literature suggests that adipocyte enlargement causes disturbances in normal adipocyte function, altering the production and secretion of adipokines and inflammatory cytokines that influence vascular function and contribute to obesity-related complications [4-6]. This study demonstrated that the t10,c12-CLA isomer reduced epididymal adipocyte size in both growing and older *fa/fa* Zucker rats (Chapter 4 and 5). The reduced adipocyte size was observed in the absence of weight loss, as discussed in Chapter 5. Prior to this study, there were few groups that had reported effects of CLA isomers on adipocyte size and all previous work has been done in growing rats. Sprague-Dawley rats fed mixed CLA isomers (0.5%, w/w) had reduced adipocyte size [7, 8], while Wistar rats fed individual (0.6%, w/w) and/or combined CLA isomers (1.3%, w/w) had increased adipocyte size with the c9,t11-CLA isomer and this was counterbalanced when isomers were provided as a mixture [9]. Different adipose depots and type of background oils used in the diets may be responsible for some of the differing observations, as discussed in Chapter 5. Additionally, it is important to note that we are the first to show beneficial effects of the t10,c12-CLA isomer on adipocyte size in older obese rats, and this was associated with positive effects on blood pressure (Chapter 4). This shift in adipocyte size which was observed in *fa/fa* Zucker rats of two ages, suggests that the adipocyte function may have improved. Thus, the next phase of the study explored the possibility that the improvement in size was associated with enhanced adipocyte function as exemplified by adipokine status.

Based on a number of key studies, the first target examined was angiotensinogen. Data from these studies showed that the local RAS is activated in obesity-related hypertension [10, 11], obese Zucker rats have increased levels of angiotensinogen in adipose tissue compared to lean rats [12], and finally, reduced angiotensinogen mRNA levels have been observed in OLETF rats fed a 0.5% (w/w) t10,c12-CLA diet [13]. Although previous work looked at angiotensinogen at the gene level, the current study was the first to report the effects of individual isomers on protein levels of angiotensinogen. The current study demonstrated that size separated adipocytes from obese *fa/fa* Zucker rats have different levels of angiotensinogen, with higher levels in large adipocytes and less in small adipocytes (Chapter 5). However, neither dietary intervention (Chapter 4) nor direct treatment of isolated adipocytes (Chapter 5) with CLA isomers appeared to influence angiotensinogen levels. This was unexpected because the t10,c12-CLA isomer had previously been reported to reduce angiotensinogen mRNA in OLETF rats [13]. On the other hand, the previous research examined peri-renal adipose tissue of OLETF rats [13], thus to ensure possible changes in other depots were not missed, we also examined angiotensinogen levels in peri-renal adipose tissue. These results also showed that CLA isomers had no effect on angiotensinogen levels in peri-renal adipose tissue of *fa/fa* Zucker rats (Appendix B). Additionally, the AngII receptors and pro-inflammatory cytokines associated with AngII were unchanged by dietary

intervention with CLA isomers (Chapter 4). Thus, we concluded that the beneficial actions of CLA must be working through an additional pathway.

In contrast to data related to angiotensinogen, we detected changes in adiponectin (Chapter 5 and 6), which prompted our next area of exploration since several studies have reported negative correlations between serum adiponectin levels and blood pressure. [14-18]. Additionally, previous work had shown increased adipose accumulation is negatively associated with adiponectin secretion from adipose tissue [19] and, in agreement with this, the current study clearly showed large hypertrophic adipocytes secrete less adiponectin than smaller adipocytes isolated from obese *fa/fa* Zucker rats (Chapter 5). Since the t10,c12-CLA isomer reduced adipocyte size (Chapter 4 and 5), it was reasonable to speculate that the t10,c12-CLA isomer may also increase circulating adiponectin levels in *fa/fa* Zucker rats. Data from the feeding study (Chapter 6) concur with previous work in younger obese rats showing increased circulating adiponectin levels with CLA treatment [20-23], and adds to existing data by demonstrating isomer-specific effects and benefits in older animals. The increased circulating adiponectin due to the t10,c12-CLA isomer was likely due to the corresponding increased adipose levels of adiponectin in *fa/fa* Zucker rats (Chapter 6). However, direct treatment of large adipocytes with CLA isomers did not alter cellular or secreted levels of adiponectin (Chapter 5), thus the reduction in adipocyte size appears to be an important step in increasing adiponectin levels. Since the t10,c12-CLA isomer did reduce adipocyte size and increased

adiponectin levels *in vivo* (Chapter 4, 5 and 6), the next step was to understand how increasing adiponectin through treatment with the t10,c12-CLA isomer may improve vascular functioning.

With established associations between serum adiponectin levels and blood pressure [14-18], the next obvious question was whether increasing circulating adiponectin levels was enough to directly influence blood pressure *in vivo*.

