The Effect of Conjugated Linoleic Acid (CLA) Isomers on Leptin, Adiponectin and Peroxisome Proliferator-Activated Receptor (PPAR) Expression and their Influence on Porcine Smooth Muscle Cells

By

Melissa Michelle Zirk

A thesis submitted to the Department of Human Nutritional Sciences in partial fulfillment of the requirements for the degree of Master of Science

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

Of

MASTER OF SCIENCE

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ABSTRACT

The Effect of Conjugated Linoleic Acid (CLA) Isomers on Leptin, Adiponectin and Peroxisome Proliferator-Activated Receptor (PPAR) Expression and their Influence on Porcine Smooth Muscle Cells

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Conjugated linoleic acid (CLA) has been shown to suppress atherosclerosis and its complications. CLA also stimulates the expression of adipokines like adiponectin, which is inversely associated with atherosclerotic risk.

Consequently, it was hypothesized that CLA reduces atherosclerotic changes by modulating adipokines and subsequent cell signalling. The primary objective of my study was to investigate the mechanistic actions of CLA and adiponectin in porcine smooth muscle cells (SMC). AMP-activated protein-kinase (AMPK), the 'metabolic master switch', has been shown to have anti-proliferative effects on SMCs, and is activated by adiponectin. Thus, AMPK activation could explain the beneficial effects of CLA and adiponectin in reducing atherosclerotic progression. The relative contribution of adiponectin (full-length and globular), leptin, and CLA to the activation of AMPK, as well as SMC proliferation and/or protein synthesis,

was therefore examined. Furthermore, their influence on the phosphorylation state of Akt, a marker of phosphoinositide-3 kinase activation, was monitored.

Both adiponectin and leptin stimulated protein synthesis in SMCs, however, adiponectin acted as a mitogen, while leptin did not. Interestingly, full-length and globular forms of adiponectin had opposite effects on SMC proliferation, with globular adiponectin reducing SMC proliferation. The c9,t11 CLA reduced protein synthesis, while t10,c12 CLA had no effect. Neither of the CLA isomers had any effect on SMC proliferation.

Platelet-Derived Growth Factor-BB (PDGF), a known SMC mitogen, and 5-aminoimidazol-4-carboxamide-1-ß-D-ribofuranoside (AICAR), a known AMPK activator, both increased pAMPK, while having opposite effects on SMC proliferation. Thus, AMPK phosphorylation does not correlate with the inhibition of SMC proliferation.

In conclusion, globular adiponectin exhibits anti-mitogenic effects in SMCs, while adiponectin and CLA do not have anti-at9heroslcerotic properties in SMCs. The mechanisms through which these agents act remain undefined.

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To my brother, Matthew, my fiancé, Gavin, my Dad and Denyse, and all my family and friends for their patience and support. I love you all and I wouldn't have been able to do this work without your help.

DEDICATION

This Master's thesis is dedicated to my mother, Darlene Zirk, who was the biggest support in my life, and a great role model in life's lessons. You taught me the importance of hard work, as well as the necessity of enjoying life. Thank you for watching over us.

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ABBREVIATIONS

ACC	Acyl-CoA
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AIN-93G Diet	American Institute of Nutrition-93 Diet for Growth
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
BIO	Bioriginals
BSA	Bovine serum albumin
c11,t13	Cis-11, trans13
c9,t11	Cis-9, trans-11
CAD	Coronary artery disease
CH	Cholesterol
CLA	Conjugated linoleic acid
CVD	Cardiovascular disease
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
gAcrp30	Globular adiponectin
GW5	GW501516
HASMC	Human aortic smooth muscle cell
HDL	High-density lipoprotein
LA	Linoleic acid
LDL	Low density lipoprotein
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic peptide-1
mM	milli-mole
OCT	Optimal cutting temperature
OLETF	Otsuka Long-Evans Tokushima fatty
Ox-LDL	Oxidized-low density lipoprotein
pAMPK	Phospho-AMP-activated protein kinase
PDGF	Platelet-derived growth factor-BB
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR-response element
PVDF	Polyvinylidene difluoride membranes
RIA	Radioimmunoassay
SEM	Standard error of mean
SMC	Smooth muscle cell
SMC PBS	Smooth muscle cell phosphate buffered saline
t8,c10	Trans-8, cis-10
t10,c12	Trans-10, cis-10
TBS	Tris buffered saline

TBST	Tris buffered saline with Tween 20
TC	Total holesterol
TCA	Trichloroacetic acid
TG	Triglycerides
TZD	Thiazolidinediones
VLDL	Very low density lipoprotein
w/w	Weight by weight

PART 1 – LITERATURE REVIEW

1. Introduction

Atherosclerosis is a major contributor to hospitalization and mortality in our country. Obesity and diabetes, two diseases also prevalent in our society, correlate with atherosclerosis and progression to cardiovascular disease (CVD). Conjugated linoleic acid (CLA), an eighteen-carbon fatty acid, has been shown to exhibit many health benefits, including possibly reducing atherosclerosis and its complications. CLA has been shown to affect expression of peroxisome proliferator-activated receptors (PPARs), as well as adipokines such as adiponectin and leptin. These cytokines, which are expressed primarily in adipose tissue, are associated with atherosclerotic risk. AMP-activated protein kinase (AMPK) functions in energy-utilizing pathways and has shown to be influenced by both adiponectin and leptin. PPARs may also play a role in the atherosclerotic process. The following literature review will focus on CLA, adiponectin and leptin, and their effects on PPARs and AMPK activation in relation to atherosclerotic progression.

2. Atherosclerosis and Diabetes

Atherosclerosis is an inflammatory process that begins at an early age. To understand the progression of this disease, one must be familiar with the structure of the vasculature. The arterial wall is made up of three main layers: the intima, media and the adventitia. The intima is the first layer (exposed to the lumen) and is made up of a single layer of tightly connected endothelial cells. Underneath this thin floor is an elastic lamina made up primarily of elastin (humans and pigs also have smooth muscle cells (SMCs) in this layer) (Newby, 2000). The media consists of SMCs within a matrix of collagen and fibronectin. The final layer, the adventitia, is made up of loose connective tissue, capillaries, lipids and fibroblasts (Newby, 2000).

Atherosclerosis involves an accumulation of lipids (both extra and intracellular), SMCs, macrophages, connective tissue and glycosaminoglycans in the intima of the arterial wall (Merck Research Laboratories, 2003). Upon damage to the intima, or upon intimal thickening, an increased amount of low-density lipoprotein (LDL) is able to enter the intima through the endothelial cells, and this LDL is subsequently oxidized (Newby, 2000). Once oxidized, LDL is not recognized by the LDL-receptor, but rather by a scavenger-receptor on the macrophage cell surface. These scavenger-receptors have no limitations on uptake, and can continuously bind and transport oxidized-LDL (ox-LDL) into the macrophage. Increased ox-LDL promotes adherence of monocytes, macrophages and T-

lymphocytes in the intima. An increase in SMC proliferation is also correlated to atherosclerotic lesions (Hedin et al, 2004). SMCs migrate from the media into the intima and proliferate in an uncontrolled manner at the surface. The alteration in phenotype which involves a transition from quiescent to synthetically active cells, allows SMCs to produce extracellular matrix proteins which further contribute to increased intimal thickening (Plutzky, 2003). As well, various inflammatory cells are attracted to the sites of endothelial injury and further stimulate cell proliferation. An accumulation of lipids in macrophages further promotes endothelial damage, leading to the visible appearance of fatty streaks. These lipid-laden macrophages develop into foam cells. The continuous accumulation of lipids leads to development of fibrous plaques in major arteries. The proliferation of SMCs and accumulation of connective tissue and additional lipid can lead to blockages, calcification, ulceration, hemorrhage, and possibly a clinical event. Thrombi can form on the surface of plaque or in already ruptured plaques with the latter being more dangerous as they have larger lipid cores (Newby, 2000). Occlusions due to thrombi can lead to myocardial infarctions and cerebrovascular events, resulting in death or physical/mental impairment.

The pathogenesis of atherosclerosis has not been fully determined, although two main hypotheses exist. The Lipid Hypothesis states that atherosclerosis is caused by elevated LDL levels. LDL attach to the arterial wall and their accumulation in macrophages promotes` the formation of foam cells. LDL is

more readily oxidized increasing the formation of fatty streaks (Merck Research Laboratories, 2003).

The Chronic Endothelial Injury Hypothesis states that atherosclerosis develops due to aggregation of platelets and their adhesion to the sub-endothelium. Monocyte-derived growth factors are released and induce migration of SMCs into the intima. This leads to the replication and accumulation of SMCs and connective tissue that later develops into fibrous plaques (Merck Research Laboratories, 2003). Although the exact cause of atherosclerosis has not been determined, each of these two hypotheses are plausible explanations and both may partly explain atherosclerotic progression. For example, endothelial injury may initiate the events that lead to plaque formation, but this process only proceeds in the presence of elevated levels of LDL.

In humans, fatty streak development begins in childhood, and atherosclerotic lesions exist as early as the second decade of life (Plutzky, 2003). Although atherosclerosis does not fully develop until later in life, early prevention is key.

Atherosclerosis is a common complication of obesity and diabetes, as well as a major risk factor for CVD. CVD (along with stroke) is a major contributor to mortality in our country, accounting for 36% of all deaths in Canada (Heart and Stroke Foundation, 2003). Eighty percent of Canadians aged 20-59 years are at risk for developing CVD. CVD greatly impacts the quality of life of those affected

and puts a great strain on the health care budget. Heart disease and stroke are the number one causes of hospitalization, accounting for 18% of all hospitalizations in Canada (Heart and Stroke Foundation, 2003). Economically, this costs Canadians $18.5 billion (data from 1998), which equates to $610.70 per capita per year.

Patients with diabetes have an increased risk of atherosclerosis due to alterations in lipid metabolism (Hotta et al, 2000), as well as increased glycosylation of connective tissue (Merck Research Laboratories, 2003). High insulin levels in the blood also increase vascular endothelium damage. The higher risk of atherosclerosis in diabetes means the incidence of CVD is also elevated in diabetes. Consequently, heart disease is one of the leading causes of death in those with diabetes (American Diabetes Association, 2004).

Worldwide, there are an estimated 135 million people with diabetes. Nationally, over 2 million Canadians are affected (Canadian Diabetes Association, 2004). In 1994, 3.7% of Canadians self-reported that they had diabetes, and in 2000, this number had increased to 4.7% (Heart and Stroke Foundation, 2003). The prevalence of diabetes increases with age, with 18.3% of those 60 years or older affected (American Diabetes Association, 2002). Metabolic syndrome is also on the rise, with an estimated 25% of the US population affected, increasing to 45% in adults over the age of 65 years (Lamarche & Desroches, 2004). The World Health Organization estimates that the number of those with diabetes will

increase to 300 million by the year 2025. Billions of dollars each year are spent treating diabetes and associated complications (Canadian Diabetes Association, 2004).

Due to the great impact that both diabetes and atherosclerosis have in our society, many studies have investigated the effects of various nutraceuticals and functional foods on these diseases. CLA is one of these food components that has shown promise.

3. Conjugated Linoleic Acid (CLA)

3a) CLA Defined

CLA was first discovered in 1935 as a component in milk fat (Khanal & Dhiman, 2004). CLA is a collection of geometric and positional isomers of linoleic acid (LA) (18:2 n-6). CLA also has 18 carbons, with two conjugated double bonds. CLA is mostly found in meats and dairy products with the highest concentrations coming from ruminant animals (Prandini et al, 2001). Dairy products have 2.9-11.3 mg CLA/g fat (Shantha & Decker, 1993), while beef has 3.1-8.5 mg/g fat (Shantha et al, 1994).

CLA is synthesized by the bacterial bio-hydrogenation of LA in the rumen (Granlund et al, 2003), and can also be produced in small amounts in the mammary gland and possibly the liver in some animal models (Mahfouz et al, 1980). CLA found in human adipose tissue is directly related to milk fat intake (Kelly, 2001). Preliminary evidence also suggests that humans can produce minute amounts of CLA, although these studies may not be scientifically sound (Adolf et al, 2000).

Cis-9, trans-11 (c9,t11) CLA is the most prominent isomer found naturally in food products (Yu et al, 2003). For example, 73-93% of CLA in dairy products is the c9,t11 form, while 3-5% is trans-10, cis-12 (t10,c12) CLA (Khanal et al, 2004). Commercial CLA supplements are also primarily made up of these isomers (Brown et al, 2003).

Human intake is estimated to be 15-430 mg CLA/day (McGuire et al, 1999), with the majority coming from c9,t11 CLA. Limited Canadian data is available, however, one study of 22 free-living Canadians indicated that average CLA consumption was 15-174 mg/day, averaging 95 mg/day (Ens et al, 2001). This intake is lower compared to other countries. Additionally, the usual intake in CLA supplementation studies with experimental animals is approximately 10 times higher than the normal human intake. However, toxicology reports indicate that CLA is safe to consume and support the short-term use of CLA. Human studies providing up to 7 grams of CLA/day for up to one year (Whigham et al, 2004) did

not adversely affect the health of subjects. Minor adverse effects such as diarrhea and constipation were reported by a few study participants, but nothing warranted stopping the supplement (Whigham et al, 2004). One major alarm was provided by Riserus et al (2002) who found that t10,c12 CLA supplementation increased insulin resistance in adults with type 2 diabetes. Although this is only one study, long-term efficacy must be proven before fully advocating this isomer.

Natural CLA in the diet is in triglyceride (TG) form, while synthetic CLA is most often in the form of a free fatty acid. In rats, both TG-CLA and free fatty acid-CLA have similar effects on leptin and lipid metabolism (Wang et al, 2003). Similarly, both natural high-CLA butter and synthetic CLA had similar effects on mammary gland development and cancer risk in rats (Ip et al, 1999). While there is a limited amount of research comparing the effects of natural and synthetic forms of CLA, those to date suggest they have similar effects in the body.

Until recently, CLA has mostly been tested as a mixture of isomers, although new research suggests that individual isomers have varying effects on health (Pariza et al, 2000). For example, t10,c12 CLA has been shown to reduce body fat in animals, while the c9,t11 isomer does not have this effect (Brown et al, 2003).

3b) CLA and Atherosclerosis

Combinations of CLA mixtures have been reported to decrease plasma lipids and aortic lesions in rabbits, hamsters, rats and mice. Unless otherwise indicated within this review, CLA mixture refers to a synthetic mixture of CLA that includes 4 isomers: predominantly c9,t11 and t10,c12, significant amounts of trans-8, cis-10 (t8,c10), and cis-11, trans-13 (c11,t13), and minimal amounts of other minor isomers. CLA in diets is expressed as % weight-by-weight (w/w) (g/100 g of diet).

i) Rabbit Model

Atherosclerosis has been extensively studied in the rabbit model. One of the first studies to show the beneficial effects of CLA in the rabbit model with regards to the progression of atherosclerosis was done in 1994 by Lee and colleagues. Rabbits were fed an atherogenic diet (coconut oil with 0.1% cholesterol (CH)) for 22 weeks, with half being supplemented with 0.5 grams CLA/rabbit/day (0.5% w/w of food). After 12 weeks, both serum total cholesterol (TC) and LDL-CH, as well as serum TG were significantly lower in the CLA-fed group, while high-density-lipoprotein (HDL) levels were unaffected (Lee et al, 1994). At termination, fewer atherosclerotic lesions (described as fatty streaks in the aortic arch and descending thoracic aorta) were detected in the CLA-fed group compared to controls (Lee et al, 1994). This study was the first to demonstrate the potential

anti-atherogenic and hypocholesterolemic properties of CLA as it lowered serum TGs, TC and LDL-CH in the serum, all risk factors for atherogenesis.

Six years later, these results were supported by another study. Kritchevsky et al (2000) fed the same atherogenic diet to rabbits supplementing half with 0.5% w/w CLA (43% c9,t11 and 44% t10,c12) for 22 weeks. CLA-fed rabbits had 27% less plaque compared to the control group, as well as less lipid deposition and connective tissue development in their aortas (Kritchevsky et al, 2000).

In a second part of the same study, 30 rabbits were provided with control diet (atherogenic diet containing 0.2% CH) with 10 rabbits receiving 1% w/w CLA (43% c9,t11 and 44% t10,c12) for 90 days (Kritchevsky et al., 2000). Serum lipids were higher in the CLA-fed group, while liver CH was lower in those fed CLA. Liver TGs were similar in the two groups. CLA-fed rabbits had 31-40% less atherosclerosis in the aorta compared to control rabbits.

The remaining rabbits were used as a regression model and were fed a CH-free diet with or without CLA for another 90 days. Serum total-CH levels fell more in the CLA-fed rabbits, and HDL-CH increased by 121% and 74% in the control and CLA-fed rabbits, respectively. Serum TGs were lower in CLA-fed rabbits, but unchanged in the control group (Kritchevsky et al, 2000). Atherosclerotic incidence was also lower in the CLA group, although not significantly. This

suggests that CLA is able to alter serum TG pre- and post-atherosclerosis but is more effective as a preventative agent than for treatment of established disease.

The required dietary levels of CLA for protective effects have also been investigated. Rabbits fed an atherogenic diet with 0.1%, 0.5% or 1.0% w/w CLA had differing amounts of atherosclerosis. The severity of atherosclerosis in the aortic arch decreased significantly by 28%, 63%, and 58% compared to baseline, respectively, and atherogenesis in the thoracic aorta was decreased by 41%, 66%, and 57%, respectively (Kritchevsky et al., 2000). It is interesting to note that 0.5% and 1% CLA had a similar effect. This suggests CLA has a threshold at which additional CLA may not help to reduce atherosclerosis.

As low as 0.05% w/w CLA shows anti-atherogenic effects in rabbits. However, 0.075-0.50% w/w CLA appears to be the best concentration as this level provided elevated HDL-CH levels, and atherosclerosis in the aorta was lowest. Serum TG levels were also higher in CLA-fed rabbits, but these results were not dose-related (Kritchevsky et al, 2002).

An abundance of atherosclerosis work has been done using the rabbit model. CLA clearly has beneficial effects, reducing risk factors such as lowering serum TC, LDL-CH, and serum TG. Direct measures of atherosclerosis such as aortic/abdominal/thoracic arch lesions have been shown to be lower when CLA is present, suggesting a role for CLA in atherosclerotic prevention. However, this

effect is less for regression of established atherosclerosis. Rabbits appear to benefit from as little as 0.05% CLA, and optimal effects are seen at levels of 0.5% CLA.

ii) Hamster Model

Results obtained with the hamster model of atherosclerosis are similar to those seen in rabbits. The hamster is a good model due to its established responsiveness to plasma CH lowering and anti-atherogenic interventions (Nicolosi et al, 1997).

Hamsters fed hypercholesterolemic diet supplemented with 1% w/w CLA had lower plasma TC and reduced aortic fatty streak formation. Those fed 1% w/w LA had lower serum TG levels compared to both control and CLA-fed groups. (Wilson et al, 2000a). It appears that both CLA and LA have the ability to lower serum CH, however, only CLA can directly decrease fatty streak formation. Nicolosi et al (1997) also demonstrated reduced TC, very low density lipoproteins (VLDL) and LDL, with no changes in HDL when feeding hamsters various levels of CLA (0% - 1.1% w/w CLA). Fatty streaks of the aortic arch were also significantly reduced (Nicolosi et al, 1997). These studies indicate that CLA and LA reduce fatty streak formation in the aorta and that serum CH is reduced only by CLA.

While there is an abundance of information in regards to CLA and its therapeutic effects as a mixture, the effects of individual isomers in animal models on atherosclerosis are not as clear.

Hamsters fed a cholesterol-rich diet with either no CLA, a mixture of CLA isomers (c9,t11 and t10,c12 CLA), c9,t11 CLA alone, or t10,c12 CLA alone at 0.66% w/w had differing effects (De Deckere et al, 1999). Those fed the t10,c12 isomer had lower body weights and lower plasma TC, LDL-CH and HDL-CH compared to those fed the c9,t11 CLA isomer, but higher VLDL-CH. This suggests a possible detrimental effect of t10,c12 CLA, as elevated VLDL-CH relates to higher serum TG levels.

Another possible detrimental effect of CLA includes its possible ability to increase peroxisome proliferation in the liver. Excess peroxisome proliferation could result in liver damage. In the hamster model, no changes in peroxisome proliferation in the liver was found when animals were given CLA, illustrating that CLA does not pose a threat to liver function in this model (De Deckere et al, 1999).

Thus, in the hamster, CLA lowers plasma TC, raises serum TG, and reduces total fatty streak formation (De Deckere et al, 1999; Nicolosi et al, 1997; Wilson et al, 2000a). More specifically, t10,c12 CLA appears to be responsible for elevations in plasma TG. T10,c12 CLA may have a higher oxidation rate

compared to c9,t11 CLA, thus allowing it to be the more active CLA isomer in relation to lipid metabolism in the hamster (De Deckere et al, 1999). These studies illustrate the varying range of effects of individual CLA isomers and their mixtures. The next sections will focus on rat and mouse models, and the effects of CLA isomers.

iii) Rat and Mouse Models

Studies with rats and mice have examined the effects of CLA on various risk factors of atherosclerosis. Otsuka Long-Evans Tokushima fatty (OLETF) rats fed the t10,c12 CLA isomer had significantly lower systolic blood pressure compared to those rats fed LA or c9,t11 CLA (Nagao et al, 2003a).

Another risk factor for atherosclerosis is an increase in plasma TG. Degrace et al (2003) identified that t10,c12 and c9,t11 CLA both individually lower plasma TG in C57BL/6J mice, although t10,c12 CLA has a greater effect (Munday et al, 1999). The serum TG lowering effects of CLA are unique to the mouse and rabbit model, and has been suggested to be due to increased lipoprotein clearance at the level of the liver. As discussed previously, CLA has been shown to both increase and decrease serum TG in hamsters and rats (De Deckere et al, 1999; Nagao et al, 2003a; Nicolosi et al, 1997; Wilson et al, 2000b).

High levels of serum VLDL and LDL is another risk factor for atherosclerosis. Mice fed t10,c12 CLA had enlarged livers compared to other groups as well as greater TG accumulation in the liver. Liver secretion of VLDL was two-fold greater in t10,c12 CLA-fed mice compared to controls, thus VLDL accumulation could not be responsible for changes in the size of livers or hepatic TG accumulation (Degrace et al., 2003).

CLA increased aortic sinus lesions in mice fed 0.25% CLA, while it reduced fatty streaks in those animals fed 0.5% CLA (Munday et al, 1999). This was the first study to show that CLA promotes atherosclerosis, but the mechanisms by which CLA promotes the development of fatty streak formation may be unique to this animal model. Munday et al (1999) conclude that as per their results, CLA cannot be referred to as anti-atherogenic. However, it is important to note that the mouse may not be the ideal model for studying atherosclerosis as mice do not have two of the major proteins affecting atherosclerosis in humans: lipoprotein (a) and cholesteryl ester transfer protein (Munday et al., 1999).

The effects of CLA in atherosclerosis seen in mice are quite different compared to other animal models. It is important to take these findings into consideration when focusing on those animal models that better relate to the human pathogenesis of atherosclerosis.

iv) Humans

Less evidence exists in regard to CLA's anti-atherosclerotic effects in humans, and the need for long-term studies is increasingly evident. However, to date, lipid-lowering effects have been obtained with as little as 0.1-1.5% w/w CLA (Stangl et al, 1999).

Noone et al (2002) provided human subjects with either 3 g/d 50:50 blend (c9,t11 and t10,c12), 80:20 blend (80% c9,t11 and 20% t10,c12) or LA for eight weeks. Plasma TG was significantly reduced by 20% in those fed 50:50 blend, while 80:20 did not reduce total plasma TG but reduced VLDL concentrations. LDL, HDL and reverse CH transport were not altered. These results suggest that in humans, t10,c12 CLA may be responsible for the hypotriglyceridemic effect (Noone et al., 2002).

Furthermore, Brown et al (2001) found that in vitro, the TG-lowering effects of CLA in 3T3-L1 preadipocytes was due to the t10,c12 CLA isomer, and that LA partly reversed the ability of CLA to lower TG content. This suggests that LA may compete with CLA for incorporation into TG. It is hypothesized that t10,c12 CLA may act by attenuating adipogenesis, increasing lipolysis or decreasing fatty acid esterification into TG (Brown et al, 2001).

While some human studies do offer promising results, some studies (especially short-term studies) show no changes in atherosclerotic parameters (Benito et al, 2001). Additionally, a smaller fraction of studies suggest that long-term CLA supplementation causes detrimental health effects such as insulin resistance (Kelly, 2001). Due to the contradictory evidence, it is important to determine which CLA isomer has beneficial effects in regards to atherosclerotic progression and its mechanistic pathway before advocating CLA's supplementation.

3c) CLA and Adiposity

Another risk factor for atherosclerosis is obesity or increased adipose mass. Thus, it is of interest that CLA has been shown to decrease adipose mass in both animals and humans. OLETF rats fed t10,c12 CLA have significantly lower adipose weight and serum leptin expression compared to rats fed c9,t11 or control (Nagao et al, 2003a). Obese Zucker rats have also shown a decrease in adipose tissue when fed CLA (Belury, 2002). Similar results were found in mice, with the t10,c12 CLA group having significantly lower body weights (Degrace et al, 2003).

Experiments with cell cultures have also shown decreased adiposity in relation to CLA. Treating 3T3-L1 preadipocytes with 100 uM of CLA decreased adipocyte size as CLA concentration increased. Cultures with CLA had over 30% less cellular TG (Evans et al, 2000). T10,c12 CLA decreases TG content more than

c9,t11 CLA (Blankenberg et al, 2001). CLA's ability to decrease adiposity is relevant as adipose tissue is responsible for secreting adipokines (Nagao et al, 2003b), which have been related to cardiovascular risk.

Contradictory results have been found in regards to CLA's anti-adiposity effects in humans. Various studies report CLA having no effect on adiposity, while others show a reduction in adipose weight (Kelly, 2001). Due to the varying lengths of these studies, as well as the varying types and concentrations of CLA ingested, it is understandable that contradictions exist.

Overall, CLA mixtures and the t10,c12 CLA isomer seem to have a modulating effect in pre-atherosclerotic processes such as aortic fatty streaks/lesions, and elevated circulating lipids. Proposed mechanisms may involve both PPARs and cytokines as both are involved with lipid metabolism and are altered in atherosclerosis.

4. Peroxisome Proliferator-activated Receptors (PPARs)

4a) PPARs

PPARs are nuclear hormone receptors that act as transcription factors and have a role in adipocyte differentiation (Hwang et al, 1997). PPARs code for enzymes

involved in the uptake of fatty acids in membranes, fatty acid oxidation, and in lipoprotein assembly and transport (Kersten et al, 2000).

For transcription to occur, a ligand is also required. Fatty acids, eicosanoids, and certain hypolipidemic and anti-diabetic drugs act as ligands and are capable of activating PPARs.

Once ligand activated, PPARs selectively form a heterodimer with the Retinoid X Receptor, the receptor of 9-cis retinoic acid. The heterodimer then binds to the promoter region, specifically to a PPAR-responsive element (PPRE), on a target gene (Kersten et al, 2000).

There are three PPAR subtypes: PPARα, PPARγ, and PPARβ. Each are found in varying tissues of the body, and have different functions. PPARα and PPARγ1 are expressed in human smooth muscle cells, while PPARγ2 is not (Zahradka et al, 2003). In humans, PPARα is found in monocytes, while PPARγ is not expressed until monocytes differentiate into macrophages (Chinetti et al, 1998). PPARβ is ubiquitously expressed in all cell types, including monocytes and macrophages (Moller & Berger, 2003; Khan & Vanden Heuvel, 2003).

PPARα is predominant in the liver, brown adipose tissue, and to a lesser extent in the kidney, heart and skeletal muscle (Kersten et al, 2000). PPARα increases key regulatory proteins in catabolic oxidation pathways. PPARα activation may

increase beta-oxidation (degradation of fatty acids) and energy expenditure. Research shows that CLA is a strong ligand and activator of PPARα (Kersten et al, 2000; Moya-Camarena et al, 1999), thus, it is suggested that CLA's anti-adipogenic effect involves PPARs (Brown et al, 2003). PPARα also modulates bile acid synthesis and catabolism in the liver (Barbier et al, 2002). PPARα, but not PPARγ, has been shown to decrease the adhesion of monocytes to endothelial cells, thus potentially slowing the progression to atherosclerosis (Marx et al, 1999a). The fibrate class of drugs are PPARα agonists.

PPARγ is found mainly in adipose tissue with small amounts being detected in the colon, immune system and retina (Kersten et al, 2000). PPARγ is a major transcription factor inducing anabolic processes (TG synthesis, glucose uptake, and fatty acid uptake). PPARγ acts either directly or indirectly by enhancing the transcription of genes (Brown et al, 2003). PPARγ has been shown to regulate plasminogen activator inhibitor-1 expression in human endothelial cells (Marx et al, 1999a; Yu et al, 2003). PPARγ has also been shown to increase reverse CH transport in foam cells (Chinetti et al, 1998), which results in an overall decrease in total serum CH. Additionally, CLA's action in PPARγ activation may be related to its downstream activities and actions of Δ6 desaturase metabolites of CLA (Belury et al, 2002). An increase in PPARγ mRNA expression was found in human coronary arteries and left ventricles in human patients with coronary heart disease and cardiomyopathy, suggesting that PPARs play a role in the progression of heart disease (Mehrabi et al, 2003).

Thiazolidinediones (TZDs) are known PPARγ agonists that induce preadipocyte differentiation and increase insulin sensitivity, thus aiding in glucose uptake and stimulating lipid accumulation in adipocytes (Kang et al, 2003). TZDs are used to treat type 2 diabetes, however, they have also been reported to increase adiposity and body weight in both rodents and humans (Granlund et al, 2003).

The majority of research has been conducted on PPARα and PPARγ due to their connection with medications, however, recently PPARβ has also been receiving attention.

PPARβ has been found to be in both human and rat, and is up-regulated in vascular smooth muscle cells during lesion formation (Zhang et al, 2002). PPARβ agonists (GW501516) have also been shown to increase reverse CH transport (Oliver et al, 2001), to down-regulate genes needed for lipid metabolism (Vosper et al, 2001) and increased expression of PPARβ has been associated with neointimal thickening (Zhang et al, 2002).

Past research in this laboratory has determined that both PPARα and PPARγ agonists have the ability to interfere with SMC proliferation in vitro (Zahradka et al, 2001), however, PPARβ's function in SMC's has not yet been determined. It is proposed that PPARβ may regulate both PPARα and PPARγ by competing for

binding to the PPRE and thus, influencing gene expression (Moya-Camarena & Belury, 1999).

Taken together, these studies illustrate that PPARβ may also play an important role in controlling atherosclerotic risk factors such as lipid and CH metabolism, and SMC proliferation.

In summary, all PPARs appear to modulate adipocyte differentiation. PPARα participates in the catabolism of fatty acids (especially in the liver), while PPARγ assists in the storage of fatty acids in adipose tissue (Kersten et al, 2000). PPARs act to control serum TG and modulate expression of proteins involved in foam cell formation and vascular inflammation. All three PPARs have been shown to regulate CH in macrophages (Barbier et al, 2002). Thus, PPARs are involved in many, if not all stages of atherosclerosis.

4b) PPARs and CLA

PPARs require a ligand for transcription to occur, and CLA acts as one of these ligands. Individual isomers of CLA including c9,t11 and t10,c12 CLA have been shown to bind to PPARs in cell culture. For example, c9,t11 CLA, LA, and, t10,c12 CLA both activate PPARγ, with c9,t11 having stronger binding affinity (Belury et al, 2002).

CLA has been shown to be a potent ligand and activator for human PPARα, with c9,t11 CLA having the strongest effect (Moya-Camarena et al, 1999). It has been suggested that c9,t11 CLA may be incorporated more into tissue lipids than t10,c12 allowing it to have an increased opportunity for ligand binding activity. Additionally, t10,c12 CLA may be preferentially oxidized which increases its use as an energy source, decreasing its ability to be stored in the tissues (De Deckere et al, 1999).

Granlund et al (2003) found that t10,c12 CLA prevents TG accumulation in adipose by modulating PPARγ. This suggests that PPARγ increases TG accumulation and that this fatty acid blocks PPARγ signaling pathways, controlling TG accumulation possibly by blocking pre-adipocyte differentiation (Kang et al, 2003). Adipocytes have significantly less lipid content when treated with t10,c12 CLA (Granlund et al, 2003). It is interesting to note that less t10,c12 CLA is needed to reduce lipid content in human adipocytes compared to mouse adipocytes.

Over a short-term period, both c9,t11 CLA and t10,c12 CLA directly antagonize PPARγ, but only t10,c12 CLA has TG lowering effects. It is suggested that t10,c12 CLA directly affects PPARγ by competing with endogenous ligands, antagonizing PPARγ activity, and PPARγ transcriptional activity. It has also been hypothesized that t10,c12 CLA is responsible for reducing fatty acid desaturation

resulting in a decrease in monounsaturated fatty acids (MUFA) and MUFA: saturated fat ratio. MUFA are the preferred source for TG synthesis, thus, a decrease would result in a lower TG synthesis. T10,c12 CLA has also been postulated to increase ß-oxidation or increase energy expenditure (Brown et al, 2003).

Brown et al (2003) suggested that t10,c12 CLA is anti-adipogenic but c9,t11 CLA promotes adipogenesis. Thus, when delivered together, their effects cancel each other out and no changes are seen.

Although the exact mechanisms and why individual CLA isomers have differing effects is unclear, much research supports the view that CLA affects PPARs and that PPARs are involved in the atherosclerotic process. Adipokines, like leptin and adiponectin, are cytokines that are synthesized in adipose tissue and have been shown to influence the atherosclerosis process. CLA may increase adiponectin expression and lower leptin expression possibly through modulating PPAR activation, thus potentially decreasing atherosclerotic risk factors.

5. Adipokines and Atherosclerosis

Adipose tissue has two very important functions: it is the largest energy reserve in the body and it acts as a regulator of energy metabolism (Hwang et al, 1997). Adipocyte differentiation is controlled by transcription factors such as PPARs. PPARs help to control the actions of adipokines, such as leptin and adiponectin.

Cytokines are proteins that have certain metabolic and endocrine functions. Adipokines are cytokines synthesized in the adipocyte, and thus are adipocyte-derived proteins.

5a) Leptin

Leptin, the product of the 'obese gene', is a 167 amino acid peptide hormone encoded by the *ob* gene. It is produced and secreted mainly by adipocytes. In the healthy adult, leptin functions to regulate fatty acid metabolism by promoting fatty acid oxidation (Atkinson et al, 2002). This effect of leptin has been seen in the liver, skeletal muscle, pancreas and the heart (Atkinson et al, 2002).

Serum leptin is correlated with subcutaneous and visceral fat (Funahashi et al, 1999), and has been shown to regulate body weight and energy balance in humans (Khan et al, 2003; Lonnqvist et al, 1995). Serum leptin levels are reduced in weight reduction (Maffei et al, 1995), and leptin has also been shown

to regulate both energy intake and expenditure (Hwang et al, 1997), as well as enhance the development of insulin resistance and atherosclerosis (Hotta et al, 2000). High serum leptin levels correlate with an increased risk of atherosclerosis in humans (Ciccone et al, 2001) and are associated with a 20% increase in the risk of coronary events (Bodary et al, 2002).

Thiazolidinediones (ligands for PPARγ) suppress the expression of the leptin gene, suggesting PPARγ plays a role in regulating leptin production (Hwang et al, 1997). T10,c12 CLA also reduces leptin secretion in adipocytes (Kang & Pariza, 2001). In preadipocytes, however, t10,c12 CLA increased leptin expression compared to c9,t11 CLA (Brown et al, 2003). This suggests that t10,c12 CLA may only be able to reduce leptin levels/expression in mature adipocytes.

5b) Adiponectin

Adiponectin is an adipose-specific, 30 kDa, 244 amino acid protein which has anti-diabetic, anti-atherogenic and anti-inflammatory properties (Ouchi et al, 2003). The structure consists of a small nonhelical region with a stalk of collagens and the signal sequence at the N terminus. The C-terminus consists of the globular head domain (gAcrp30), which is a large part of the protein (Onay-Besikci et al, 2004). Adiponectin can be found as both the globular form, and the full-length form in the serum.

Adiponectin is abundant in the circulation, with average plasma concentrations of 3-30 μg/mL, contributing about 0.1% of the total plasma protein (Arita et al, 1999). Adiponectin is produced in the adipose, but is able to target other tissues (Brakenhiemlm et al, 2004), modulating both glucose and lipid metabolism. Serum adiponectin levels are lower in those with coronary artery disease (CAD), diabetes, and in obese individuals (Ouchi et al, 2004; Kumada et al, 2003; Hotta et al, 2000). Adiponectin administration has been shown to increase fatty acid oxidation, decrease TG levels in the muscle, and increase insulin sensitivity (Tsuda et al, 2004).

Serum adiponectin levels also correlate with vascular function in humans (Fernandez-Real et al, 2004). Adiponectin has been shown to inhibit monocyte adhesion to endothelial cells and lipid accumulation in human monocyte-derived macrophages, as well as down-regulate expression of intracellular adhesion molecules. Adiponectin also has been shown to inhibit vascular SMC proliferation (Fernandez-Real et al, 2004).

Adiponectin has 2 receptors, ADIPOR1 and ADIPOR2. ADIPOR1 is expressed mostly in skeletal muscle and binds primarily to the globular form of adiponectin. ADIPOR2 is found mostly in the liver and primarily binds to the full-length form (Wang et al, 2004). Cell lines from African-American humans with diabetes have

lower levels of ADIPOR1 mRNA, suggesting a role for ADIPOR1 in metabolic syndrome (Wang et al, 2004).

The globular head of adiponectin (gAcrp30) has also been shown to affect the atherosclerotic process. Proteolytic cleavage of adiponectin produces a 16kDa protein, gAcrp30. The gAcrp30 protein is about 60% the size of its precursor and it contains the entire C-terminal globular domain of adiponectin (Fruebis et al, 2003)

In mice, gAcrp30 administration produces an increase in fatty acid oxidation in muscle, and a decrease in plasma free fatty acids, glucose and TG (Fruebis et al, 2003). Full-length adiponectin had similar effects, however, it did not alter plasma TG. Thus, it is hypothesized that gAcrp30 increases removal of free fatty acids from the circulation by increasing cellular uptake. Other studies in mice have shown that gAcrp30 reduces body weight, while full-length adiponectin increased weight (Fruebis et al, 2003). While the mechanisms of both the globular domain and full-length adiponectin are not known, both forms appear to have the ability to stimulate fatty acid oxidation.

Many similar functions of the two forms of adiponectin have been found. Both increase fatty acid oxidation, PPARα ligand binding, phosphorylation of AMP-activated protein kinase (AMPK), acyl-CoA carboxlyase (ACC) and p38 mitogen-activated protein kinase (MAPK). ERK1/2 phosphorylation was not altered by

either form of adiponectin, which suggests that adiponectin does not play a role in protein/DNA synthesis (Tsao et al, 2002).

Nagao et al (2003b) showed that serum adiponectin was significantly increased in *fa/fa* Zucker rats fed CLA (1% CLA mixture for 8 weeks), while serum leptin concentrations were unchanged. Similar increases in adiponectin are seen when TZDs are administered in mice. Since TZDs activated PPARγ (Maeda et al, 2000), CLA may increase adiponectin in a process involving increased PPARγ activation (Ouchi et al, 2003).

Adiponectin has been shown to have insulin-sensitizing effects that may be attributed to its ability to increase fatty-acid oxidation through either AMPK or PPARα. PPARγ agonists have also been shown to be able to increase plasma adiponectin levels (Iwaki et al, 2003).

Although adiponectin is a newly studied protein, available research clearly illustrates that it is elevated in many diseases, including diabetes, CVD, and obesity. Due to these strong correlations, it is important to determine the exact mechanisms of adiponectin as it could possibly be a modulator in certain disease states. Various studies have linked adiponectin's actions to AMPK.

6. AMP-activated protein-kinase (AMPK)

AMPK has been referred to as the metabolic master switch (Hardie & Pan, 2002). It acts as a low-fuel warning signal for the cell, and its activation helps to provide energy to these cells.

AMPK is a serine/threonine kinase (Dyck et al, 1996) and its role is to inhibit the energy-utilizing pathways, as well as promote the use of other fuels to provide energy (Munday, 2002). AMPK acts by turning off processes that utilize ATP, and turning on alternative catabolic pathways to create more ATP (Hardie et al, 2002; Hegyi et al, 2004). Structurally, AMPK is made up of a catalytic α subunit and regulatory β and γ subunits (Yin et al, 2003).

Lipids, after entering cells, are converted into acetyl-CoA, and then are either β-oxidized and used for energy or stored as triglycerides. AMPK is activated when there is a decrease in cellular ATP which alters the AMP:ATP ratio. ACC has two isoforms (Hardie et al, 2002) and is a regulator of fatty acid metabolism. ACC-1 (alpha) is believed to regulate fatty acid synthesis, while ACC-2 (beta) is involved with fatty acid oxidation (Hardie et al, 2002). AMPK regulates both forms of ACC, thus increasing beta oxidation and decreasing lipid biosynthesis in both muscle and liver (Hegyi et al, 2004; Muoio et al, 1999).

AMPK has been shown to regulate many processes in muscle metabolism including increasing Glulcose-transporter-4, glycogen synthase, fatty acid oxidation, lipolysis and protein synthesis by controlling key enzymes and altering gene expression (Wojtaszewski et al, 2003).

Adiponectin and leptin have both been shown to activate AMPK, which is further associated with increased fatty acid oxidation and decreased TG accumulation in muscle (Tsuda et al, 2004; Yin et al, 2003). Adiponectin has been shown to be effective in both the liver and skeletal muscle (Yin et al, 2003), although its effects have not yet been seen in cardiac muscle (Carling et al, 2003). Exercise and the diabetic drug metformin both elevate AMPK activity (Leclerc et al, 2004; Wu et al, 2003; Buhl et al, 2002). AMPK is believed to function in the following way:

↑ AMPK ⟶ ↓ACC ⟶ ↓ Malonyl-CoA synthesis ⟶ ↓ fatty acid synthesis

7. 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)

AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), a potent (but not specific) activator of AMPK, affects both muscle and lipid metabolism, and may modulate cell growth (Buhl et al, 2002; Wu et al, 2003).

Animal studies show that AICAR reduces plasma triglyceride levels, free fatty acids, and increases HDL-CH levels potentially by enhancing GLUT4 protein expression (Buhl et al, 2002). AICAR has also been shown to lower both blood glucose and insulin concentrations.

Both AICAR and rosiglitazone are pharmacological AMPK activators (Xiang et al, 2004). AICAR is well-tolerated (at doses of 10-100 mg/kg) and shows beneficial effects in regards to decreased plasma glucose. However, due to its short half-life and the ability to cause an increase in uric acid concentrations, it is not a viable option for pharmacological treatment of diabetes (Musi & Goodyear, 2002).

8. Akt

Akt (also termed protein kinase B(PKB)) is a kinase which operates downstream of phosphoinositide-3 kinase (PI3K), and could possibly be regulated by AMPK activation. PI3K is found in many of the body's tissues including vascular SMCs (Oudit et al, 2004). This pathway responds to growth factors and regulates various cellular functions including cell growth and survival.

The following is a schematic representation of the PI3K/Akt pathway:

PI3K → Akt → mTOR → p70 S6K→ Cell Growth/Proliferation
or
Inhibition of Apoptosis

PI3K is an up-regulator of Akt and mTOR. Activation of these kinases leads to cell growth and hypertrophy. The Akt/PKB pathway acts to inhibit apoptosis, and promote cell survival. Phosphorylation of threonine 308 partially activates Akt/PKB, while full activation requires the phosphorylation of Serine 473. Dysfunction of this pathway has been related to both diabetes and cancer (Song et al, 2005).

9. The Zucker Rat Model

The majority of this literature reviewed has focused on animal models and emphasizes rabbits and hamsters as they have traditionally been used to study atherosclerosis. The *fa/fa* Zucker rat is a popular model used to study physiologic changes in metabolic syndrome and may also be a useful model for investigating pre-atherosclerotic changes.