Previous adenoviral work in mice suggested that adiponectin treatment may positively influence blood pressure [24], however, the amount of adiponectin required to elicit a response was unclear. Also whether this concept could be expanded to other species remained to be determined. Therefore, obese *fa/fa* Zucker rats were infused with a known amount of adiponectin and blood pressure was monitored over both the short and long term. Chronic infusion of adiponectin attenuated blood pressure in *fa/fa* Zucker rats (Chapter 6), demonstrating adiponectin had a direct affect on the vascular system.

Low levels of circulating adiponectin are associated with impaired endothelium-dependent vasorelaxation in aortic rings of adiponectin-KO mice [25, 26], suggesting a link exists between plasma adiponectin levels and endothelial dysfunction. Altered eNOS activity is characteristic of endothelial dysfunction and is associated with the development of hypertension [24, 27]. Since adiponectin has been suggested to activate eNOS in endothelial cells [24, 28], the possibility that CLA isomers may influence eNOS activity within adipose tissue was



explored. As suspected, the t10,c12-CLA isomer increased eNOS phosphorylation in adipose tissue of *fa/fa* Zucker rats (Chapter 6) leading to the question of which cells within the adipose tissue CLA was targeting, and whether CLA was directly affecting adipocytes or acting through other cells within the adipose tissue.

Primary adipocytes isolated from obese *fa/fa* Zucker rats and human aortic endothelial cells were used to address this issue. Direct treatment of large adipocytes with CLA isomers did not alter the cellular or secreted levels of adiponectin (Chapter 5), and the CLA isomers did not alter phosphorylation of eNOS in endothelial cells (Chapter 6). However, direct treatment of endothelial cells with adiponectin confirmed previous reports of eNOS activation in cultured endothelial cells (Chapter 6) [29]. Additionally, chronic infusion of adiponectin in *fa/fa* Zucker rats increased eNOS phosphorylation in the epididymal adipose tissue (Chapter 6). Thus, since CLA isomers have no direct effect on isolated adipocytes or endothelial cells yet in whole adipose tissue the t10,c12-CLA isomer increases adiponectin and eNOS phosphorylation, it seems reasonable that there may be cross-talk between cells within the adipose tissue that are responsible for the effects of CLA. More research is needed to understand how CLA may influence signaling between cells of the adipose tissue. Since the current study showed that the t10,c12-CLA isomer increased adiponectin (Chapter 5 and 6) and direct treatment with adiponectin increased eNOS activity

(Chapter 6), it seems a likely mechanism by which CLA can improve blood pressure.

Finally, this study showed that the t10,c12-CLA isomer provides beneficial effects on systolic blood pressure during the onset of obesity-related hypertension in growing animals (Chapter 6) and in an older animal model with established hypertension (Chapter 5). This study is in agreement with previous work which showed beneficial effects of the t10,c12-CLA isomer in growing rats [13] and added to the current knowledge by demonstrating benefits of the t10,c12-CLA isomer in older animals. Furthermore, we showed that the c9,t11-CLA was able to attenuate the development of obesity-related hypertension (Chapter 5), however, this isomer was ineffective in older animals (Chapter 4). A very recent study in young SHR fed a mixture of CLA isomers for 8 weeks confirmed earlier work in growing rats, showing benefits of a mixture of CLA isomers on systolic blood pressure [30]. Additionally, the present study was the first study to show that the t10,c12-CLA isomer is as effective as commonly used anti-hypertensive medication (Chapter 6).

A lack of research in humans makes it difficult to draw conclusions on the blood pressure lowering capabilities of CLA supplementation, however, CLA may be more effective in disease conditions such as obesity-related hypertension. From the limited number of studies, it appears that normal weight or overweight but otherwise healthy individuals do not benefit from taking CLA supplements for

blood pressure [31, 32], but obese men and women who are hypertensive may respond better to CLA supplementation [33]. Despite the lack of research in humans, results from this study (Chapters 5 and 6) and others [13, 22, 30] clearly demonstrate beneficial effects of CLA isomers on blood pressure in animal models of chronic diseases such as hypertension. Overall, these results suggest that the ability of the t10,c12-CLA isomer to reduce adipocyte size and thereby increase circulating adiponectin and eNOS activity would promote the observed beneficial effects on systolic blood pressure in *fa/fa* Zucker rats fed the t10,c12-CLA isomer.

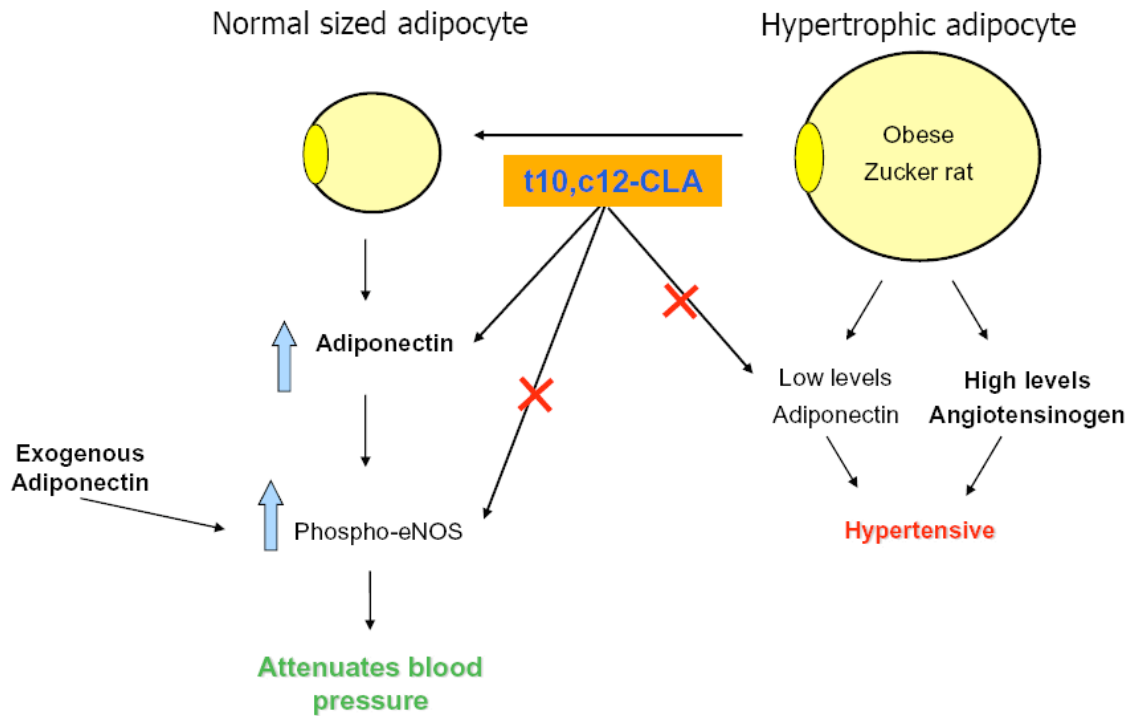


Figure 7.1 Summary of main findings.

## 7.2 Summary & Implications

In summary, these findings suggest that the t10,c12-CLA isomer attenuates blood pressure and reduces adipocyte size in both younger and older *fa/fa* Zucker rats. Smaller adipocytes have lower angiotensinogen levels and higher adiponectin levels, thus it was assumed that reducing adipocyte size would improve the adipokine profile of the adipose tissue by reducing angiotensinogen levels and increasing adiponectin levels. However, the beneficial effects of CLA isomers on hypertension were not linked with changes in the levels of adipose angiotensinogen. On the other hand, reduction in adipocyte size was associated with increased adipose and circulating adiponectin. In large isolated adipocytes, CLA treatment *in vitro* did not alter adiponectin secretion. Thus, some inter-cellular signaling system within the adipose tissue must be responsible for regulating adiponectin release. The t10,c12-CLA isomer also increased eNOS phosphorylation in adipose, but not by affecting the endothelial cells directly, again suggesting the need for the cross-talk between cells in adipose tissue. Finally, adiponectin directly increased eNOS phosphorylation in primary endothelial cells as well as adipose tissue and improved blood pressure in *fa/fa* Zucker rats, suggesting a sequence of events whereby the t10,c12-CLA isomer may be able to increase adiponectin which can subsequently act on endothelial cells to increase eNOS phosphorylation and improve blood pressure. The c9,t11-CLA isomer also resulted in significantly reduced systolic blood pressure values in younger rats, however, there was no indication that the same pathway

involving adiponectin and eNOS was activated. Thus there must be a different mechanism responsible for the actions of the c9,t11-CLA isomer and further investigation is required.

In closing, the burden of obesity-related complications such as hypertension is a widespread problem that requires new prevention and treatment approaches.

This study investigated the use of CLA isomers for the prevention and treatment of obesity-related hypertension. Results from this study have contributed, through the use of *in vivo* and *in vitro* approaches, to the current state of knowledge by demonstrating a clear role of the t10,c12-CLA isomer in attenuating blood pressure by reducing adipocyte size and consequently increasing adiponectin production which activates the enzyme responsible for production of the vasodilator endothelial NO. The larger contribution of this study to the nutrition field is the demonstrated capability that a food component, such as CLA, has the ability to beneficially influence adipocyte size and adipokine production, and ultimately improve obesity-related cardiovascular risk factors such as hypertension.