The *fa/fa* Zucker rat develops three-fold higher serum CH and TG compared to other rat models (St.John & Bell, 1991). Concentrations of CH in the aorta in *fa/fa* Zuckers is also significantly higher than in lean Zuckers or Sprague Dawley counterparts. This is significant as high arterial CH concentrations is one of the early signs of atherosclerosis. Alterations in lipid profiles in *fa/fa* Zuckers is comparable to changes seen in the human artery (St. John & Bell, 1991).

The *fa/fa* Zucker rats visually appear obese at 5 weeks of age (Argiles, 1988), exhibit hyperinsulinemia by 4-5 weeks of age and are hypertensive at six weeks (Banz et al, 1996). Hyperinsulinemia represents an increased concentration of insulin in the blood indicating inadequate utilization (insulin resistance) by the body's cells, an early sign of type 2 diabetes. Thus, taken together, the *fa/fa* Zucker rat exhibits metabolic syndrome as they have insulin resistance, obesity, elevated TGs and hypertension (Nagao et al, 2003b).

The *fa/fa* Zucker rat displays specific characteristics of metabolic syndrome, which would suggest it is at greater risk of developing atherosclerosis. Thus, the *fa/fa* Zucker may be an appropriate model to investigate pre-atherosclerotic changes and effects of dietary treatments early in the life cycle.

However, the *fa/fa* Zucker rat may not be the best animal model for identifying visible signs of vascular damage (Amy & Dolphin, 1988). For example, after 6 months of age, *fa/fa* Zucker rats still exhibit normal endothelial surfaces in the

aortic arch. Some abnormal 'knobby' endothelial surfaces (swelling of endothelial surfaces) are found in these rats, suggesting that *fa/fa* Zuckers exhibit changes in endothelial cells different from other animals, and this may represent early stages of endothelial damage (Amy et al, 1988). It is also important to note that individual litters of *fa/fa* Zucker rats can vary considerably.

Jiang et al (1999) found that 14 week-old *fa/fa* Zucker rats have insulin-induced impaired vasodilatory properties in the aorta, with concomitant accelerated pro-atherogenic mechanisms (as controlled by MAP-kinase) (Jiang et al, 1999a).

Arguments can be made that the atherosclerosis-specific models, such as the JCR:LA-cp rat is a superior choice as it exhibits hyperlipidemia, insulin resistance, hyperinsulinemia, and atherosclerosis in early adulthood (O'Brien et al, 1999; Richardson et al, 1998). However, this is not the goal of the present investigation.

At 14 weeks of age, the *fa/fa* Zucker exhibits metabolic syndrome (Nagao et al, 2003b), as well as insulin-induced changes in the aorta (Jiang et al, 1999b). Thus, taken together, the *fa/fa* Zucker may be an appropriate model to investigate pre-atherosclerotic changes and the effects of dietary treatments early in the life cycle.

10. Porcine Smooth Muscle Cell Culture

As discussed above, SMCs make up a large component of the media as well as the intima of the artery wall. Porcine SMCs grown in cell culture have similar characteristics compared to the SMCs found in the arterial wall and both are able to alter their phenotype (Saward & Zahradka, 1997).

In response to vascular injury, SMCs change from a contractile state to a synthetic state (Komalavilas et al, 1999). SMC proliferation is a preliminary step in the initiation of atherosclerosis (Arita et al, 2002), and thus a reliable marker for tracking disease initiation.

PART 2 - STUDY RATIONALE

1. Limitations of Previous Research

The literature displays an abundance of research in regards to the beneficial effects of CLA. However, all these studies share two major discrepancies, which make them difficult to compare. Firstly, all look at different animal models, which result in varying sensitivities to CLA and to adipokines.

Secondly, new research points out that individual CLA isomers have different effects in the body, although there is limited data on these independent changes and their mechanisms. To make the literature more confusing, some early research does not even distinguish between the major isomers used making it even more difficult to reach an appropriate conclusion.

The same problem exists when looking at adiponectin. This newly studied adipokine has different oligomeric forms. It is now recognized that different forms of adiponectin (full-length vs. globular) function differently in the body and respond differently to adiponectin receptors. This further confounds the findings, as one must distinguish between different forms of adiponectin prior to looking at results.

CLA, adiponectin and leptin have all been shown to relate to the progression of atherosclerosis, however, their exact modes of action cannot be fully determined. Some support that PPARs may play a role in reducing atherosclerotic progression through these agonists, however, their mechanism and exact function is still undetermined. While many mechanisms have been proposed, they require further experimental testing.

2. The Importance of this Study

Serum levels of both leptin and adiponectin are related to obesity, insulin resistance and atherosclerosis in human subjects. CLA, a natural food component, may further affect these adipokines.

Obesity, atherosclerosis and diabetes are all commonly seen in our hospitals and are all interrelated. Although treatment of disease has been a focus, the future of health care requires that we begin to practice more preventative strategies.

Science, media and consumer groups are increasingly promoting the use of various functional foods and nutraceuticals for the prevention/treatment of disease. Unfortunately, some of these avenues disregard scientific evidence or promote products which have not been proven effective.

CLA is one of these functional foods/nutraceuticals that may offer health benefits in many disease states, including atherosclerosis. However, CLA's major sources are foods traditionally termed 'high fat' and current health recommendations advise limiting their daily consumption.

While most animal studies agree that CLA (both mixtures and individual isomers) has a preventative role in atherosclerosis, the exact mechanisms of action must be determined to further distinguish their function in the human body. As well, differences in the actions of individual isomers must also be identified to ensure appropriate recommendations (as dairy/animal products predominantly are made up of the c9,t11 isomer). The exact functions of PPARs and adipokines must also be made clear as it is known that they all play a role in lipid metabolism and have been related to atherosclerosis progression.

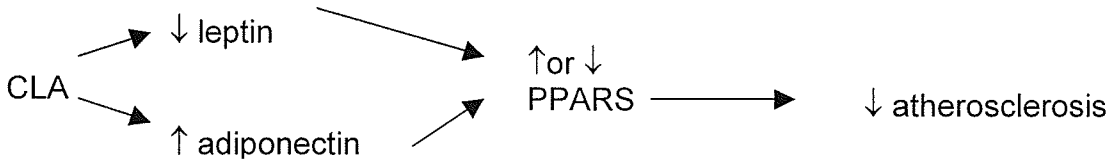
CLA, taken either as a mixture or individual isomers, may be a food component that has been providing us with atherosclerotic protection for years. Its high availability in our diet makes it a great option as a functional food, but its functionality must first be discovered. This will provide missing pieces to the puzzle and help us to unravel the potential beneficial effects of this fatty acid in regards to atherosclerosis.

3. Hypotheses

The overall hypothesis of this research is that CLA reduces atherosclerotic changes by modulating adipokines (leptin and adiponectin) and PPARs.

1 Rats fed CLA diet will have more adiponectin and less leptin which will alter PPAR expression and reduce early atherosclerotic progression in the *fa/fa* Zucker rat model.

2 Rats fed the t10,c12 isomer or the CLA mixture will have less leptin, more adiponectin and less atherosclerotic progression than rats fed the c9,t11 isomer.



4. Objectives

1 To determine the effect of dietary CLA isomers (singly and in combination) on serum leptin and adiponectin of *fa/fa* and lean Zucker rats.

2 To investigate interactions of CLA, adipokines and PPARs in porcine SMC culture.

3 To investigate the mechanisms by which CLA, adiponectin and leptin act in porcine SMCs.

Originally, one of the goals was to investigate PPAR expression and early atherosclerotic lesions in the abdominal aortas of *fa/fa* and lean Zucker rats using immunohistochemistry. However, this was beyond the scope of this thesis project. Emphasis was placed on cell culture work, focusing on the possible mechanisms of some of the interesting findings.

PART 3 - MATERIALS AND METHODS

Animal Study

1. Animals and Diet

One hundred forty 5-week-old male lean and *fa/fa* Zucker rats were purchased from Harlan (Indianapolis, IN). Rats were acclimatized for 5-7 days. Ten lean and 10 *fa/fa* rats were randomly assigned to one of seven dietary treatment groups (*Table 1*).

All diets were nutritionally adequate as they were based on the AIN-93G diet (Reeves et al, 1993), with fat contributing 8.5% (w/w) of the diet. All CLA was purchased in free fatty acid form. CLA diets contained 0.4% CLA (*Tables 2-5)* based on past experiments conducted in Dr. C. Taylor's lab (Noto, 2004). Dry ingredients were mixed and stored at -20°C. Oil was added weekly to ensure freshness and the diets were stored at -20°C.

Sample size (n=10) was chosen based on past experiments. Power calculations for serum adiponectin determined a sample size of 9 was needed to provide a significant difference with a 2-tailed t-test and a Power of 0.80, α=0.05 (SISA, http://home.clara.net/sisa). Leptin values used in this calculation were obtained from a previous experiment in this laboratory (Noto, 2004). Our sample size of 10

in each group provided an extra animal in case of accidental loss or contaminated samples. A pair-weighed (PW) group was also fed control diet. This group was fed amounts of diet to maintain body weights equivalent to the lightest *fa/fa* or lean Zucker rats allowing us to account for differences in parameters due to body weight.

Throughout the study, animals were housed individually in stainless steel wire-bottomed cages. Rats were maintained in a controlled environment with a 14-hour light, 10-hour dark cycle. Humidity was maintained at 55%, and temperatures at 21-23°C. Water was provided ab libitum, and feed was changed three times a week. Feed intake adjusted for spillage was recorded. Body weights were recorded once a week.

Table 1 – Experimental Design

Diet	*fa/fa* Rats[b]	Lean Rats[b]
c9,t11 CLA	10	10
t10,c12 CLA	10	10
TOG (c9,t10 and t10,c12 CLA)	10	10
BIO (CLA 2 isomer mixture)	10	10
NUCHEK (CLA 4 isomer mixture)	10	10
Control	10	10
Pair-Weighed[a]	10	10
TOTAL	**70**	**70**

[a] Rats were fed control diet in restricted amounts to maintain body weight equal to the CLA-group with the lowest body weight. This was done to ensure that body weight differences would not be responsible for changes in measured parameters.

[b] Number indicates how many animals per group.

Table 2 - Composition and Sources of CLA

Group	Amount (w/w) and Composition	Source
c9,t11 CLA[a]	0.4% c9,t11 CLA	Natural ASA, Hovdebygda, Norway
t10,c12 CLA[a]	0.4% t10,c12 CLA	Natural ASA, Hovdebygda, Norway
TOG[a]	0.4% c9,t11 CLA and 0.4% t10,c12 CLA	Natural ASA, Hovdebygda, Norway
BIO (CLA 2 isomer mixture)[a]	1.2% CLA mixture containing 0.4% c9,t11 CLA and 0.4% t10,c12 CLA	Bioriginals, Saskatoon, SK
NUCHEK (CLA 4 isomer mixture)	2.2% CLA mixture containing 0.4% c9,t11 CLA and 0.4% t10,c12 CLA)	Nuchek-Prep, Elysian, MN
Control[a]	8.5% Soy oil	Harland Teklad, Madison, WI
Pair-Weighed[a,b]	8.5% Soy oil	Harland Teklad, Madison, WI

[a] Fatty acid and CLA composition is presented in Table 4a and 4b.

[b] Fed control diet in restricted amounts to maintain body weight equal to the CLA–group with the lowest body weight within each genotype. This was done to ensure that body weight differences would not be responsible for changes in measured parameters.

Table 3 - Diet Formulation[a]

Ingredients	c9,t11 CLA	t10,c12 CLA	TOG	BIO	NUCHEK	CONTROL
Dry Mix						
Cornstarch	363	363	363	363	363	363
Maltodextrin	132	132	132	132	132	132
Sucrose	100	100	100	100	100	100
Egg White	212.5	212.5	212.5	212.5	212.5	212.5
Cellulose	50	50	50	50	50	50
Mineral Mix	35	35	35	35	35	35
Vitamin Mix	10	10	10	10	10	10
Choline	2.5	2.5	2.5	2.5	2.5	2.5
Biotin Mix[b]	10	10	10	10	10	10
TBH[c]	0.014	0.014	0.014	0.014	0.014	0.014
Oil[d]						
Soy oil	80.7	80.7	76.3	73	63	85
C9,t11 CLA	4.3	0	4.3	0	0	0
T10,c12 CLA	0	4.3	4.3	0	0	0
BIO	0	0	0	12	0	0
NUCHEK	0	0	0	0	22	0

[a] Number indicates how many grams/ kg of diet.

[b] Biotin mix contains 200 mg biotin/kg of cornstarch.

[c] TBH = Tert-butylhydroquinone

[d] Total oil in all diets= 85 grams/kg of diet.

Table 4a - Percentage of CLA in Each Oil

	c9,t11	t10,c12	BIO	NUCHEK
% CLA	96.0	99.0	73.2	87.3
% of other fatty acids	4.0	1.0	26.8	12.7

Table 4b - Fatty Acid Composition (%) of Each Oil

	c9,t11	t10,c12	BIO	NUCHEK
% c9,11	92.7	4.3	32.9	17.8
% t10,c12	0.6	92.3	33.2	19.5
% t8,c10	0	0	0	18.6
% c11,t13	0	0	0	18.7
Other CLA isomers[a]	2.7	2.4	7.1	12.7
Total CLA Isomers	**96**	**99**	**73.2**	**87.3**
18:1 c9(oleic)	4.0	0.8	12.6	N/D
18:2 c9,c12 (linoleic)	<0.1	<0.1	4.1	N/D
Other	0	<0.1	10.1	N/D
Total	**100**	**100**	**100**	**100**

[a] Actual composition has not been determined.

ND- Not Determined.

Table 5 - Total Amount of CLA in Each Diet (g/kg diet)

Diet	c9,t11 (g)	t10,c12 (g)	Other CLA Isomers (g)	Total CLA (g)
c9,t11 diet	4.00	0.03	0.12	4.15
t10,c12 diet	0.19	4.00	0.10	4.29
TOG	4.19	4.03	0.22	8.44
BIO	3.94	3.99	0.84	8.77
NUCHEK	3.91	4.28	2.81	11.00

2. Tissue Collection

Rats were euthanized according to the Canadian Council on Animal Care Guidelines (Olfert et al, 1993). At 8 weeks, rats were fasted overnight and then euthanized by carbon dioxide asphyxiation and weighed. Blood was collected via cervical dislocation and stored on ice until centrifuged to collect serum. Serum samples were frozen in aliquots at –80°C.

3. Serum Biochemistry

Radioimmunoassay (RIA's) for leptin (Linco ^{125}I Rat Leptin RIA Kit Cat# RL-83K), and adiponectin (Linco ^{125}I Rat Adiponectin RIA Kit Cat# MADP-60HK) were used. All assays were conducted with termination serum collected immediately after euthanization.

3a) Leptin Radioimmunoassay (RIA) Kit

Linco's Rat Leptin RIA Kit was used to quantify leptin in the serum of our animals. This kit used labeled tracer antigen with an anti-serum, and measured the amount of tracer bound to antibody. The amount of labeled leptin bound to the antibody is inversely proportional to the concentration of unlabeled leptin in the serum.

To determine the percentage of total binding of each sample, the following calculation was used:

$$\%\text{Bound/Total Bound} = \frac{(\text{Average Count} - \text{Non specific Binding Tube})}{\text{Total Bound Tube}} \times 100\%$$

Using the standard curve from the assay, individual leptin concentrations were determined using Graph Pad Prism 2.01 Software (Intuitive Software for Science, San Diego, CA) and multiplied by a dilution factor (if required). Serum from *fa/fa* rats was diluted 10x based on previous experiments.

3b) Adiponectin Radioimmunoassay (RIA) Kit

At the time of testing, there was no adiponectin kit made specifically for the rat species. Thus, the mouse kit was used as it has similar specificity. This kit identified full-length adiponectin only.

The basic principles of procedure are the same as per the rat leptin RIA kit. Serum from *fa/fa* and lean rats was diluted 500x based on previous experiments.

Porcine Smooth Muscle Cell Cultures

4. Cell Culture

SMCs extracted from porcine coronary arteries were used. Porcine hearts were obtained from a local abattoir immediately after being removed from the animal and stored on ice. The left descending coronary artery was dissected and cleaned with SMC-Phosphate Buffered Saline (SMC PBS), containing 10% antibiotic-antimycotic (Invitrogen Corporation).

The coronary artery was cut into sections 2-5 mm thick and placed into a single 100-mm dish containing Dulbecco's Modified Eagles Medium (DMEM; Gibco/BRL) with 20% fetal bovine serum (FBS, Gibco/BRL) and 2 mM glutamine.

After 7-14 days of migration, SMCs were present (Saward & Zahradka, 1997). Cells used for thymidine or leucine incorporation studies were cultured in 24-well plates, while those used for Western blotting were set up in 12-well plates. When 75% confluent, growth medium was replaced with serum-free media (0.25 mL pyruvate/transferrin/selenium/abscorbic acid (PTSA), 0.25 mL antibiotic-antimycotic (AB/AM) and 24.5 mL leucine-free media per 24-well plate) for 4-5 days.

Various agonists/antagonists (*Table 6*) were added to both quiescent and stimulated SMCs for various time periods (15 minutes-72 hours) and used for ^{3}H-thymidine incorporation, ^{3}H-leucine incorporation or Western blots.

Table 6 - Treatments added to Smooth Muscle Cell Cultures

Treatment	Concentration	Source	Product #	Reconstituted in
AICAR	1 mM	Calbiochem	123040	Water
Adiponectin (mouse) (globular)	1.5 µg/mL	Alexis Biochemicals	ALX-201-306	Water
Adiponectin (recombinant, mouse) (full-length)	1ug/uL	Alexis Biochemicals	ALX-522-059-0000	Water
c9,t11 CLA	10,25,60,100, 120,240 uM	Cayman Chemical Company	90140 Lot#22561-106195	Ethanol
c9,t11 CLA and t10,c12 CLA	30 uM c9,t11 30 uM t10,c12	Cayman Chemical Company	90140 Lot#22561-106195 90145 Lot#22250-105524	Ethanol
Clofibrate	250 uM	Calbiochem	231405 Cat# 637-07-0	Ethanol
GW501516	10 uM	Calbiochem	370710	DMSO
GW9662	10 uM	TOCRIS	1508 CAS # 22978-25-2	Ethanol
L165041	1uM	TOCRIS	Cat# 1856	Sodium Hydroxide (100 mM)
Pioglitazone	20 uM			DMSO
Platelet-Derived Growth Factor-BB (Human)	0.1ug/mL	PeproTech Incorporated	100-14B Lot#020304-A	Water
Leptin (human, recombinant)	0.1ug/mL	Peprotech Canada, Inc	300-27 Lot#11890	Water
Rosiglitazone	1,4,10 uM	Smith Kline Beecham	BRL#49653-C	DMSO
t10,c12 CLA	10,25,60,100, 120,240 uM	Cayman Chemical Company	90145 Lot#22250-105524	Ethanol
WY14643	20, 100, 250 uM	Cayman Chemicals	70730	DMSO

4a) Preparation of Protein Samples

Media was removed from SMCs, and washed with PBS. Two-times sample buffer (200 µL) was added to each well for 5 minutes, and the liquid was then transferred to individual microfuge tubes and stored at –20°C until needed for Western blotting.

4b) Membrane Preparation

SDS/Polyacrylamide gels (10%) were prepared according to standard protocols (Yau et al, 1999). Protein assays were conducted on all samples to determine the volume required to load 5 mg of protein into each well.

Bromophenol blue (1 µL of 10% stock solution) and β-mercaptoethanol (1 µL) were added to each sample, and boiled for 10 minutes to denature protein. Samples and molecular mass markers were loaded onto the gel, and electrophoresis was conducted at 20 mA per gel for approximately 90 minutes.

The protein was then transferred from the gel to a polyvinylidene difluoride (PVDF) membrane and ran at 100 volts for 70 minutes (1.5 mm wells) or 60 minutes (1.0 mm wells). PVDF membranes were stored in 1X Tris-buffered saline with Tween-20 (TBST) at 4°C until use.

4c) Western Blotting

Membranes were blocked with 3% BSA (Bovine Serum Albumin) in TBST for one hour. Specific primary antibodies were subsequently added at varying concentrations (*Table 7*) and the membrane was incubated for one hour. Membranes were then washed with TBST, and secondary antibody (diluted 1:10,000) was added with 1% BSA in TBST for an additional 1 hour, followed by a final wash in TBST. The membranes were agitated in ECL Western Blotting Detection Reagents (Amersham Biosciences), or ECL plus Western Blotting Detection System (Amersham Biosciences) luminescent reagent and the relative chemiluminescence for each sample was documented by autoradiography.

Relative intensity was quantified with a GS-800 Calibrated Densitometer and Quantity One software (Bio-Rad Laboratories, 1-D Analysis Service).

Table 7 - Antibodies used in Western Blotting

Antibody	Actual Name	Source	Clone #	Source	Dilution	Mol Wt (kDa)
Adiponectin	Anti-Acrp30	Calbiochem	Cat#107920, Lot#D19586	Rabbit	1:1000	30
Akt	Akt	Cell Signaling	9272	Rabbit	1:1000	60
AMPKα	AMP-activated protein kinase alpha	Cell Signaling	2532	Rabbit	1:1000	62
MAPK p44/42	p44/42 MAP Kinase	Cell Signaling	9102	Rabbit	1:1000	42,44
pAkt	Phospho-Akt (Ser473)	Cell Signaling	9271	Rabbit	1:1000	60
p-AMPKα	Phospho-AMPK-α (Thr172)	Cell Signaling	2531	Rabbit	1:1000	62
pMAPK p44/42	Phospho-p44/42 MAP Kinase (Thr202/Tyr204)	Cell Signaling	9101	Rabbit	1:1000	42, 44
PPARα	Peroxisome Proliferator Activated Receptor - alpha	Cedarlane Laboratories Limited	Cat#MA1-822	Mouse	1:1000	52
PPARβ	Peroxisome Proliferator Activated Receptor - beta/delta	Cedarlanes Laboratories Limited	Cat#PA1-823	Rabbit	1:2000	49
PPARγ	Peroxisome Proliferator Activated Receptor - gamma	Cedarlane Laboratories Limited	Cat#PA3-821	Rabbit	1:1000	55

4d) Thymidine Incorporation

^{3}H-thymidine incorporation was used to assess DNA synthesis in SMC. ^{3}H-thymidine (Perkin Elmer Life Sciences) was added to SMC culture media and allowed to incorporate into newly synthesized DNA.