## 7.3 Strengths & Limitations

### Strengths:

- Use of single isomers – allowed for evaluation of isomer-specific responses.
- Length of study – previous studies have shown 8 weeks to be a long enough time to observe physiological changes and the time period would be equivalent to approximately 5-6 human years (in adulthood, 1 rat month is ~2.5-3 human years).
- Animal model – the *fa/fa* Zucker rats is a well-characterized model of obesity that develops hypertension as discussed in Chapter 2.
- Age of animals – both younger growing rats and older rats with established hypertension were studied.
- Combination of approaches – *in vivo* and *in vitro* approaches were used to examine isomer-specific effects of CLA. Additionally, recombinant protein infusion in an animal model was used to test the proposed blood pressure lowering pathway.
- Primary cell culture model - considered more physiologically relevant and closely mimics the *in vivo* state. As well, the culture model allows direct treatment of CLA isomers on cells in a controlled environment, and thus eliminates confounding factors such as feed intake, digestion, absorption, and environmental stresses related to handling and restraints.

- Size separated cells – adipocytes separated by size allowed use to directly test the effect of CLA isomers on hypertrophic cells compared to normal sized cells.
- Blood pressure measured by telemetry – this technology allows for continuous measurements in an un-restrained animal and does not require long periods of training and acclimatization of the animals.

**Limitations:**

- Animal model – the *fa* mutation has only been found in rodents, thus data from this study may not be applicable to humans.
- Gender – the current study examined the effects of CLA isomers only in male rats, therefore data cannot be extrapolated to females.
- Most measurements were only evaluated at week 8 – it would have strengthened the study to have baseline measurements for data such as adipocyte size, serum adiponectin concentrations and levels of adipokines within the adipose tissue.
- Other angiotensin peptides were not investigated – it may have been useful to investigate the angiotensin pathway further, exploring the conversion of AngII to Ang-(1-7) via the ACE2 enzyme and the possibility that Ang-(1-7) can bind to the MAS receptor and activate eNOS.
- Dose of CLA – the amount of CLA used in the studies would be unrealistic to consume as the equivalent ruminant products, especially since the



predominant isomer found in ruminant products is c9,t11-CLA and most of the beneficial effects were observed with the t10,c12-CLA isomer.

- Use of single isomers - CLA mixture group would have been of interest to see if isomers work synergistically or oppose each other.
- Tail-cuff method for blood pressure measurements – this method can be very unreliable unless proper training with the animals is done prior to starting the study. Proper training can be extremely time consuming and includes getting the animals familiarized with handling, restraints, and tail cuffs to ensure that are as calm as possible at time of measurement. However, studies have shown that with proper training the tail-cuff method is comparable to telemetry.
- Primary cell culture model – can only be used for a limited number of passages before characteristics of cells begin to change and cannot be easily used to examine interactions between cell types.
- Primary adipocyte methodology –advancements in this area have been made since this study was originally conducted and new methods would allow the study of visceral adipocytes from early stages of precursor cells through differentiation and proliferation into mature adipocytes.

## 7.4 Future Directions

- Individual as well as combined CLA isomers in different obese hypertensive models.
- Different species, sexes, and ages need to be examined.
- Examination of both total and size separated adipocytes is warranted to better understand the effects of CLA isomers on adipocyte function.
- Use of new culture techniques, including both organ culture to study the interactions among cells that can influence adipocyte function as well as isolated adipocyte culture to understand the effects of CLA isomers throughout all stages of cell growth.
- Exploration into pathways involving the beneficial effects of the c9,t11-CLA isomer on blood pressure in growing *fa/fa* Zucker rats.
- Dose and time studies with adiponectin infusion to optimize doses and gain a better understanding of how long it takes before benefits of adiponectin can be detected and how long they last.
- Changes in adipocyte size distribution over time with CLA treatment - characterization of changes in adipocyte size every week or month.
- Histology - co-stain for macrophage accumulation or vascular smooth muscle cells in adipose tissue.
- Staining to localize eNOS in vascular structures and inflammatory molecules in adipose tissue.

- Co-culture system to address issue of cross-talk between adipocytes and endothelial cells.
- Functional studies looking at the effects of CLA isomers in contraction-relaxation of aortic rings from obese models as an additional way to assess endothelial function.

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# Appendices

## Appendix A: Method Details

### Isolation and Treatment of Adipocytes

#### *Equipment and Reagents*

- Surgical scissors
- Scalpel and blades
- Phosphate buffered saline (PBS)
- 50 ml conical tubes
- Minimum Essential Media (Gibco #16170-086)
- Collagenase, Type A (Roche #10 103 578 001)
- Calf Serum (Gibco #12360))
- Antimicrobial (Gibco #15240-096)
- Nitrex mesh (50, 70, and 250  $\mu\text{m}$ ) (Dynamic Aqua-Supply #NTX50, #NTX 70, and #NTX250)
- Culture plates (6 and 24-well)
- 20 and 5000  $\mu\text{l}$  pipette with tips
- CO<sub>2</sub> incubator
- Fatty acids (Cayman; linoleic acid #390150; c9,t11-CLA #90140; and t10,c12-CLA #90145)
- 2x sodium dodecyl sulfate (SDS)

## ***Procedure***

1. Prepare media
  - a. MEM, 4% calf serum - Mix 60 ml of Minimal Essential Media with 0.6 ml of antimicrobial, and 2.4 ml of calf serum.
  - b. Isolation media - Mix 60 ml of Minimal Essential Media with 0.6 ml of antimicrobial, 2.4 ml of calf serum, and 63 mg of collagenase.
2. Make an incision in the abdominal cavity to expose visceral adipose tissues.
3. Dissect epididymal adipose tissue, rinse with PBS and weigh.
4. Mince 10 grams of adipose tissue with scissors, then place in a 50 ml conical tube containing isolation media.
5. Incubate tube at 37°C in CO<sub>2</sub> incubator for 60 minutes, with gentle inversions by hand every 15 minutes.
6. Filter digested adipocytes through a 250 µm nitrex mesh.
7. Wash the nitrex mesh 3 times with 2.5 ml MEM 4% calf serum, collecting the flow through in a 50 ml conical tube.
8. Gently invert conical tube 3 times by hand and allow cells 30 seconds to resurface.
9. Collect the cells that resurface using a 5 ml pipette and then filter through a 70 µm nitrex mesh.

10. To collect large adipocytes, invert the 70  $\mu\text{m}$  nitrex mesh over a new conical tube and wash mesh with 2.5 ml of fresh media, collecting large adipocytes in the new tube. Repeat wash step 2 times.
11. For small adipocytes, filter dense cells that did not surface after 30 seconds (step 8) through 50  $\mu\text{m}$  nitrex mesh, collecting 'flow through' for use as the small adipocytes.
12. Count large adipocytes by pipetting 100  $\mu\text{l}$  of cell suspension into a well of a 24-well plate. Count small adipocytes on a regular hemocytometer using 20  $\mu\text{l}$  of cell suspension.
13. Treat adipocytes with 60  $\mu\text{m}$  linoleic acid, t10,c12-CLA or c9,t11-CLA isomers and place in a  $\text{CO}_2$  incubator for 48 hours.
14. Change media at 48 hours by carefully removing media from under adipocytes and adding fresh MEM 4% calf serum plus fatty acid treatments, then put back in the  $\text{CO}_2$  incubator until the completion of the experiment (96 hours).
15. Following incubation, remove media from the wells, transfer to 1.5 ml tubes and store at  $-80^\circ\text{C}$ .
16. To lyse cells, add 200  $\mu\text{l}$  2x SDS and incubate for 15 minutes at room temperature, then collect sample using a pipette and store at  $-80^\circ\text{C}$ .

## **Human Aortic Endothelial Cell (HAEC) Culture**

Standard cell culture procedures were followed. Media and procedures specific to HAECs are listed below.

### ***Special Equipment and Reagents***

- HAEC-aortic endothelial (Lonza #CC-2335)
- EGM-2 endothelial cell growth media-2 (Lonza #CC3162)
- Endothelial cell basal media (Lonza #CC3156)
- Reagent pack (trypsin, neutralizing solution, HEPES-buffered saline) (Lonza #CC-5034)
- Culture plates (NUNC)

### ***Procedures***

1. Thaw cells in a water-bath then seed onto 100 mm dishes with 10 ml of pre-warmed growth media.
2. Change media every 24 hours media.
3. Once cells have reached approximately 70% confluency, passage onto a 140 mm plate with 20 ml of growth media.
4. To passage cells, remove media, rinse with HEPES-buffered saline and then add 2 ml of trypsin.

5. Incubate cells at 37°C until cells lift, then neutralize with equal volume of growth media and transfer to 15 ml conical tube.
6. Centrifuge at 220 rcf for 5 minutes, then remove supernatant.
7. Resuspend in 1 ml growth media, then transfer to 140 mm plate containing 20 ml pre-warmed media.
8. Again, grow cells to 70% confluency, then passage.
9. Remove media and rinse with 5 ml HEPES-buffered saline.
10. Add 3 ml of trypsin and incubate at 37°C until cells lift.
11. Neutralize cells with equal volume of growth media.
12. Transfer cells to 15 ml conical tubes and centrifuge at 220 rcf for 5 minutes.
13. Remove supernatant and resuspend cells in 2-3 ml of growth media.
14. Count cells on hemocytometer and then seed cells on 12-well plates ( $3 \times 10^4$  cells/well) for treatments with CLA isomers.

## **Cloning and Expression of Adiponectin**

Standard cloning and recombinant protein expression procedures were used for the cloning and expression of adiponectin, with specifications noted in Chapter 6.