Quiescent SMCs were pre-treated with various agonists (in triplicate) for 10 minutes, and then stimulated with PDGF (*Table 6*). The cells were incubated for 24 hours, after which ^{3}H-thymidine (10 µL of 1 mCi/mL) was added, and incubation was continued for an additional 68-72 hours. Media was then removed and replaced with 1 mL cell lysis buffer (10 mM Tris/HCL pH 7.4, 100 mM NaCl, 1mM EDTA, 0.5% SDS) for 24 hours. DNA was precipitated by adding 1 mL of cold 20% trichloroacetic acid (TCA), collected on GF/A filters, and washed 3 times with 5% TCA. Once dried, the filters were put into scintillation vials with CytoScint scintillation fluid. Incorporation of ^{3}H-thymidine was quantified with a Beckman ls6500 Multi-Purpose Scintillation Counter.

4e) Leucine Incorporation

^{3}H-Leucine incorporation was used to assess protein synthesis in SMC. ^{3}H-Leucine (Perkin Elmer Life Sciences) was added to SMC culture media and allowed to incorporate into newly synthesized proteins.

Quiescent SMCs were placed into a leucine-free medium for 24 hours.

After 24 hours, cells were pre-treated with various inhibitors (*Table 6*) for 1 hour, after which PDGF was added. After 10 minutes ^{3}H-Leucine (10 µL of 1 mCi/mL) was added and incubation was continued for 5 hours.

After the incubation period, cells were rinsed with PBS, and 1 mL cold 10% TCA was added per well. The plate was left on ice for 15 minutes, rinsed with TCA, and iced for another 15 minutes. TCA was then removed and 0.5 mL of lysis buffer (0.5 M NaOH in 0.1% Triton X-100) was added to each well (for 15-20 hours). The lysis buffer was then transferred to scintillation vials along with 3 mL EcoLume and quantified with a Beckman ls6500 Multi-Purpose Scintillation Counter.

5) Statistical Analysis

Animal Study

All statistical analysis was carried out using SAS (The SAS System 8e for Windows, SAS Institute Inc., Cary, NC: USA). Results were compiled and analyzed using two-way ANOVA (Analysis of Variance) for main effects (genotype, diet, and genotype x diet interactions). Individual groups were compared using Duncan's Multiple Range or Contrasts for specific preplanned comparisons. A significant difference was accepted at a p-value <0.05. All values were reported as means $\pm$ standard error of the mean (SEM).

Cell Culture

All statistical analysis was carried out using ORIGIN Data Analysis & Technical Software (Microcal Software Incorporated, copyright 1991-1999). Student t-tests were conducted among means of data to detect significant differences. Values were reported as mean $\pm$ SEM.

PART 4 - RESULTS

1. Animal Study

1a) Serum Adiponectin

Serum adiponectin was analyzed two ways. Primarily, it was taken as total µg/mL of serum. This showed that lean animals had less serum adiponectin (4.47 ± 0.14 µg/mL) compared to *fa/fa* rats (6.15 ± 0.19 µg/mL) (*Figure 1).* Further, diet effects were seen with rats fed the t10,c12 isomers having higher serum adiponectin concentrations (5.82 ± 0.35 µg/mL) compared to both the c9,t11 isomers (4.87 ± 0.25 µg/mL) and control (4.69 ± 0.26 µg/mL). There were no significant differences among the other diet groups (*Figure 2).* There was no significant diet x genotype interaction.

Due to the surprising finding that lean animals had less serum adiponectin levels compared to *fa/fa* animals, serum adiponectin was determined as a ratio of serum adiponectin/grams of adipose tissue (sum of epididymal and peri-renal fat pads). This accounted for the drastic differences in specific fat-pad weights, and allowed us to assess total serum adiponectin per gram of fat pad weight.

Lean Zucker rats had significantly higher serum adiponectin levels (0.47 ± 0.02 µg/mL/g) compared to *fa/fa* Zucker rats (0.14 ± 0.01 µg/mL/g) based on fat pad

weight (*Figure 3)*. This equates to a 3.4-fold higher serum adiponectin (based on adiposities) in lean rats compared to *fa/fa* rats.

As discussed previously, these results correspond to previous data indicating that lean animals/humans have higher serum adiponectin levels.

Taking diet groups together, there were no differences in serum adiponectin (per gram of adipose tissue (*Figure 4)*. There were no significant diet x genotype interactions.

Figure 1 - Serum Adiponectin in Lean and *fa/fa* Zucker Rats

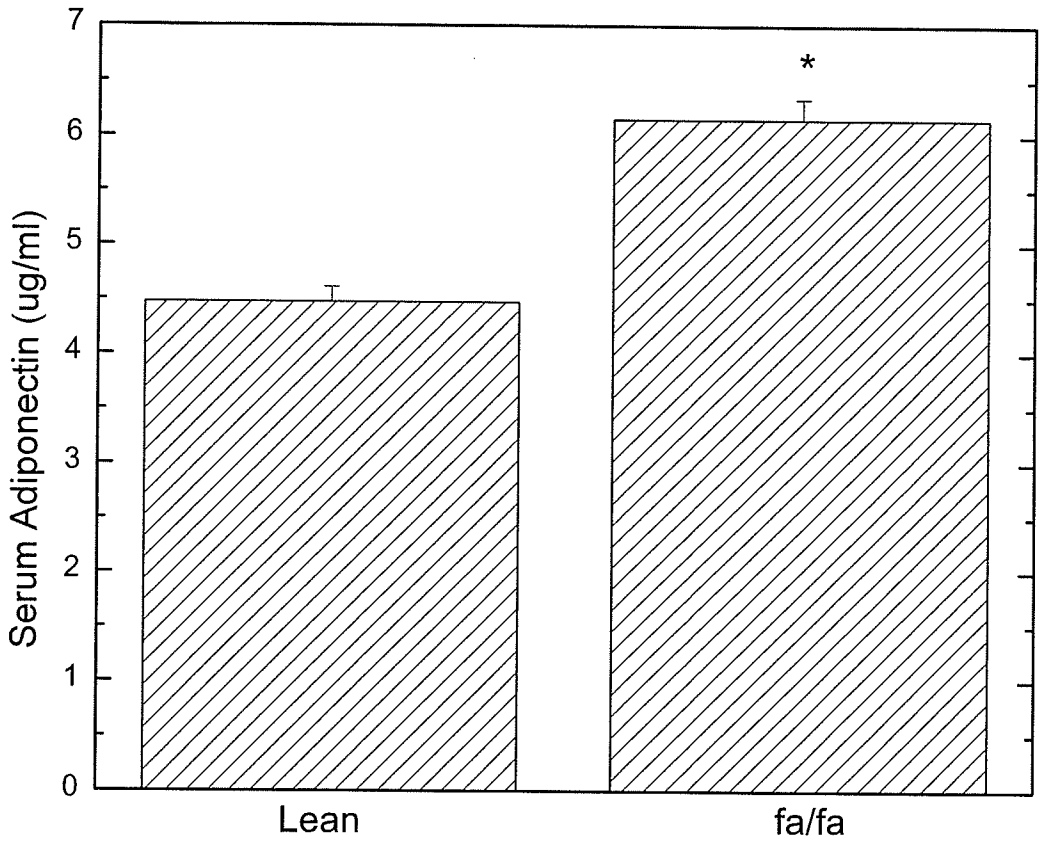


Figure 1 – Changes in serum adiponectin in Zucker rats. The *fa/fa* rats had significantly higher serum adiponectin concentrations compared to lean rats. Data are expressed as mean ± SEM (n=59 per genotype).

* Significantly different from lean at $p<0.05$.

Figure 2 - Serum Adiponectin by Diet Group

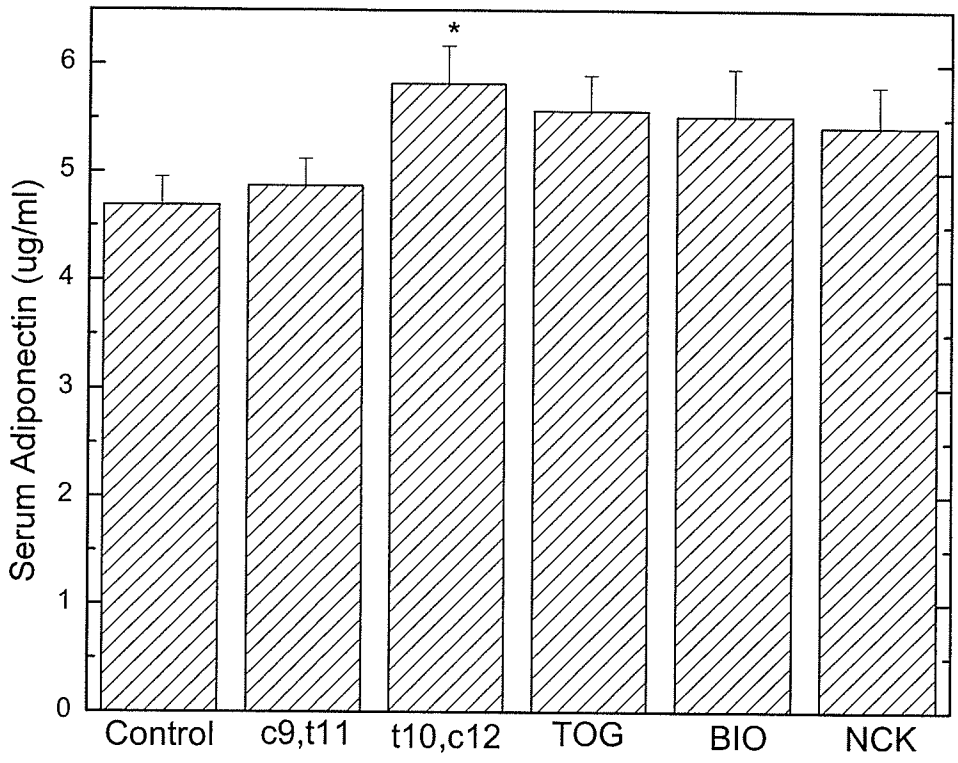


Figure 2- The effect of Control, *cis*-9, *trans*-11 (c9,t11 CLA), trans-10, cis-12 CLA (t10,c12 CLA), TOG, BIO and NCK diets on serum adiponectin concentrations in Zucker rats. Data are expressed as mean ± SEM per diet (n=19-20).

* Significantly different from control and c9,t11 CLA.

Figure 3 - Serum Adiponectin (per gram of adipose tissue) in Lean and *fa/fa* Zucker Rats

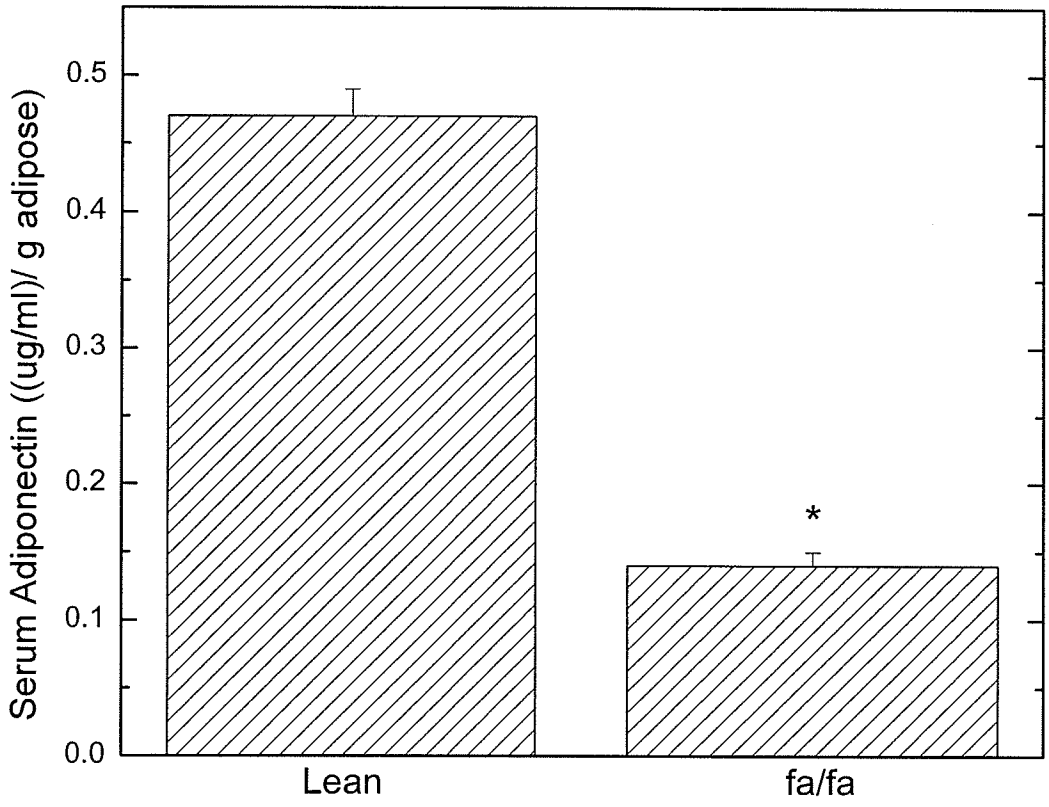


Figure 3 – Changes in serum adiponectin in Zucker rats. The *fa/*fa rats had significantly lower serum adiponectin concentrations compared to lean rats. Data are expressed as mean ± SEM (n=59 per genotype).

* Significantly different from lean at $p<0.05$.

Figure 4 - Serum Adiponectin (per gram of adipose tissue) by Diet Group

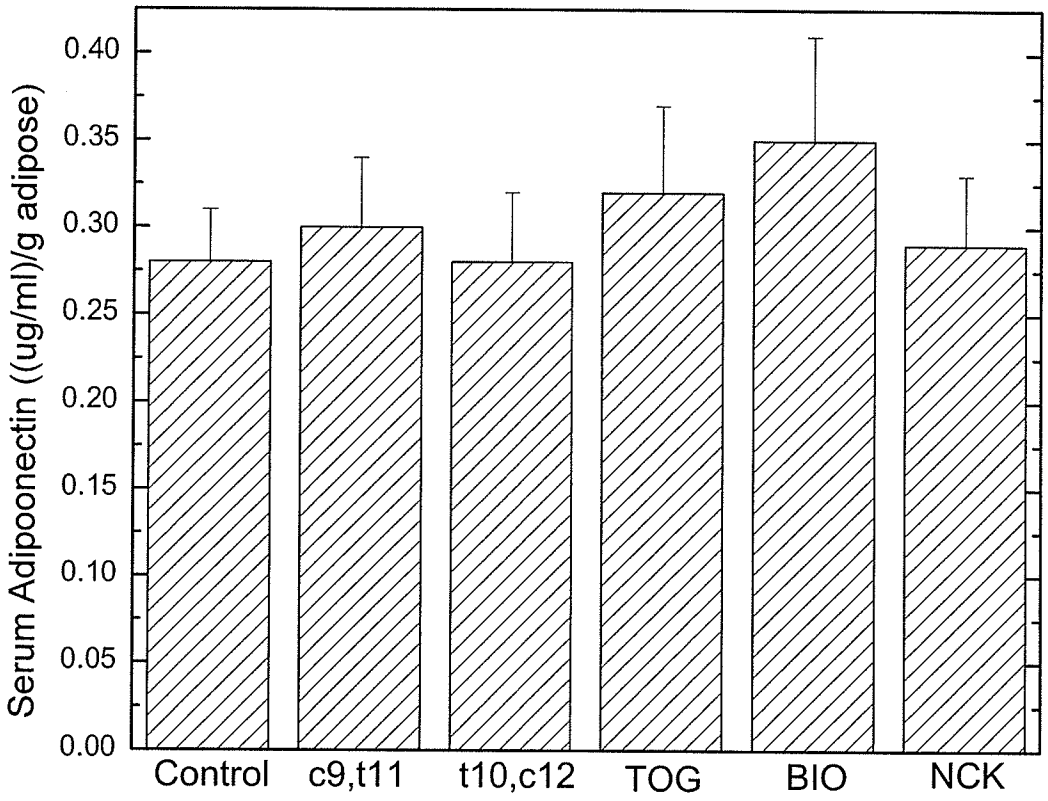


Figure 4 - The effect of Control, *cis*-9, *trans*-11 (c9,t11 CLA), trans-10, cis-12 CLA (t10,c12 CLA), TOG, BIO and NCK diets on serum adiponectin levels in Zucker rats. Data are expressed as mean ± SEM (n=19-20 per diet). There were no significant differences among dietary groups.

1b) Serum Leptin

Lean Zucker rats had 26-fold lower serum leptin concentrations (4.3 $\pm$ 0.3 ng/mL) compared to *fa/fa* Zucker rats (112.60 $\pm$ 2.5 ng/mL) (*Figure 5)*.

There was no significant main effect of diet *(Figure 6)*. Genotype x diet interactions did occur, with t10,c12 CLA, TOG, BIO and NCK being significantly lower than control in the *fa/fa* group (*Figure 7)*. In the *fa/f*a rats, the group fed the c9,t11 CLA isomer had significantly higher serum leptin than all other CLA groups and was equivalent to the CTRL group.

Figure 5 - Serum Leptin in Lean and *fa/fa* Zucker Rats

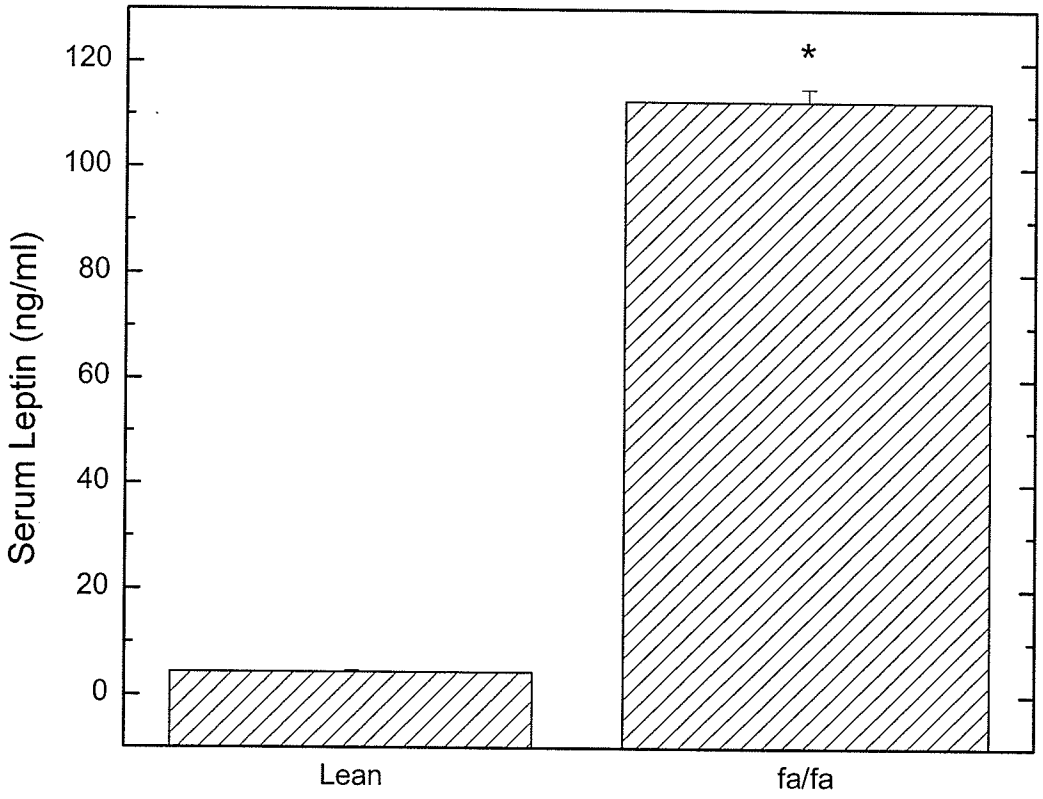


Figure 5 – Changes in serum leptin in lean Zucker rats compared to *fa/*fa Zucker rats. *Fa/*fa had significantly higher serum leptin concentrations compared to lean rats. Data are expressed as mean ± SEM per genotype (n=60).

* Significantly different from lean at $p<0.05$.

Figure 6 – Serum Leptin by Diet Group

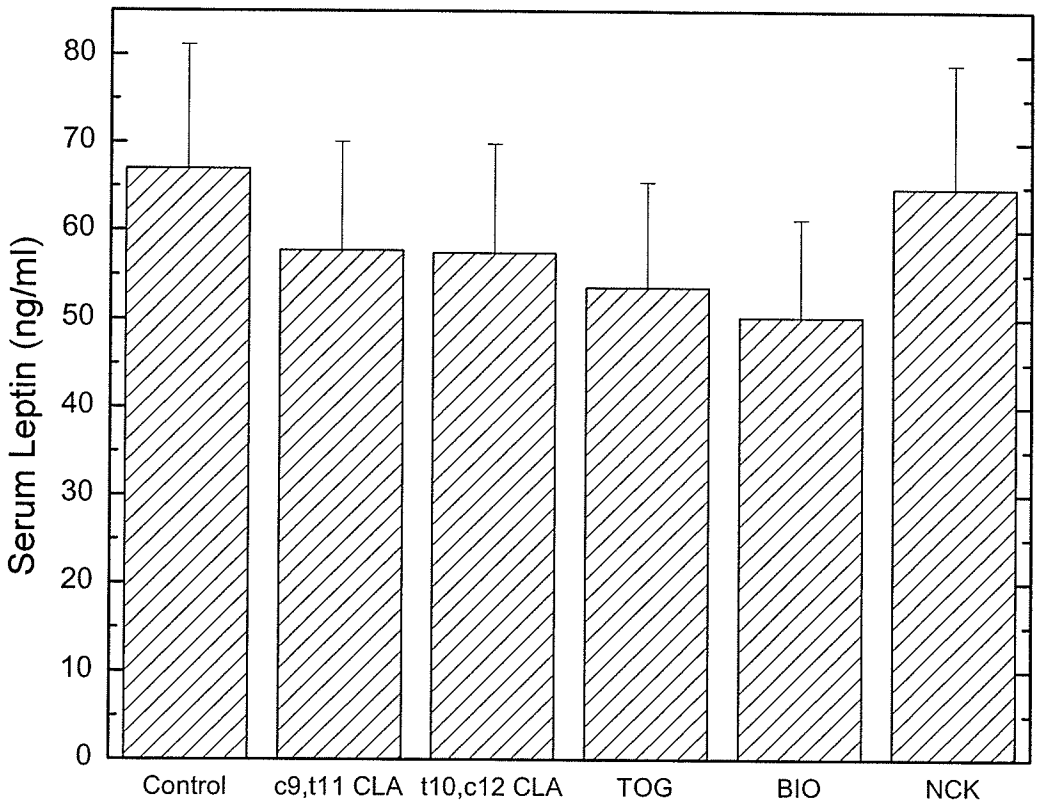


Figure 6 -The effect of Control, *cis*-9, *trans*-11 (c9,t11 CLA), trans-10, cis-12 CLA (t10,c12 CLA), TOG, BIO and NCK diets on serum leptin concentrations. Data are expressed as mean ± SEM (n=20 per diet). There were no significant effects on leptin concentrations when considering diet alone.

Figure 7 - Serum Leptin in Lean and *fa/fa* Zucker Rats by Diet Group

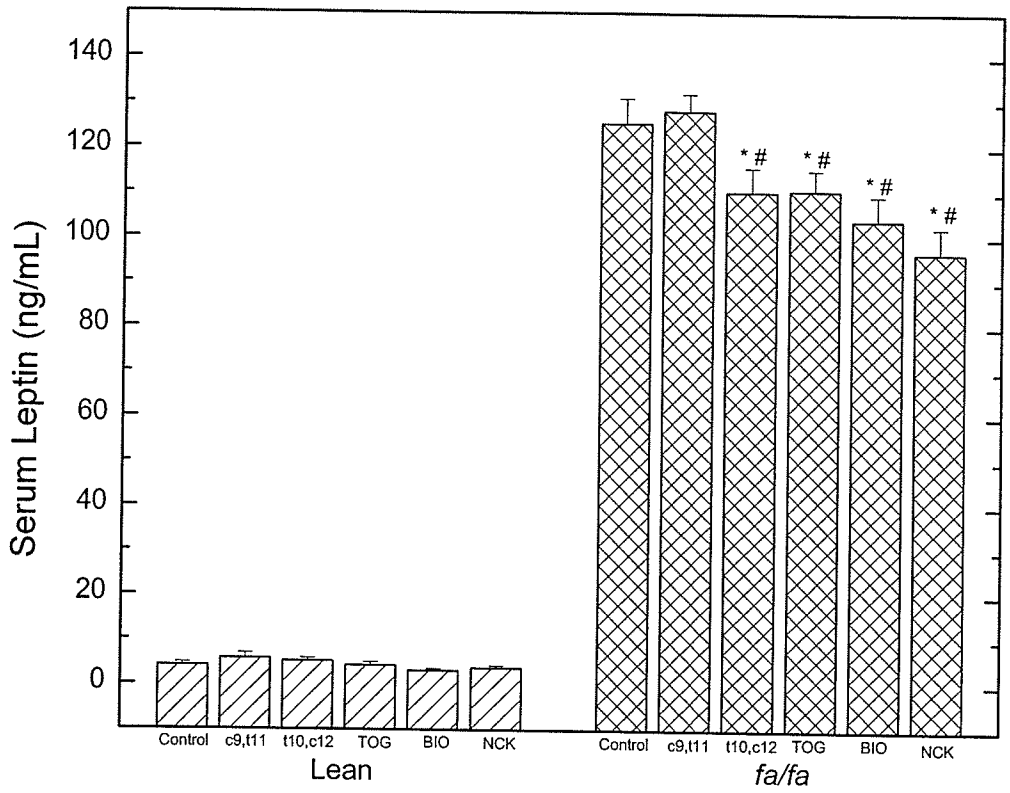


Figure 7 - The effect of Control, *cis*-9, *trans*-11 (c9,t11 CLA), trans-10, cis-12 CLA (t10,c12 CLA), TOG, BIO and NCK diets on serum leptin concentrations in lean and *fa/fa* Zucker rats. Data are expressed as mean ± SEM (n=10). Diet did not significantly affect leptin concentrations in lean animals. The t10,c12, TOG, BIO, and NCK were all significantly different from control and c9,t11 CLA.

* Significantly different from *fa/fa* control at $p<0.05$.
# Significantly different from c9,t11 CLA at $p<0.05$.

2. Porcine Smooth Muscle Cell Culture

2a) Adiponectin and AICAR

While adiponectin is fairly new, it has garnered significant interest in the scientific literature. Adiponectin has been detected in the muscle, liver, and pancreas (Wang et al, 2004), and has also been shown to influence different cell types including endothelial cells (Chen et al, 2003). Recent work in our laboratory determined that adiponectin receptors are also present in porcine SMCs (unpublished data, Natasha Yurkova), consequently it is likely that adiponectin will influence SMCs.

^{3}H-Leucine

^{3}H-Leucine incorporation was used to assess protein synthesis in SMCs after treatment with adiponectin. ^{3}H-Leucine was added to SMC culture media and allowed to incorporate into newly synthesized proteins (as described in Methods).

PDGF increased protein synthesis. PDGF is a known-mitogen, thus, the activation of processes such as protein synthesis is expected. Compared to control, adiponectin increased protein synthesis in quiescent SMCs (*Figure 8).* When adiponectin was added to PDGF-stimulated SMCs, there was no change in protein synthesis (*Figure 8).* Thus, it was concluded that adiponectin stimulates protein synthesis in quiescent SMCs, but does not affect PDGF-stimulated cells.

^{3}H-Thymidine

^{3}H-Thymidine incorporation was used to assess DNA synthesis in SMCs, since SMC proliferation is a central feature of vascular disease.

PDGF increased DNA synthesis, as expected. In quiescent SMCs, adiponectin also increased DNA synthesis (*Figure 9*). Adiponectin did not reduce SMC proliferation in PDGF-stimulated cells.

This suggests that in healthy cells, adiponectin acts as a mitogen, stimulating cell division and growth. However, in proliferative SMCs, adiponectin has no effect. Thus, adiponectin is a mitogen and does not exhibit anti-proliferative properties in vascular SMCs.

AMPK

To investigate the possible pathway(s) by which adiponectin operates as a mitogen, changes in AMPK levels and phosphorylation state were assessed.

Total AMPK levels represent the total amount of AMPK in SMCs available for activation. AMPK levels were standardized using a ratio of AMPK/MAPKp44. MAPKp44 was used to standardize and control for loading variabilities.

Western blots for total amounts of AMPK were conducted on samples prepared from SMCs, which had been treated with adiponectin overnight. Results show

that adiponectin did not significantly change levels of AMPK over this time period (*Figure 10*). Treatment with either PDGF or AICAR also did not significantly influence the levels of AMPK compared to control.

Western blots examining the phosphorylation state of AMPK were conducted on extracts prepared from cells that had been treated with adiponectin for 15 minutes, as changes in phosphorylation typically occur within 5-30 minutes. MAPKp44 was used as a control to correct for loading variability.

Adiponectin stimulated AMPK phosphorylation, which is correlated to the activation of AMPK (*Figure 11*). AICAR, which is a potent AMPK activator, did not significantly increase pAMPK. A strong trend was seen in AICAR's ability to increase pAMPK. While it was not found to be significant in these groups of cells, it is generally assumed that given identical cells, a significant increase would have been seen.

Akt

To examine the mechanism by which adiponectin causes an increase in pAMPK and promotes SMC proliferation, pAkt (Ser473) was measured by Western blotting. pAkt promotes cell survival and growth, and is located downstream in the PI3-kinase pathway.

While not significant, adiponectin and PDGF both increase the phosphorylation of Akt (*Figure 12*). Based on all available data, adiponectin consistently increased pAkt levels. Due to the great variances in each gel, this was not shown to be statistically significant. However, visually, it appears that adiponectin does have the ability to increase pAkt levels. This suggests that adiponectin-dependent increases in pAMPK, may be linked to increased pAkt, resulting in an increase in SMC proliferation in healthy cells.

These data suggest that adiponectin increases SMC proliferation by the following possible pathways:

Adiponectin→ pAMPK→ pAkt→ increased SMC proliferation

Or

Adiponectin→ pAkt→ pAMPK→ increased SMC proliferation

To determine which scheme was correct, AICAR was also tested.

AICAR

AICAR is a potent activator of AMPK (Buhl et al, 2002; Wu et al, 2003). As shown in the above figures, AICAR did not change overall amounts of AMPK, but did increase the phosphorylation of AMPK (*Figure 10, Figure 11*).

However, AICAR did not increase the phosphorylation of Akt (*Figure 12*).

Thus,

AICAR→ pAMPK→ X pAkt→ increased SMC proliferation

Adiponectin→ pAkt→ X pAMPK→ increased SMC proliferation

Although both AICAR and adiponectin appear to have similar effects on pAMPK and on SMC proliferation, they do not both activate Akt. It was concluded that pAkt (a measure of P13-kinase) is not the mechanism through which adiponectin increased SMC proliferation.

Figure 8 – Effect of Adiponectin on Protein Synthesis in Porcine Smooth Muscle Cells

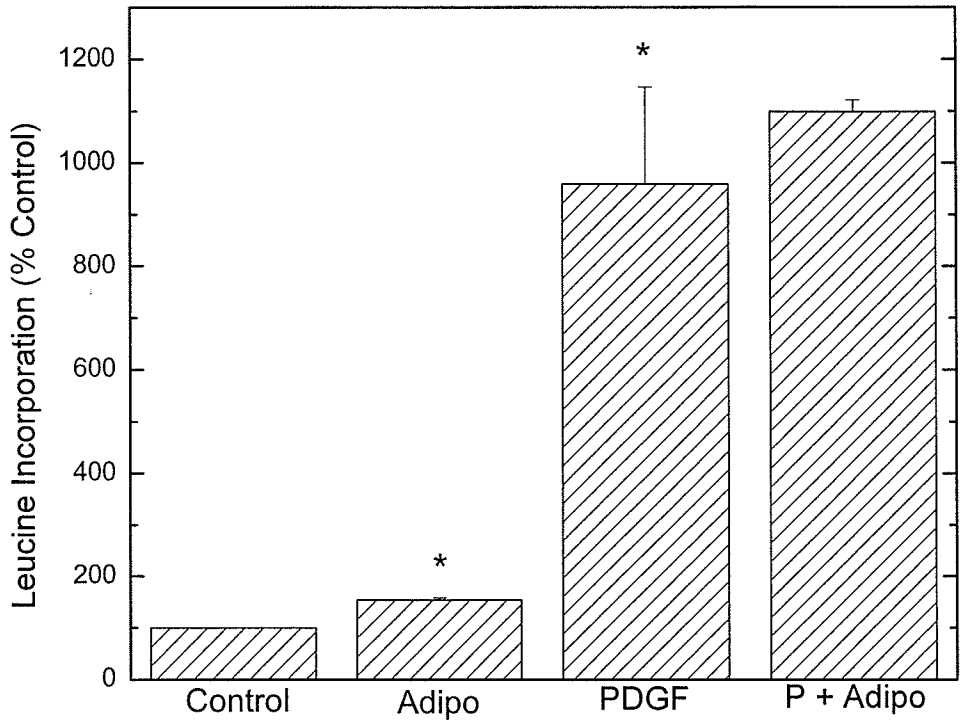


Figure 8 - The effect of 10 ug/mL adiponectin (Adipo) on ^{3}H-Leucine incorporation was compared to control and to the mitogenic effects of 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. Adipo significantly increased protein synthesis in quiescent cells, but had no effect on PDGF-stimulated cells (P+ Adipo= PDGF + Adiponectin).

* Significant differences from control at $p<0.05$.

Figure 9 – Effect of Adiponectin on Smooth Muscle Cell Proliferation

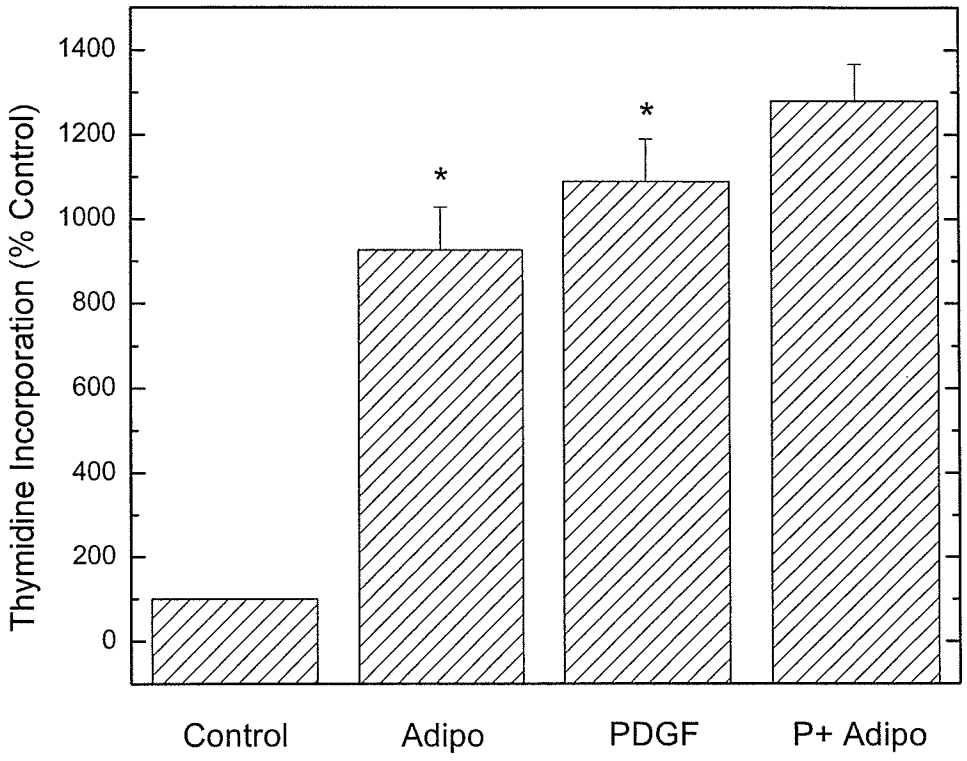
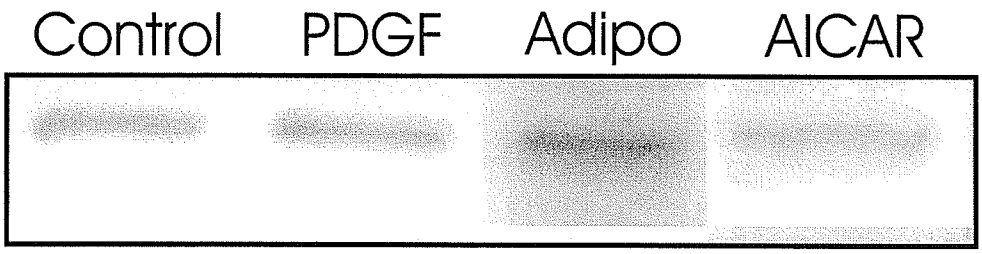


Figure 9 - The effect of 10 µg/mL adiponectin (Adipo) on ^{3}H-Thymidine incorporation was compared to control and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. Adipo significantly increased DNA proliferation in quiescent cells, but had no effect on PDGF-stimulated cells (P+ Adipo = PDGF+ Adiponectin).

* Statistically significant differences from control (unstimulated) at $p<0.05$.

Figure 10 – Effect of Adiponectin and AICAR on Total AMPK Levels in Porcine Smooth Muscle Cells



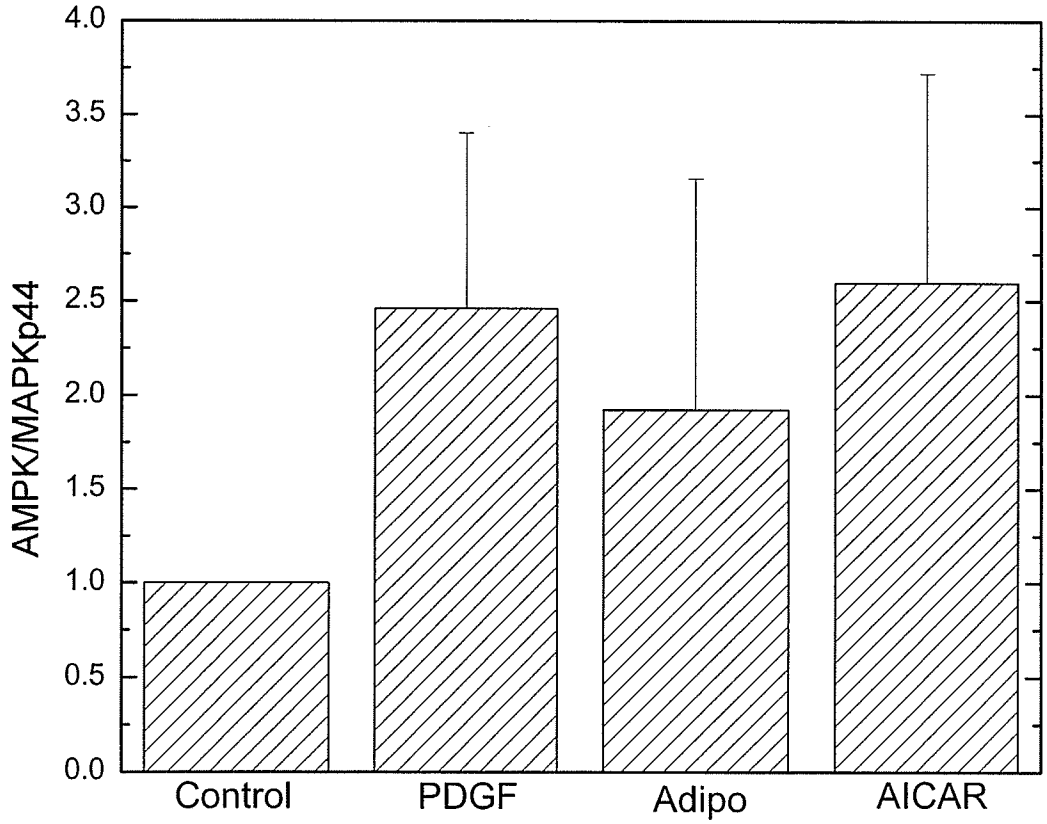


Figure 10 - The effect of 10 µg/mL adiponectin (Adipo) and 1 mM AICAR. AMPK levels were compared to control and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. Neither of the agonists (PDGF, Adipo, AICAR) had a significant effect on total AMPK levels.

Figure 11 – Effect of Adiponectin on the Phosphorylation of AMPK in Porcine Smooth Muscle Cells

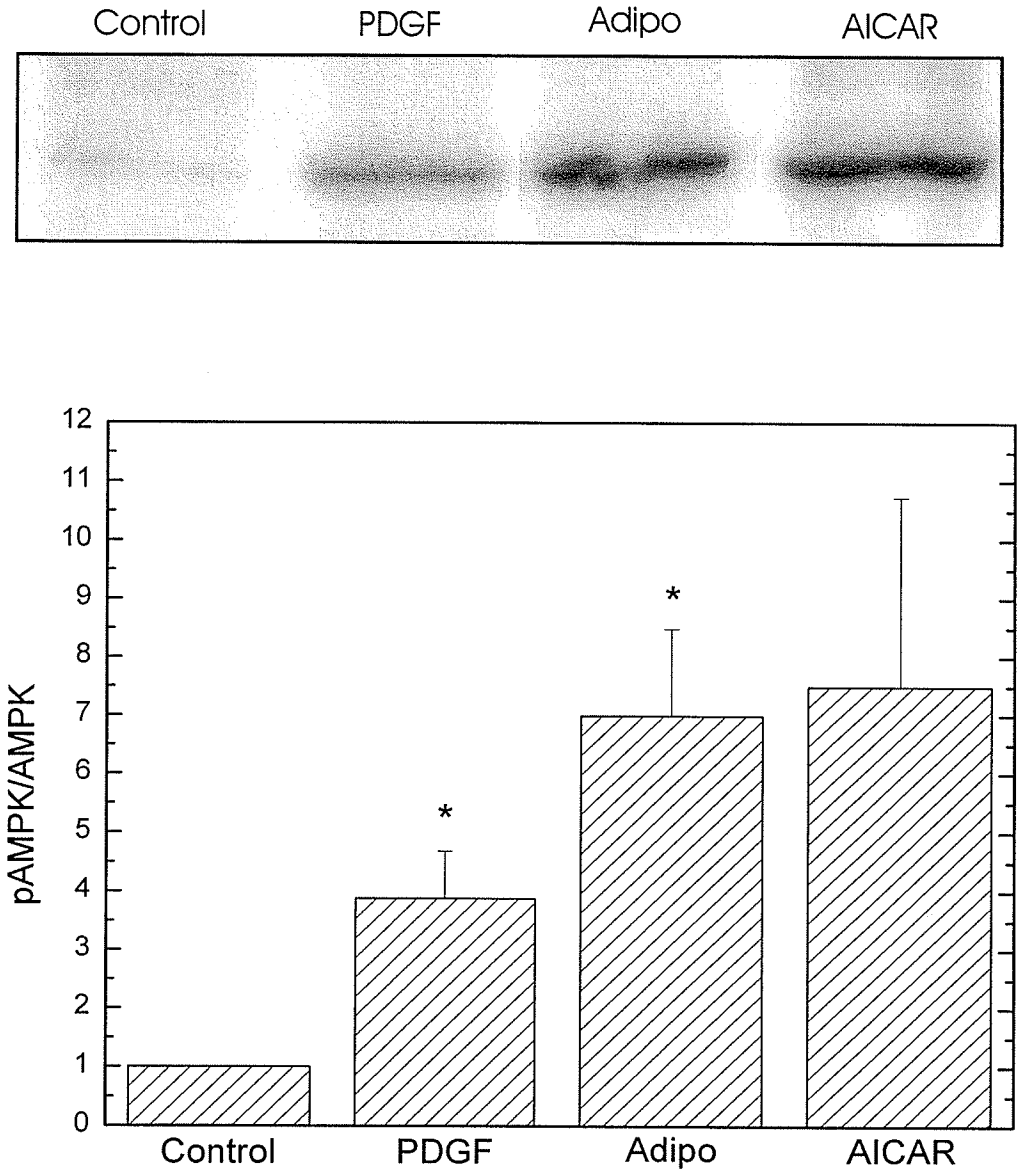
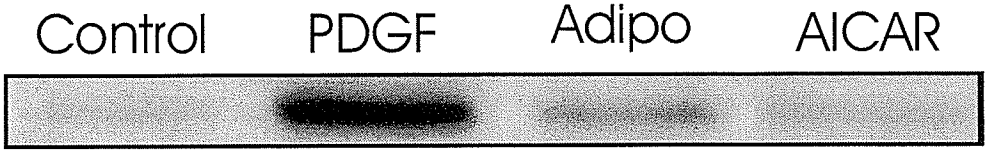