## **Purification of Adiponectin**

### ***Special supplies***

- Bug Buster plus Benzonase Nuclease (Novagen #70750-3)
- Protease Cocktail Inhibitor Set II (Calbiochem #539131)
- Profinity IMAC Ni-Charged Resin (Biorad #156-0133)
- MCAC buffer (4 ml-20mM TrisHCl, 20 ml-0.5M NaCl, 2 ml-1mM PMSF, 20 ml glycerol, up to 200 ml ddH<sub>2</sub>O)
- MCAC buffer with imidazole (4 ml-20 mM Tris-HCl, 20 ml-0.5 M NaCl, 2 ml-1 mM PMSF, 20 ml glycerol, 40 ml-5 M imidazole, up to 200 ml ddH<sub>2</sub>O)

### ***Procedures***

1. Following induction of protein expression, spin down cultures in 50 ml conical tubes and subsequently stored at -80°C until ready to purify.
2. Once thawed, resuspend pellets in 5 ml Bug Buster, 50 µl of Benzonase Nuclease and 500 µl of 100× Protease Cocktail Inhibitor, vortex and then incubate at room temperature for 15 minutes on an orbital shaker.
3. After incubation, centrifuge at 16 000 g for 20 minutes at 4°C.

4. Collect supernatant and use for column purification.
5. For column purification, first wash Ni-charged resin according to manufacturer's instructions.
6. Resuspend resin in regular MCAC buffer at a ratio of 1:1 in a 50 ml conical tube (Note: To purify 100 ml of supernatant (or 10 pellets), a column bed of 50 ml of resin in MCAC is needed).
7. Allow resin to settle in tube, then remove as much MCAC buffer as possible.
8. Add a small volume of ddH<sub>2</sub>O to resin in order to form a slurry (just enough liquid so the resin can be transferred to an Erlenmeyer flask).
9. Once transferred to flask, add the supernatant from step 4 and seal flask with parafilm.
10. Incubate at 4°C for 30 minutes on orbital shaker set to a high enough speed to ensure resin from settling to the bottom of the flask.
11. Transfer contents from flask to column and allow resin to settle and liquid to flow through.
12. Wash column with 150 ml of regular MCAC buffer.
13. Elute protein from column with 100 ml of MCAC buffer with imidazole.
14. To concentrate sample, load 15 ml of purified protein in an Amicon Ultra 15 Ultracel 10K Spin Column and centrifuge at 5000 g for 15 minutes at 4°C.
15. Pool concentrated samples together and concentrate a second time following the same method as above.



16. Determine the purity of the protein by running SDS-PAGE followed by Coomassie blue staining (Appendix B).

**Table A-1 Composition of experimental diets.**

Ingredients (%)	Control/ Captopril/ Lean	c9,t11-CLA	t10,c12-CLA
Cornstarch <sup>1</sup>	36.3	36.3	36.3
Maltodextrin <sup>2</sup>	13.2	13.2	13.2
Sucrose <sup>2</sup>	10.0	10.0	10.0
Egg white <sup>2</sup>	21.3	21.3	21.3
Cellulose <sup>2</sup>	5.0	5.0	5.0
Mineral Mix <sup>2</sup>	3.5	3.5	3.5
Vitamin Mix <sup>2</sup>	1.0	1.0	1.0
Choline <sup>2</sup>	0.3	0.3	0.3
Biotin Mix <sup>2,3</sup>	1.0	1.0	1.0
TBHQ <sup>4</sup>	<0.01	<0.01	<0.01
Soy oil <sup>2</sup>	8.5	8.1	8.1
c9,t11-CLA oil <sup>5</sup>	0.0	0.4	0.0
t10,c12-CLA oil <sup>5</sup>	0.0	0.0	0.4

1) Purchased from Harlan Best Foods, Etobicoke, ON.

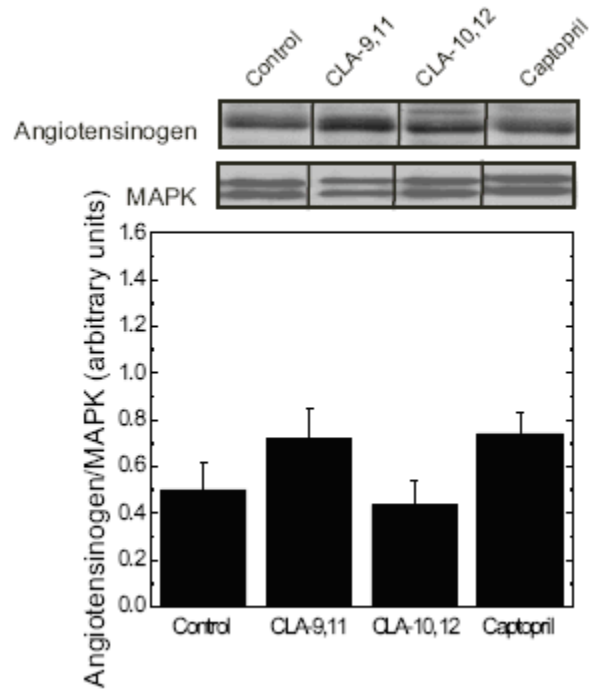
2) Purchased from Harlan Teklad, Madison, WI.