Figure 11 - The effect of 10 µg/mL adiponectin (Adipo) and 1 mM AICAR on pAMPK. Activation of AMPK was compared to control and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. PDGF and Adipo significantly increased pAMPK in SMCs. AICAR also appears to increase activation of AMPK, although there was too much intercellular variability for this to be significant.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

Figure 12 – Effect of Adiponectin on the Phosphorylation of Akt in Porcine Smooth Muscle Cells



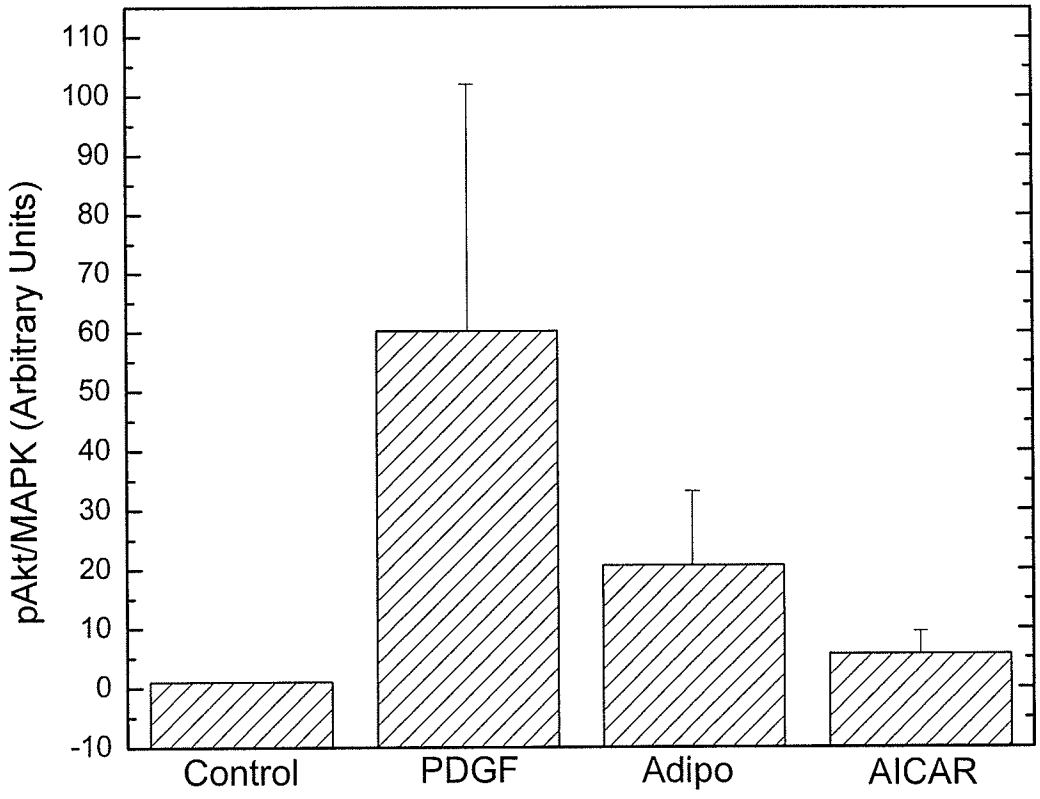


Figure 12 - The effect of 10 µg/mL adiponectin (Adipo) and 1 mM AICAR on pAkt was compared to control and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. Both adipo and PDGF increased pAkt, although due to the high variability of individual cells, significant differences were not found.

2b) Leptin Results

Leptin is an important hormone as it affects both storage and metabolism of fatty acids (Atkinson et al, 2002). This effect of leptin has been seen in the liver, skeletal muscle, pancreas and the heart, however, its effects in SMCs have not been fully determined. Thus, both protein synthesis and SMC proliferation were investigated.

^{3}H-Leucine

^{3}H-Leucine incorporation was used to assess protein synthesis in SMCs after treatment with leptin. ^{3}H-Leucine was added to SMC culture media and allowed to incorporate into newly synthesized proteins (as described in Methods).

Compared to control, leptin significantly increased protein synthesis in quiescent SMCs (Figure 13).

PDGF increased protein synthesis. PDGF is a known-mitogen, thus, an increase in protein synthesis is expected. When leptin was added to PDGF-stimulated SMCs, no significant changes in protein synthesis were seen (*Figure 14*). Thus, it is concluded that leptin influences protein synthesis only in quiescent cells.

^{3}H-Thymidine

^{3}H-Thymidine incorporation was used to assess DNA synthesis in SMCs, since DNA synthesis increases when SMC proliferate. PDGF increased DNA

synthesis, as expected. Leptin did not affect SMCs proliferation in quiescent or proliferating SMCs (*Figure 15*).

AMPK

Total AMPK levels represent the total amount of AMPK in SMC available for activation.

AMPK levels were standardized using a ratio of AMPK/MAPKp44, with MAPKp44 used to control for loading variation.

Western blots for total amounts of AMPK were conducted on samples prepared from SMCs that had been treated with leptin overnight. Results show that leptin did not significantly change levels of AMPK over this time period (*Figure 16*). Treatment with PDGF also did not significantly influence the levels of AMPK compared to control.

Western blots for phosphorylation of AMPK were conducted on gels that had been treated with leptin for 15 minutes. MAPKp44 was used as a control to correct for loading variability.

Leptin stimulated AMPK phosphorylation (*Figure 17*). This indicates that leptin activates AMPK in SMCs.

Akt

To examine the mechanism by which leptin causes an increase in pAMPK, pAkt (Ser473) was measured by Western blotting. pAkt promotes cell survival and growth, and works downstream of PI3K.

Results show that both leptin and PDGF increase the phosphorylation of Akt (*Figure 18*). AICAR also appears to increase pAkt, although this was not found to be significant. At first glance, AICAR's effects on pAkt do not appear to correlate with changes seen prior (*Figure 12)*. However, both experiments did show that AICAR does not significantly alter pAkt levels.

Figure 13 – Effect of Leptin on Protein Synthesis in Porcine Smooth Muscle Cells

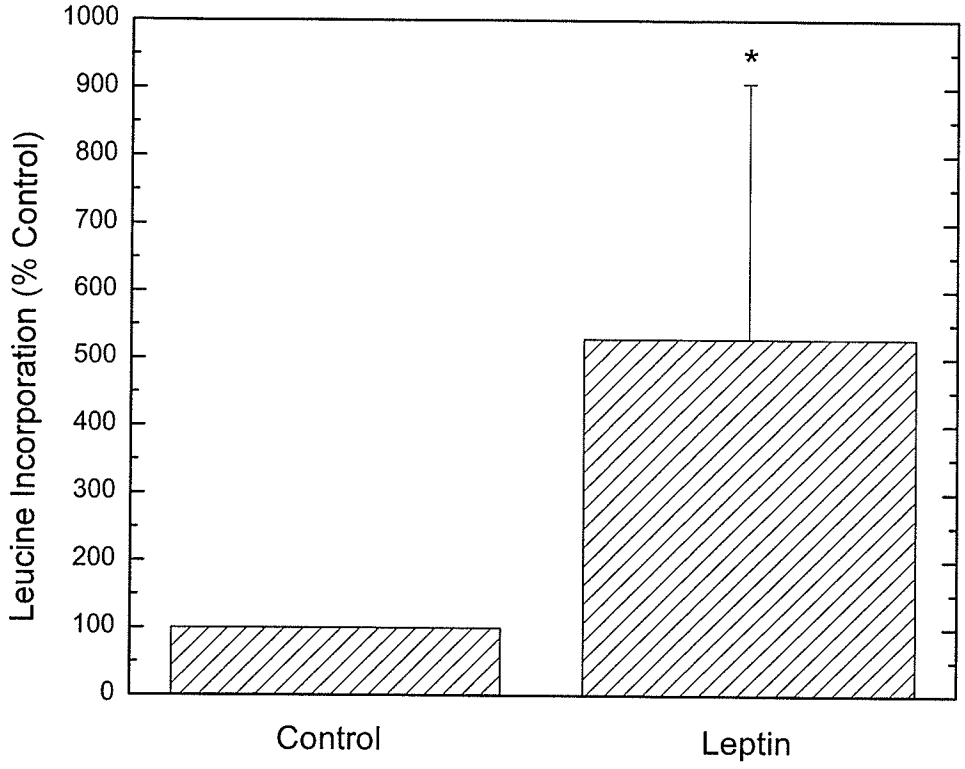


Figure 13 - The effect of 0.1µg/mL leptin on ^{3}H-Leucine incorporation was compared to control. Data are expressed as mean ± SEM of samples in triplicate. Leptin significantly increased protein synthesis in quiescent cells.

* Statistically significant differences from control at $p<0.05$.

Figure 14 – Effect of Leptin on Protein Synthesis in PDGF-Stimulated Porcine Smooth Muscle Cells

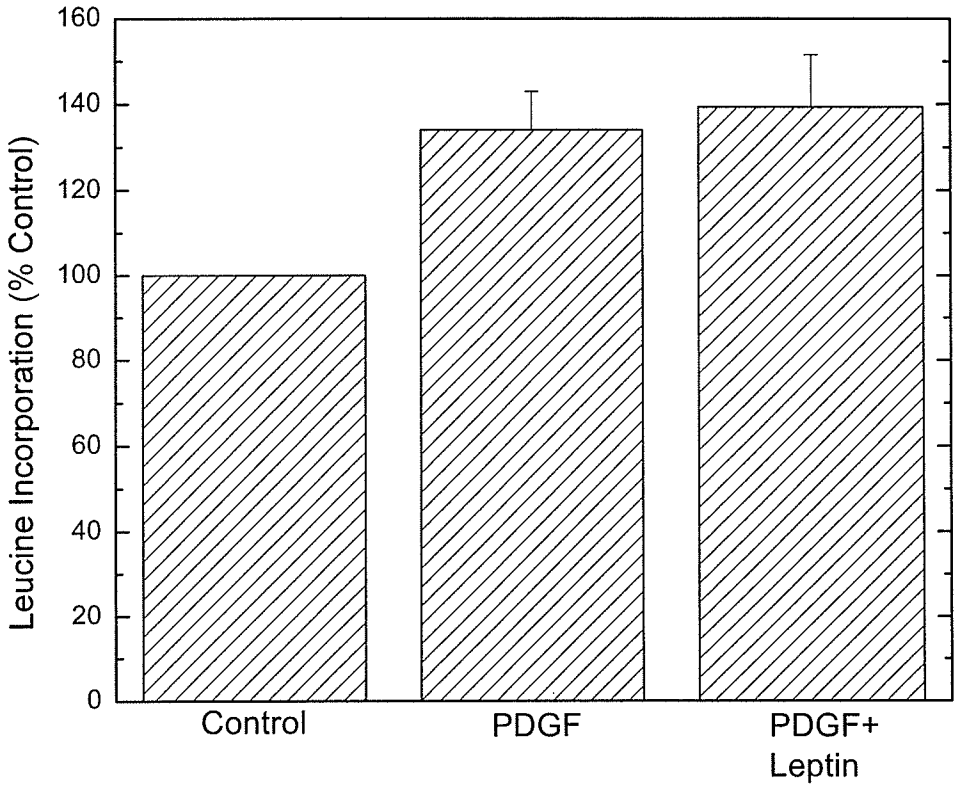


Figure 14 - The effect of 0.1 µg/mL leptin on ^{3}H-Leucine incorporation was compared to control and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. Leptin did not affect protein synthesis in PDGF-stimulated cells.

Figure 15 – Effect of Leptin on Smooth Muscle Cell Proliferation

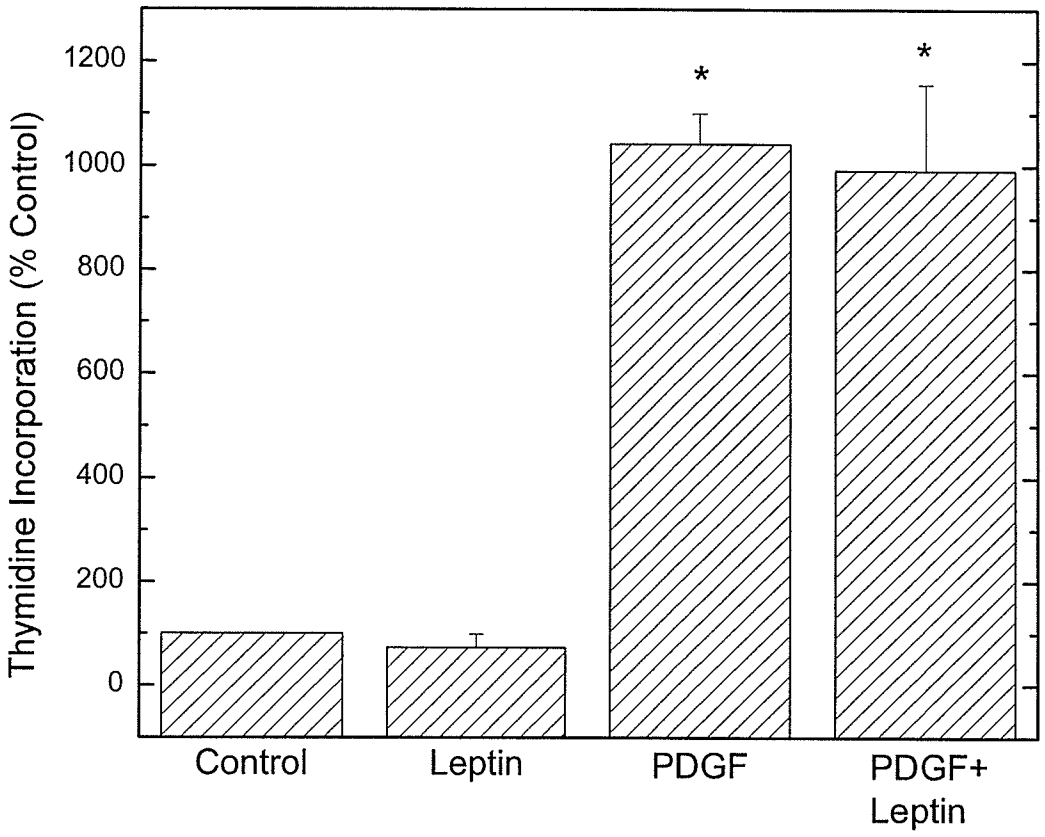
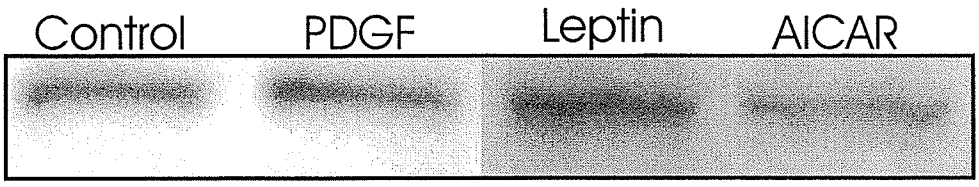


Figure 15 - The effect of 0.1µg/mL leptin on ^{3}H-Leucine incorporation was compared to control and to the mitogenic effects on 0.1µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. Leptin did not affect DNA synthesis in quiescent PDGF-stimulated cells.

* Statistically significant differences from control at $p<0.05$.

Figure 16 – Effect of Leptin on Total AMPK Levels in Porcine Smooth Muscle Cells



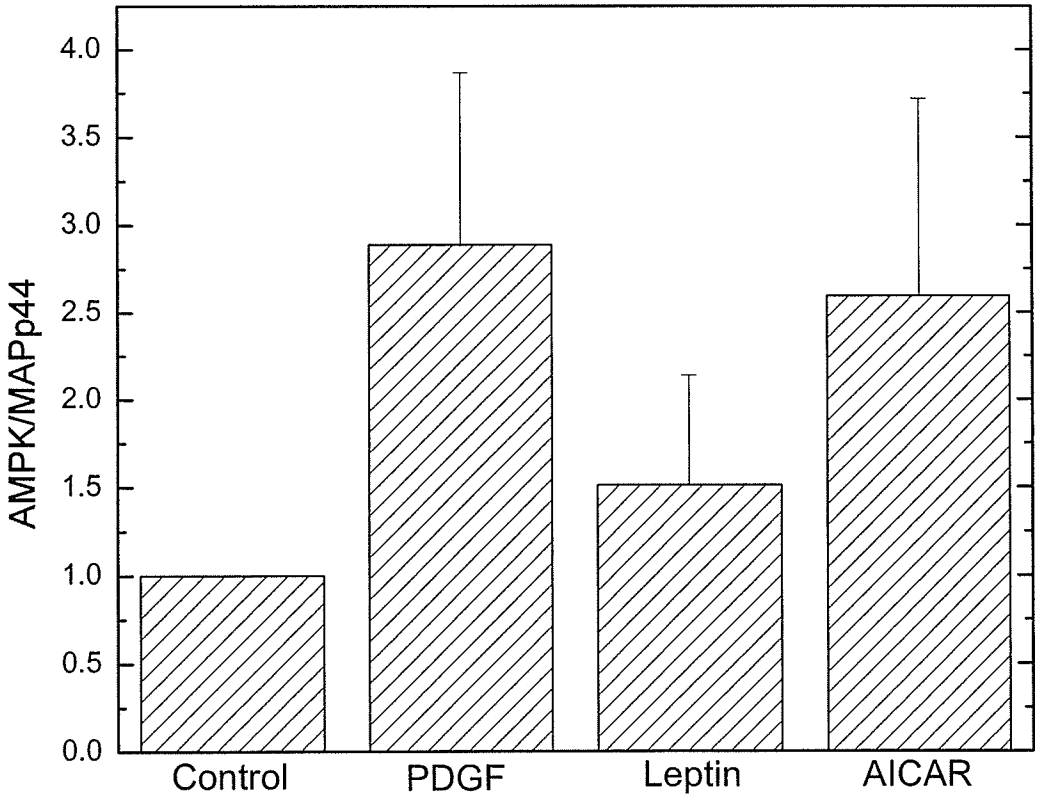
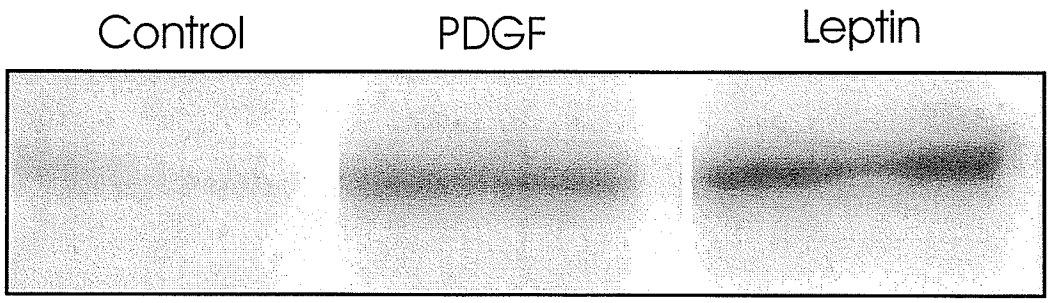


Figure 16 - The effect of 0.1 µg/mL leptin on AMPK levels was compared to control and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF) and AICAR. Data are expressed as mean ± SEM of samples in triplicate. Leptin and AICAR both did not affect AMPK levels.

Figure 17 - Effect of Leptin on the Phosphorylation of AMPK in Porcine Smooth Muscle Cells



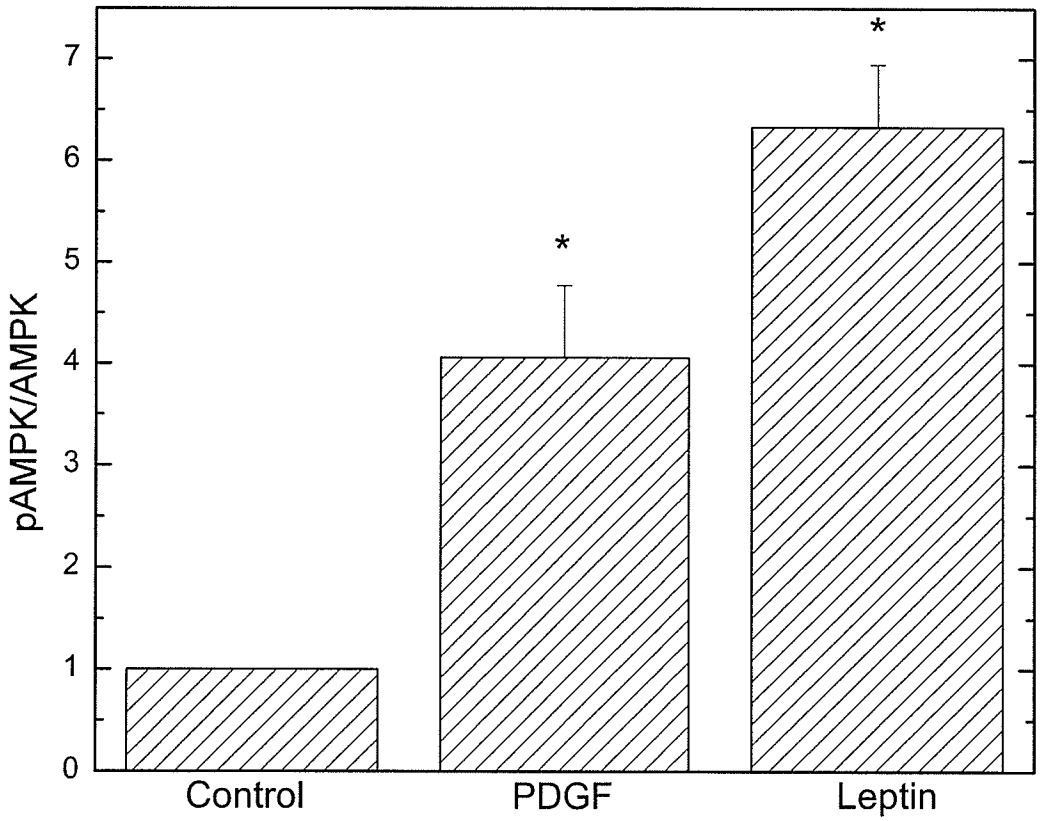


Figure 17 - The effect of 0.1 µg/mL leptin on pAMPK was compared to control and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. PDGF and leptin both significantly increased the activation of AMPK.

* Statistically significant differences from control at $p<0.05$.

Figure 18 – Effect of Leptin on the Phosphorylation of Akt in Porcine Smooth Muscle Cells

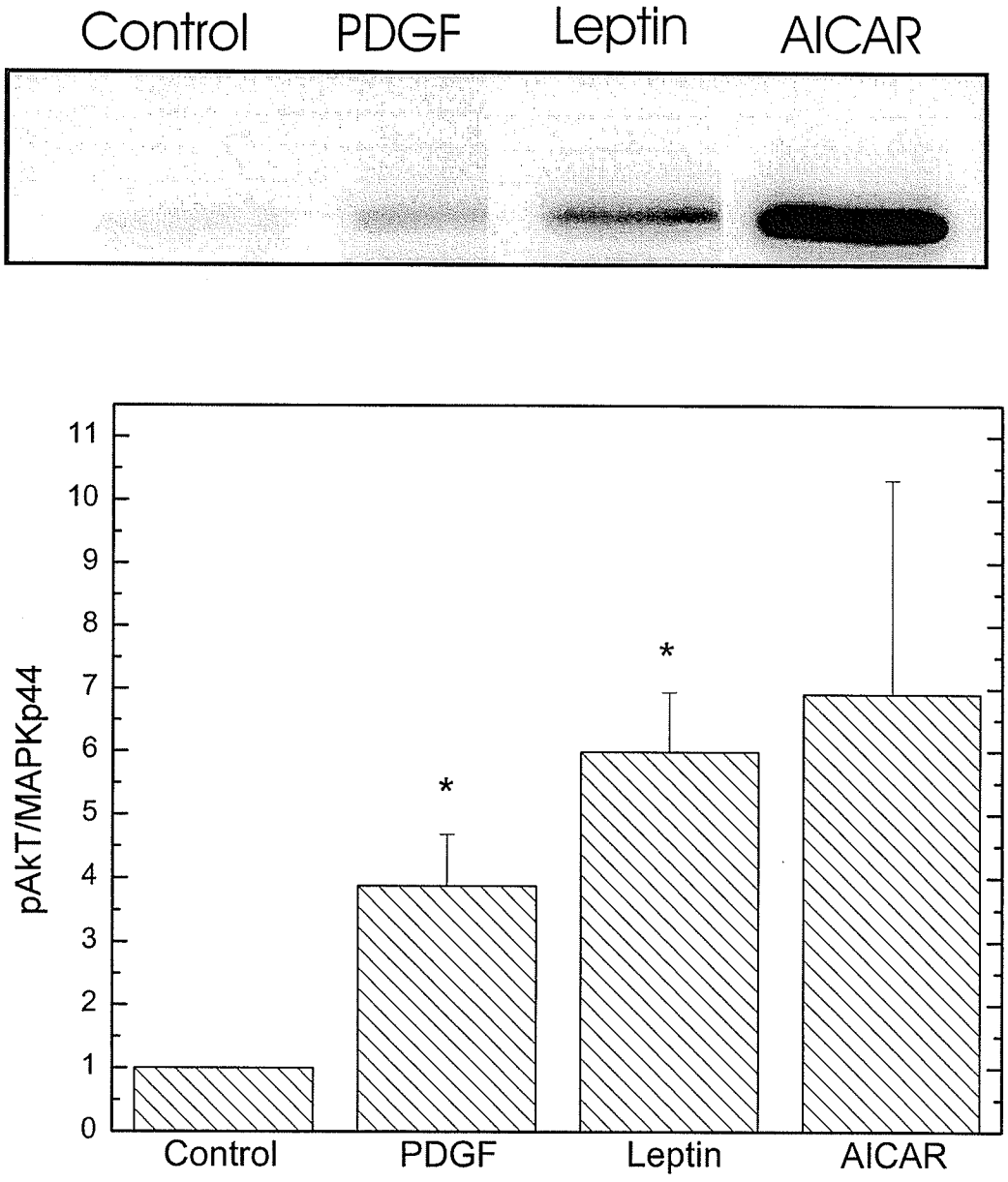


Figure 18 - The effect of 0.1 µg/mL leptin and 1 mM AICAR on the phosphorylation of Akt compared and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. PDGF and leptin both significantly increased pAkt. AICAR also appeared to increase pAkt, although no significant differences were found.

* Statistically significant differences from control at $p<0.05$

2c) Conjugated Linoleic Acid

Existing data on CLA and its effects on atherosclerotic progression has looked at CLA as one component. However, it has recently been discovered that different isomers of CLA (mainly c9,t11 and t10,c12 CLA) have different effects in the body.

Additionally, the effects of CLA on SMCs have not been investigated. This research focussed on CLA, its effects on SMCs, and its potential mechanisms of action.

Leucine Incorporation

^{3}H-Leucine incorporation was used to assess protein synthesis in SMCs after treatment with CLA isomers. ^{3}H-Leucine was added to SMC culture media and allowed to incorporate into newly synthesized proteins (as described in Methods).

Compared to control, 60 μM c9,t11 CLA decreased protein synthesis, while 60 μM t10,c12 CLA did not have an effect (*Figure 19*).

PDGF increased protein synthesis (*Figure 20*). PDGF is a known-mitogen, thus the activation of building blocks required for protein synthesis is expected. When cells were stimulated with PDGF, neither of the CLA isomers had an inhibitory effect. Concentrations of 10-240 μM CLA were tested with no concentration

dependent changes being detected (data not shown). Similarly, mixtures of CLA did not have a significant effect (data not shown).

Thymidine Incorporation

^{3}H-Thymidine incorporation was used to assess DNA synthesis in SMCs, since SMC proliferation is a central feature in vascular disease.

The c9,t11 CLA isomer had no effect on SMC proliferation at any concentration (25, 60, or 120 μM). This was true for both quiescent and PDGF-stimulated cells (*Figure 21, Figure 22*).

Likewise, the t10, c12 CLA isomer had no effect on SMC proliferation at any concentration (25, 60, or 120 μM), for both quiescent and PDGF-stimulated cells (*Figure 21, Figure 23*). Thus, it is concluded that CLA does not act as a mitogen in SMCs.

Although the data are not presented, a mixture of CLA isomers (60 μM total) also had no effect on SMC proliferation.

AMPK

CLA's effects on AMPK were also assessed. Total AMPK levels represent the total amount of AMPK in SMCs available for activation. AMPK levels were

standardized using a ratio of AMPK/MAPKp44. MAPKp44 was used to control for loading variabilities.

Western blots for total AMPK were conducted on samples prepared from CLA-treated SMCs (following a 24-hr incubation period). CLA did not significantly change levels of AMPK over this time period (*Figure 24)*.

Western blots examining the phosphorylation of AMPK were conducted on protein samples obtained from cells which had been treated with CLA isomers for 15 minutes. MAPKp44 was used as a control to correct for loading variability.

The CLA isomers did not have a significant effect on pAMPK levels. However, my work consistently did show that c9,t11 CLA increased pAMPK, while t10,c12 had no effect. Although cell to cell variability caused no significant increase to be seen, the graph clearly shows that the c9,t11 CLA isomer does have an effect on AMPK activation (*Figure 25)*.

Akt

Due to the differences seen in activation of AMPK with the two isomers, Akt was monitored as a possible down-stream target for pAMPK. The phosphorylation of Akt was assessed as it promotes cell survival and growth, and works downstream in the PI3-kinase pathway.

Neither of the CLA isomers affected the activation of Akt (*Figure 26)*. This suggests that the c9,t11 CLA-dependent increases in pAMPK do not directly affect the Akt pathway.

C9, t11 CLA→ pAMPK ✗→ pAkt

Similarily, hypothesizing that pAkt is a down-stream activator of pAMPK is also incorrect since these results indicate that pAkt does not independently stimulate pAMPK.

C9,t11 CLA→✗ pAkt→ pAMPK

Figure 19 – Effect of Conjugated Linoleic Acid Isomers on Protein Synthesis in Porcine Smooth Muscle Cells

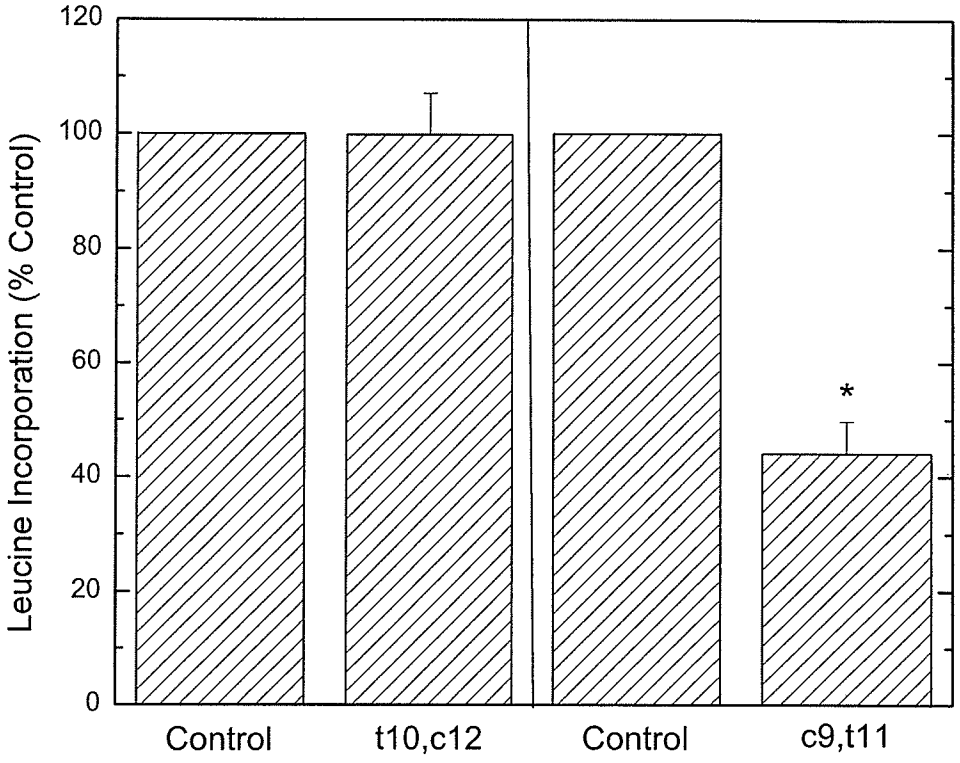


Figure 19 - The effect of 60 uM CLA on ^{3}H-Leucine incorporation was compared to control. Data are expressed as mean ± SEM of samples in triplicate. *Trans*-10, *cis*-12 CLA (t10,c12) had no significant effect in quiescent cells. The *cis*- 9, *trans*- 11 CLA (c9,t11) significantly decreased protein synthesis in unstimulated cells.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

Figure 20 – Effect of Conjugated Linoleic Acid on Protein Synthesis in PDGF-stimulated Porcine Smooth Muscle Cells

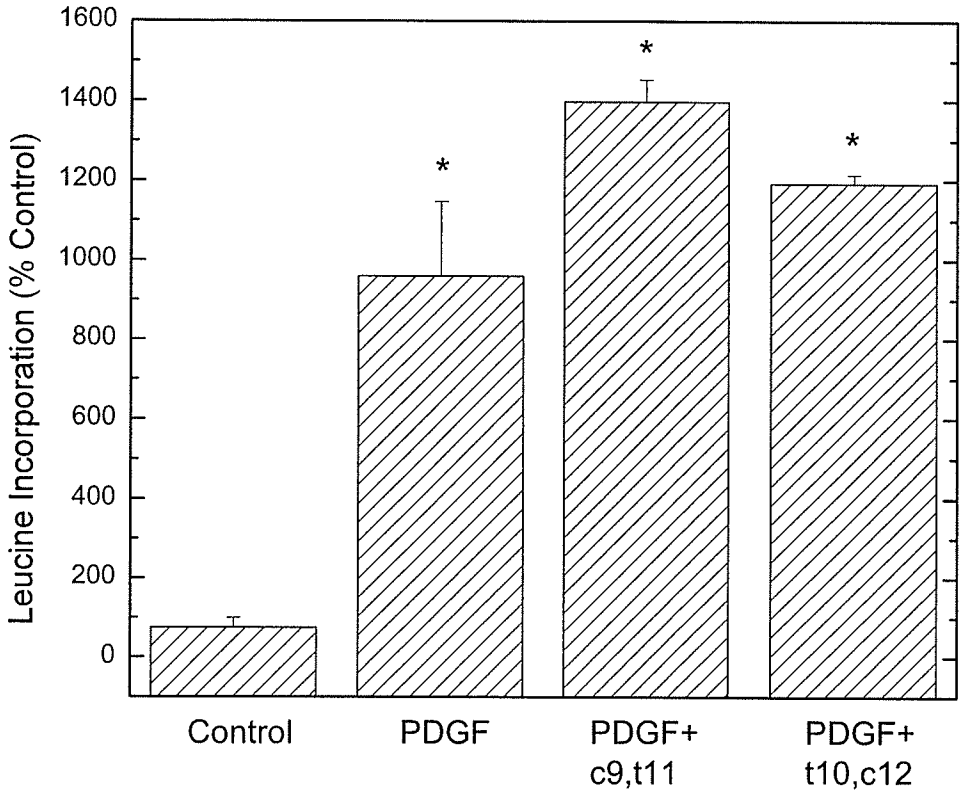


Figure 20 - The effect of 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF) stimulation on ^{3}H-Leucine incorporation was compared to control and to the mitogenic effects PDGF alone. Data are expressed as mean ± SEM of samples in triplicate. Neither of the CLA isomers had any effect on protein synthesis in PDGF-stimulated cells.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

Figure 21 – Effect of Conjugated Linoleic Acid on Smooth Muscle Proliferation

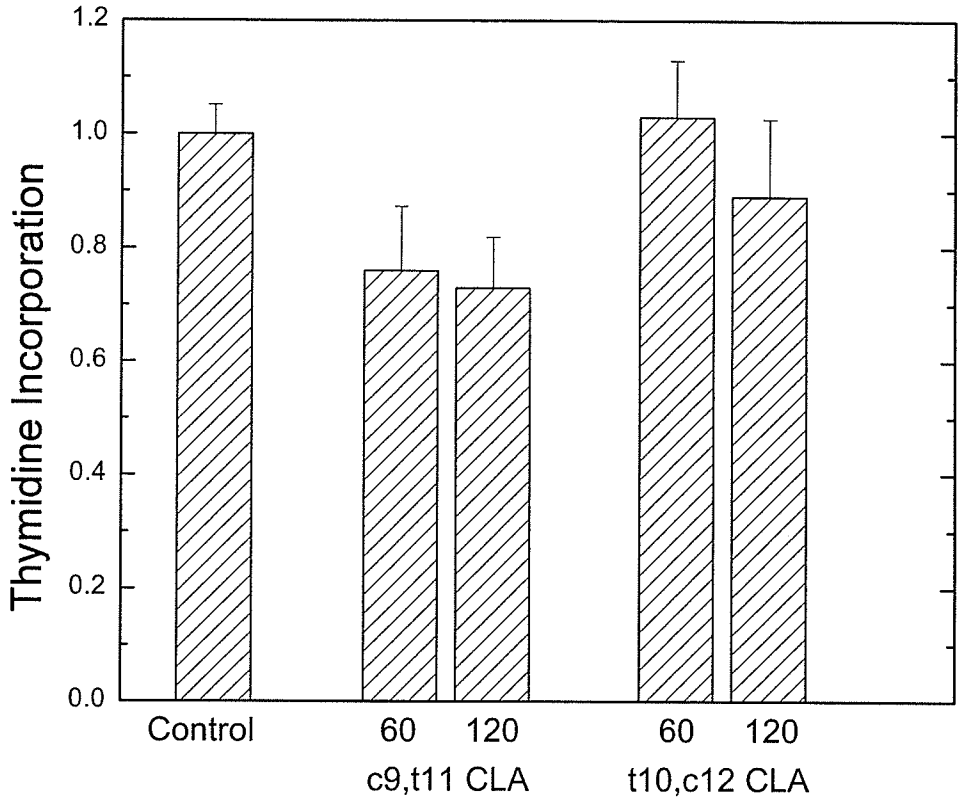


Figure 21 - The effects of 60 μM and 120 μM CLA on ^{3}H-thymidine incorporation was compared to control. Data was standardized to control and are expressed as mean ± SEM of samples in triplicate. Neither of the CLA isomers had any effect on DNA synthesis in unstimulated cells.

Figure 22 – Effect of c9,t11 Conjugated Linoleic Acid on Proliferation in PDGF-Stimulated Porcine Smooth Muscle Cells

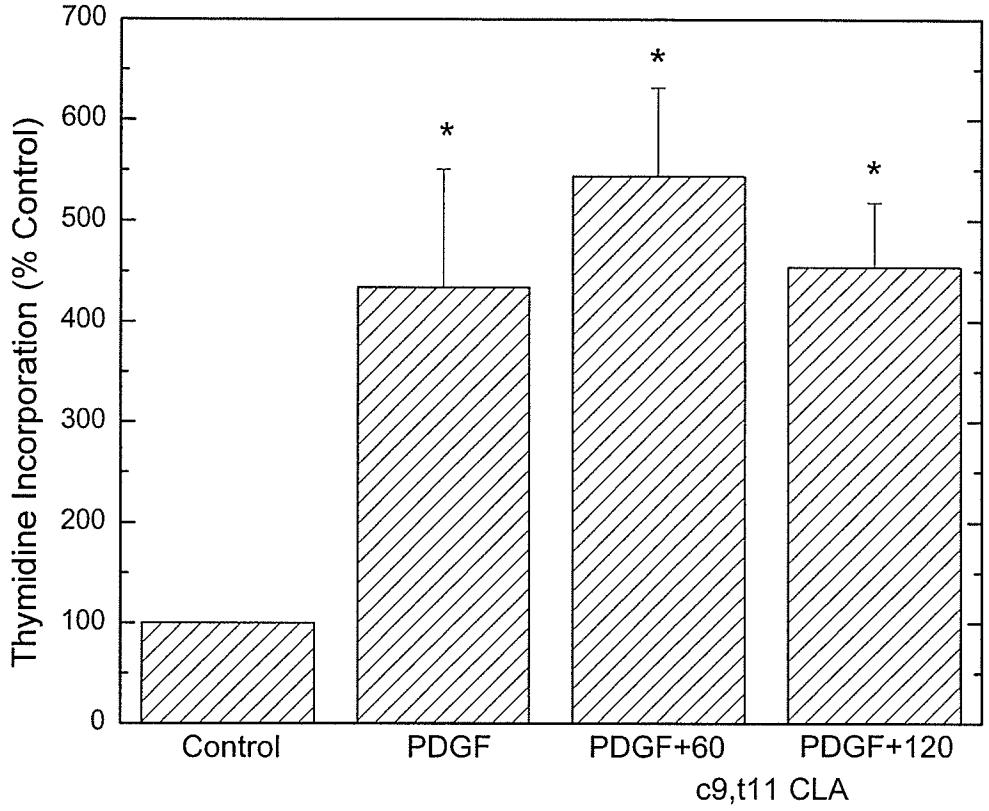


Figure 22 - The effects of 60 µM and 120 µM *cis*-9, *trans*-11 CLA (c9,t11 CLA) on ^{3}H-Thymidine incorporation was compared to control and to 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. The c9,t11 CLA isomer had no effect on DNA proliferation in PDGF-stimulated cells.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

Figure 23 –Effect of t10,c12 Conjugated Linoleic Acid on Proliferation in PDGF-Stimulated Porcine Smooth Muscle Cells

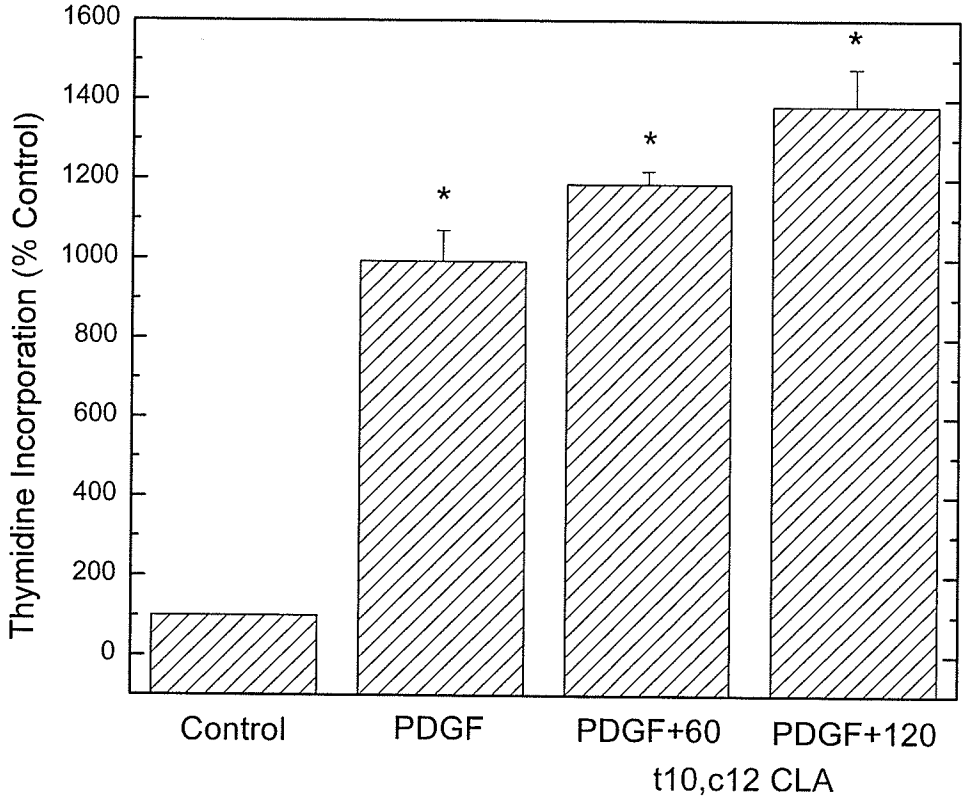


Figure 23 - The effects of 60 µM and 120 µM *trans*-10, *cis*-12 CLA (t10,c12 CLA) on ^{3}H-Thymidine incorporation was compared to control and to 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are expressed as mean ± SEM of samples in triplicate. The t10,c12 CLA isomer had no effect on cell proliferation in PDGF-stimulated cells.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