3) Biotin Mix = 200 mg/kg cornstarch

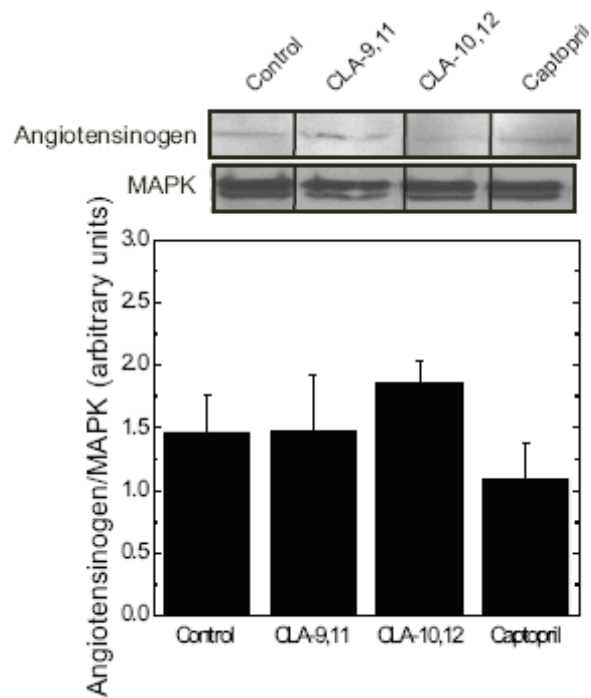
4) Tert-butylhydroquinone; purchased from Sigma-Aldrich, St. Louis, MO

5) CLA oil purchased from Natural ASA, Hovdebygda, Norway.

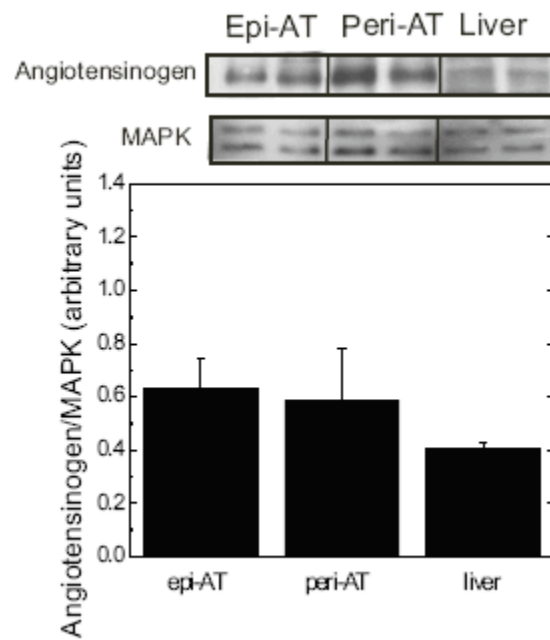
## Appendix B: Extra Figures



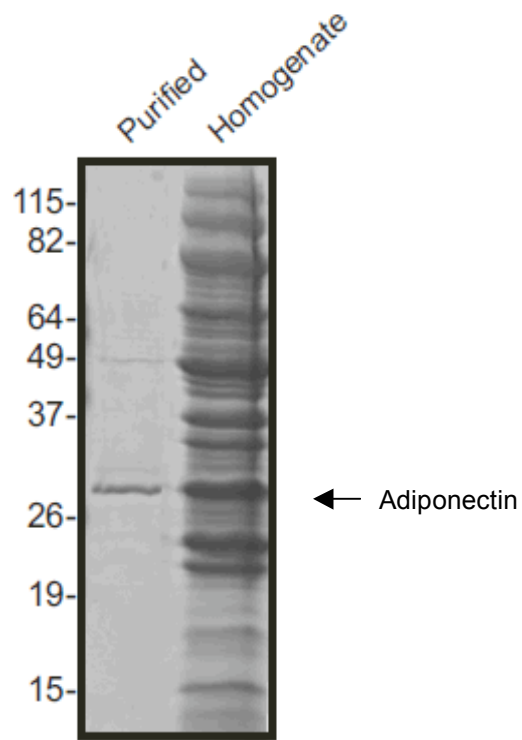
**Figure B1. Angiotensinogen levels in peri-renal adipose tissue of younger 6-week old *fa/fa* Zucker rats fed CLA isomers for 8 weeks.** Relative intensities of the bands were quantified by densitometry and normalized to MAPK. Data are presented as means  $\pm$  SEM (n=6/group). No significant differences were detected by ANOVA ( $p > 0.05$ ). Control = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; Captopril = *fa/fa* Zucker rats fed 0% CLA + 50 mg/kg/day captopril in drinking water.



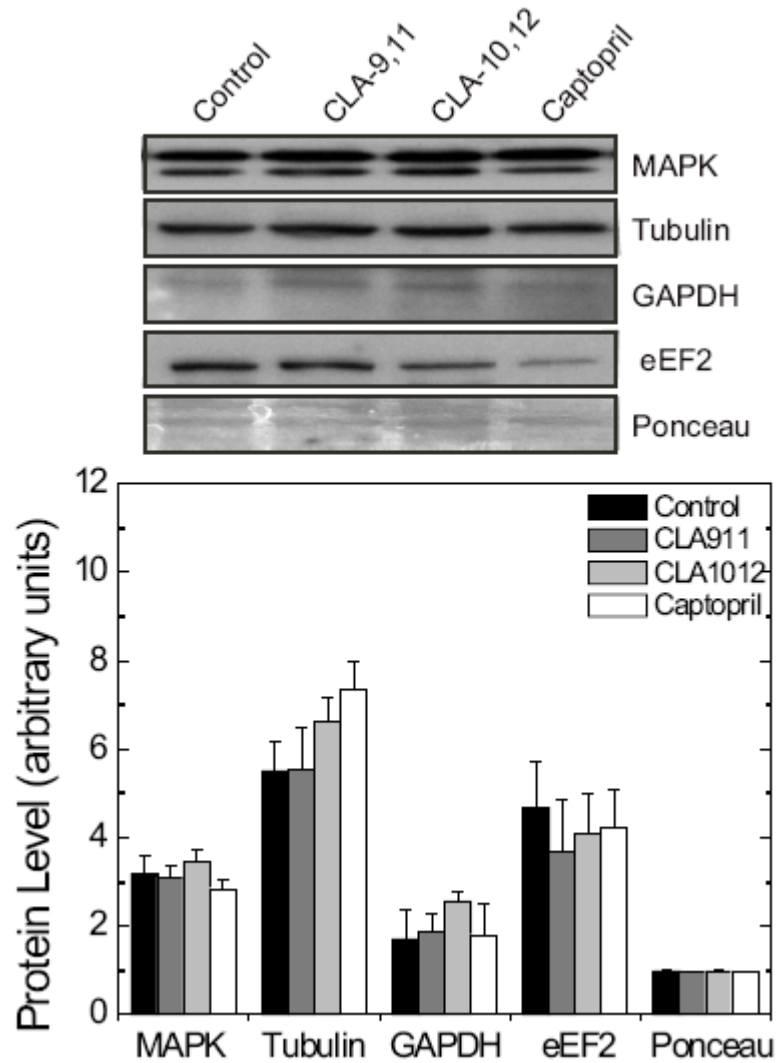
**Figure B2. Angiotensinogen levels in the aorta of younger 6-week old *fa/fa* Zucker rats fed CLA isomers for 8 weeks.** Relative intensities of the bands were quantified by densitometry and normalized to MAPK. Data are presented as means  $\pm$  SEM (n=5/group). No significant differences were detected by ANOVA ( $p > 0.05$ ). Control = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; Captopril = *fa/fa* Zucker rats fed 0% CLA + 50 mg/kg/day captopril in drinking water.



**Figure B3. Comparative levels of angiotensinogen in epididymal adipose, peri-renal adipose and liver tissue of younger 6-week old *fa/fa* Zucker rats.** Relative intensities of the bands were quantified by densitometry and normalized to MAPK. Data are presented as means  $\pm$  SEM (n=4/group). No significant differences were detected by ANOVA ( $p>0.05$ ). Epi-AT= epididymal adipose tissue; Peri-AT= peri-renal adipose tissue.



**Figure B4. Coomassie blue staining of Ni-resin column purified adiponectin and bacterial homogenate.**



**Figure B5. Comparison of loading controls in epididymal adipose of younger 6-week old *fa/fa* Zucker rats.** Relative intensities of the bands were quantified by densitometry. Data are presented as means  $\pm$  SEM (n=4/group). No significant differences were detected by ANOVA ( $p > 0.05$ ). Control = *fa/fa* Zucker rats fed 0% CLA; CLA-9,11 = *fa/fa* Zucker rats fed 0.4% c9,t11-CLA; CLA-10,12 = *fa/fa* Zucker rats fed 0.4% t10,c12-CLA; Captopril = *fa/fa* Zucker rats fed 0% CLA + 50 mg/kg/day captopril in drinking water. MAPK = Mitogen-activated protein kinases; Tubulin =  $\beta$  tubulin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; eEF2 = Elongation factor 2; Ponceau = Ponceau S Staining Solution