Figure 24 – Effect of Conjugated Linoleic Acid on Total AMPK Levels in Porcine Smooth Muscle Cells

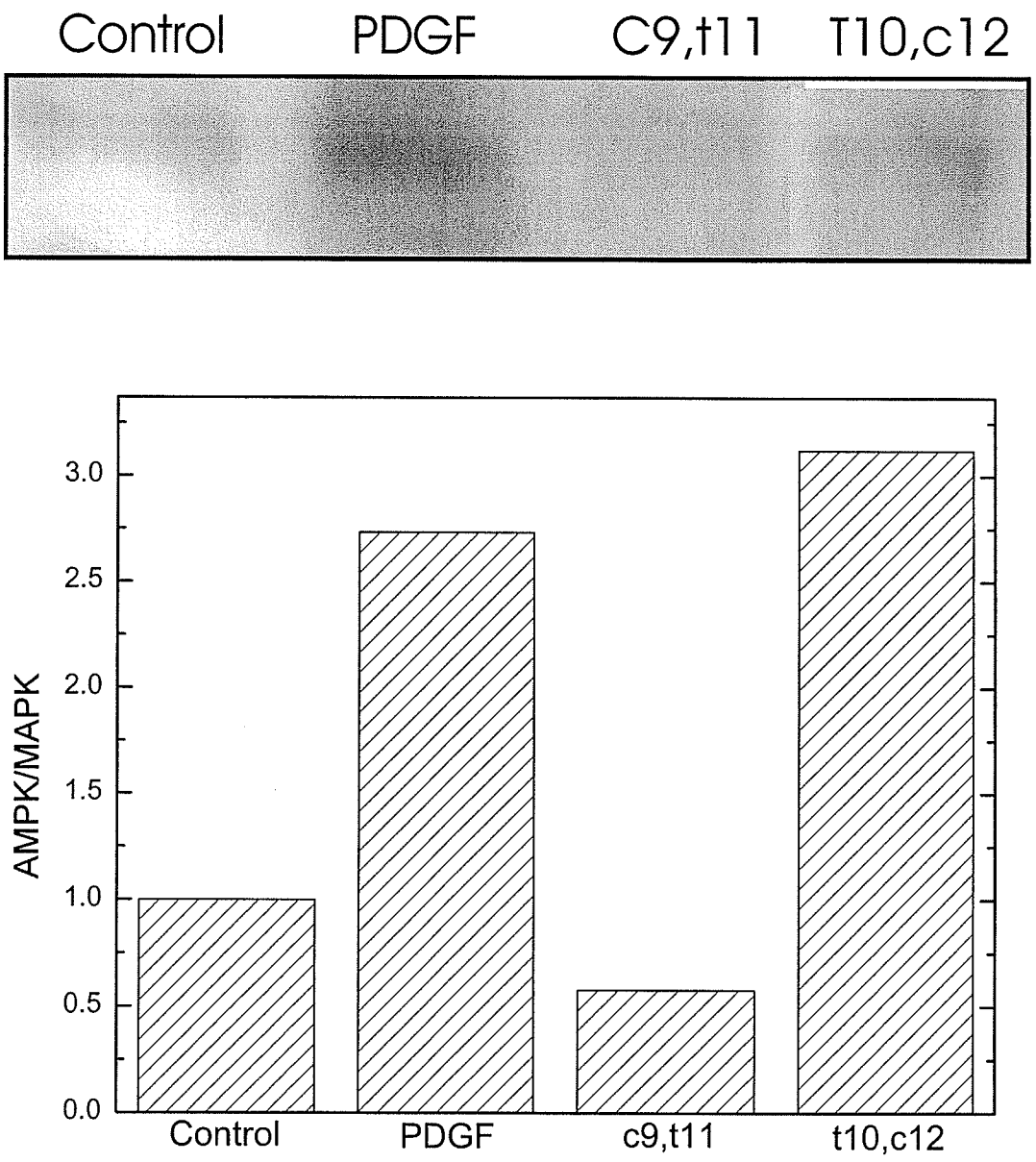


Figure 24 - The effect of 60 µM *cis*-9, *trans*-11 CLA (c9,t11) and 60 µM *trans*-10, *cis*-12 CLA (t10,c12 CLA) on AMPK levels was compared to control. Neither of the CLA isomers had a significant effect on AMPK levels in unstimulated SMCs. This figure illustrates a representative blot of samples in triplicate.

Figure 25 – Effect of Conjugated Linoleic Acid on the Phosphorylation of AMPK in Porcine Smooth Muscle Cells

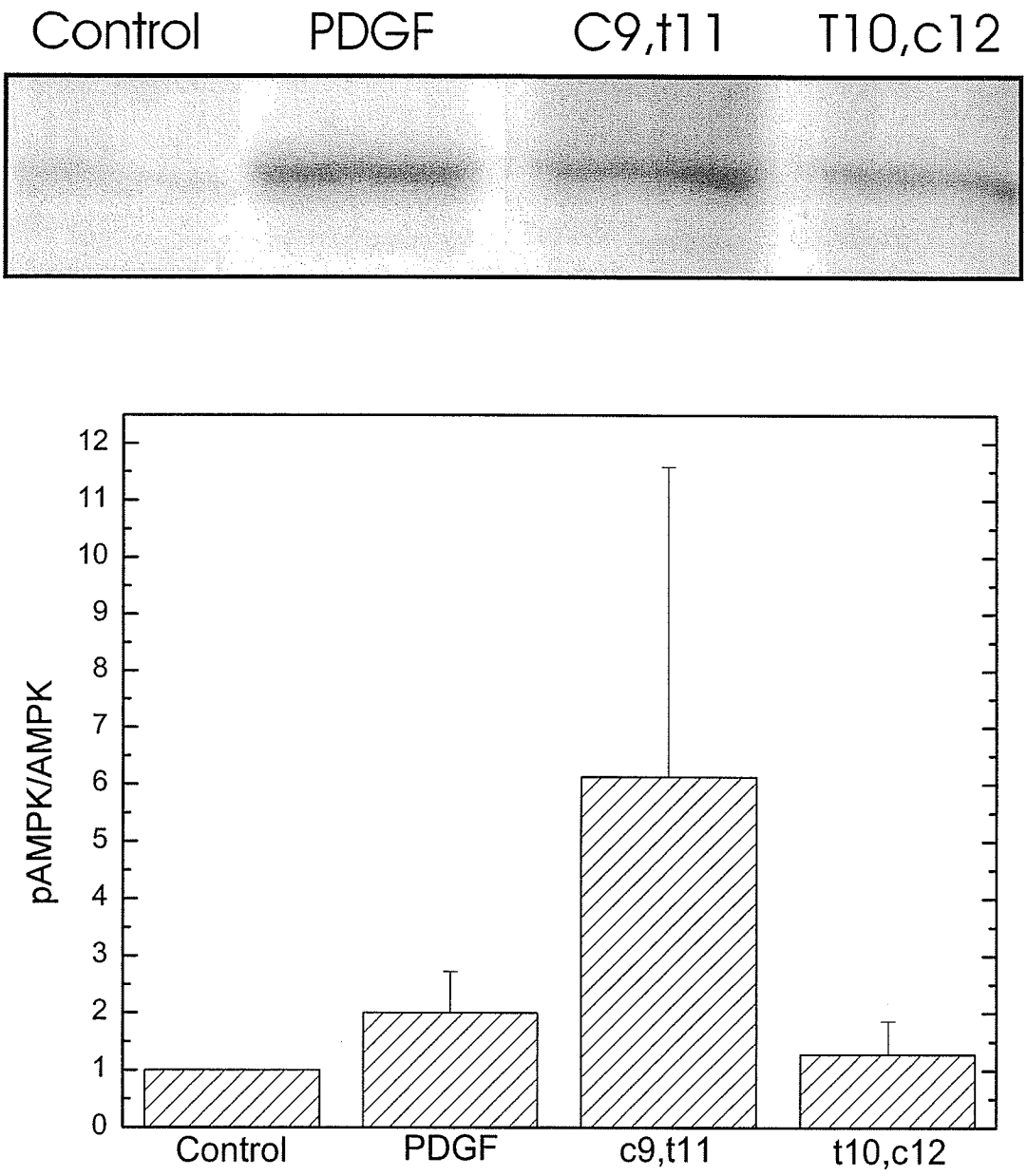


Figure 25 - The effect of 60 µM *cis*-9, *trans*-11 CLA (c9,t11) and 60 µM *trans*-10, *cis*-12 CLA (t10,c12 CLA) on activation of AMPK was compared to control and to 0.1µg/mL platelet-derived-growth factor BB (human) (PDGF). Neither of the CLA isomers had a significant effect on the phosphorylation of AMPK levels in SMCs. However, c9,t11 CLA seems to have a greater stimulatory effect compared to t10,c12 CLA. Data are expressed as mean ± SEM of samples in triplicate.

Figure 26 – Effect of Conjugated Linoleic Acid on the Phosphorylation of Akt in Porcine Smooth Muscle Cells

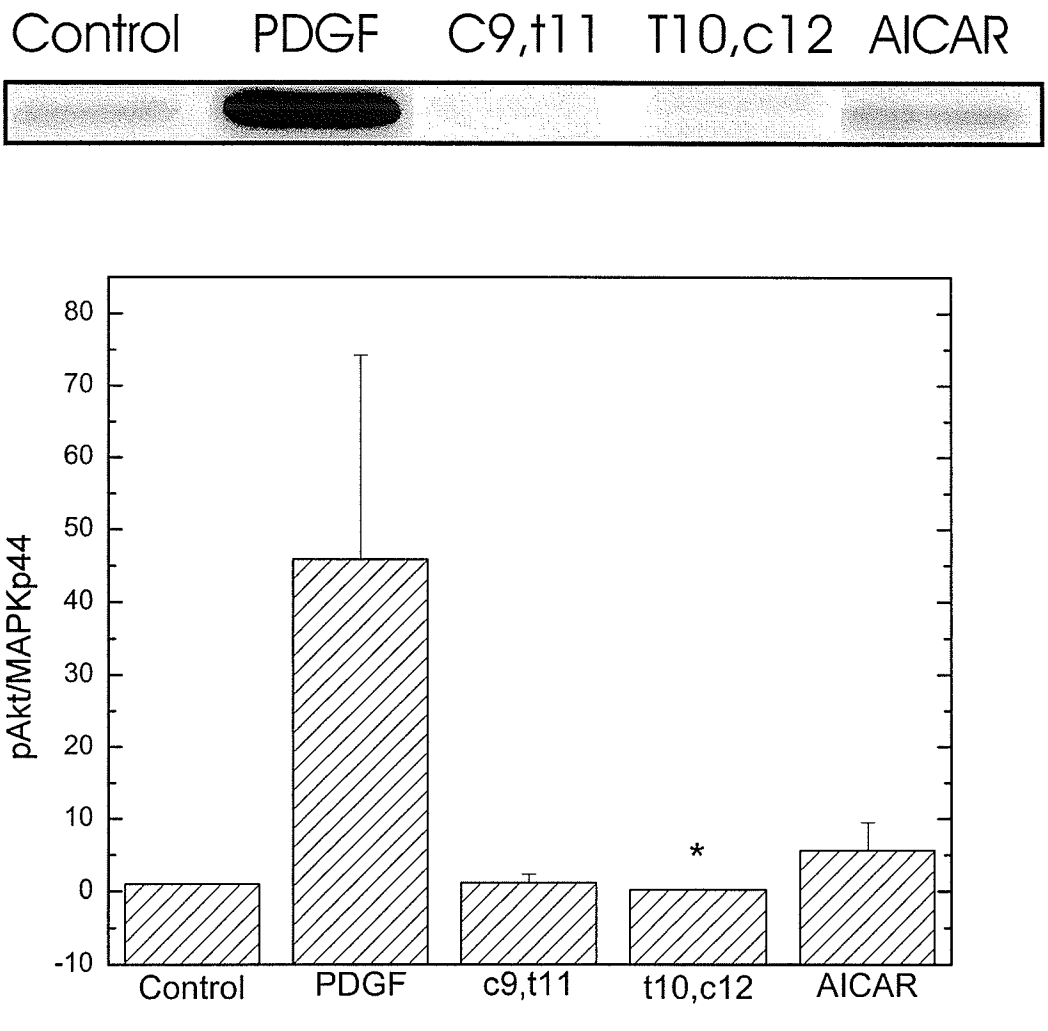


Figure 26 - The effect of 60 µM *cis*-9, *trans*-11 CLA (c9,t11), 60 µM *trans*-10, *cis*-12 CLA (t10,c12), and AICAR on activation of Akt was compared to control and to 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Neither of the CLA isomers increased pAkt activity, and t10,c12 actually decreased pAkt compared to control. Data are expressed as mean ± SEM of samples in triplicate.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

2d) Globular Adiponectin

New research suggests that different forms of adiponectin function differently, especially in regards to full-length adiponectin and its globular domain (Tsao et al, 2003).

Due to this information, the differences in globular adiponectin versus full-length adiponectin were examined in regards to SMC proliferation. The phosphorylation of AMPK was also addressed.

Globular adiponectin significantly decreased SMC proliferation in both quiescent and PDGF-stimulated cells (*Figure 27*). These data suggests that globular adiponectin has anti-mitogenic properties. This is opposite of the effects found with full-length adiponectin.

Globular adiponectin also increased the phosphorylation of AMPK. AICAR also was tested to ensure cells responded to AMPK, and as seen consistently in my work, AICAR also increased pAMPK levels (F*igure 28)*.

Figure 27 – Effect of Globular Adiponectin on Proliferation in Porcine Smooth Muscle Cells

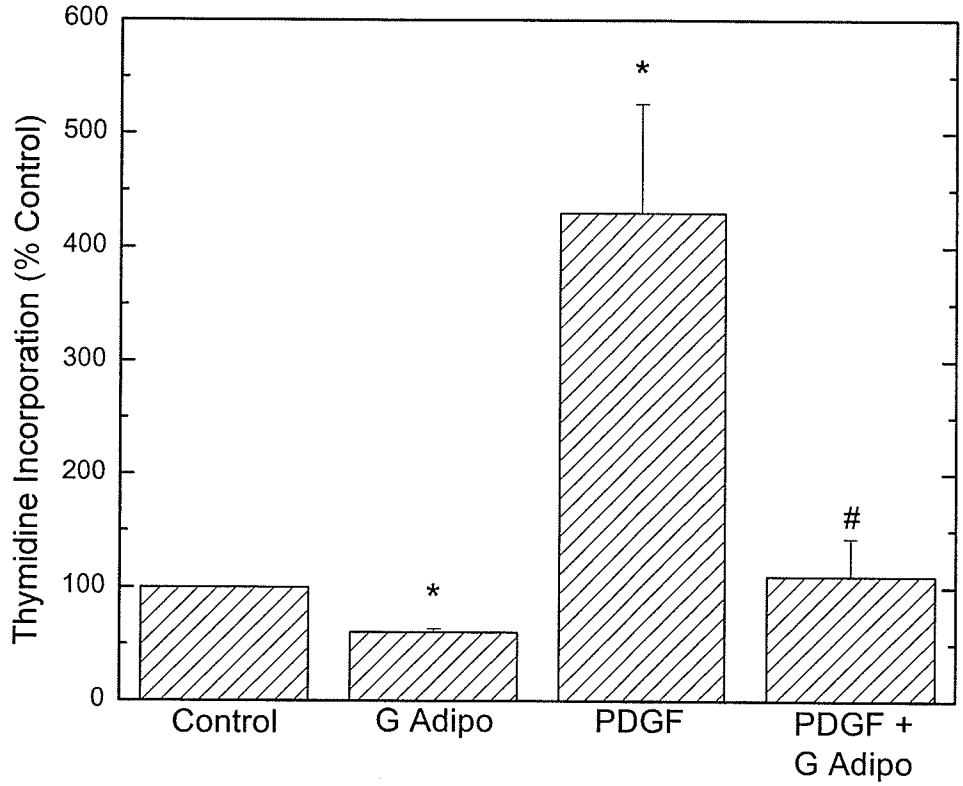
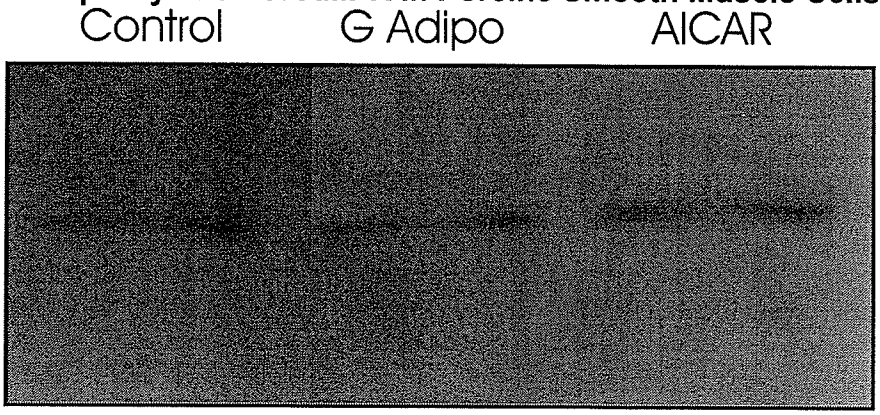


Figure 27 - The effect of 1.5 µg/mL globular adiponectin (G Adipo) on ^{3}H-Thymidine incorporation was compared to control and to the mitogenic effects on 0.1 µg/mL platelet-derived-growth factor BB (human) (PDGF). Data are standardized to Control and expressed as mean ± SEM of samples in triplicate. G Adipo significantly decreased DNA synthesis in quiescent and PDGF-stimulated cells.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

\# Statistically significant differences from PDGF-stimulated at $p<0.05$.

Figure 28 – Effect of Globular Adiponectin and AICAR on the Phosphorylation of AMPK in Porcine Smooth Muscle Cells



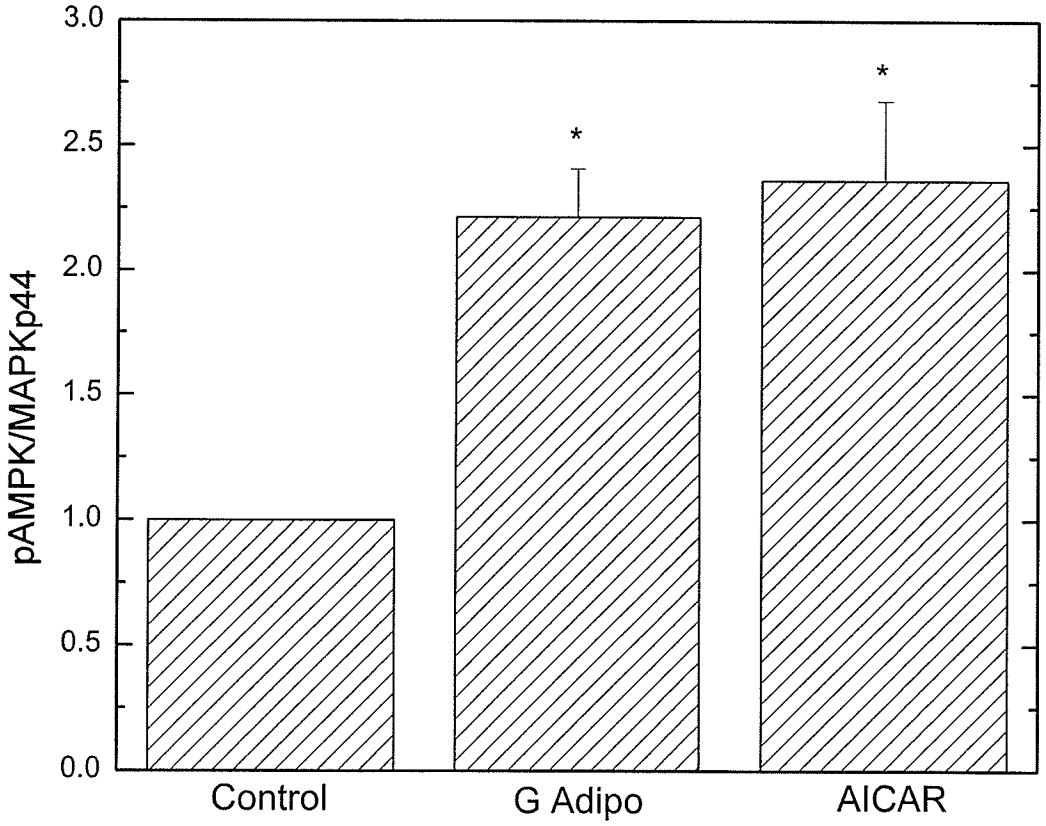


Figure 28 - The effect of 1.5 µg/mL globular adiponectin (G Adipo) and 1 mM AICAR on the phosphorylation of AMPK. Data are expressed as mean ± SEM of samples in triplicate. G Adipo and AICAR both significantly increased pAMPK.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

2e) PPARs

Studies in other laboratories have led to the premise that CLA works through PPARs (as described previously). To test CLA's ability to affect PPARs in SMCs, Western blots were performed.

Quiescent SMCs were incubated with varying concentrations of CLA isomers and left to incubate overnight. After gels were prepared, PPAR antibodies were used (PPARα, PPARβ and PPARγ).

No significant changes in any of the PPARs were seen with CLA treatment in SMCs as determined by Western blotting. In fact, PPAR levels were not significantly affected by any of the agonist treatments, those of which are known to target specific PPARs (*Table 8*). A summary table illustrating the degree of PPAR expression follows (*Table 9).*

Table 8 – Agonists and Antagonist tested on Porcine Smooth Muscle Cells treated with CLA

Agonist[a]	Target
Clofibrate	PPARα
L165041	PPARβ
Pioglitazone	PPARγ
WY14643	PPARα
Rosiglitazone	PPARγ
GW501516	PPARβ

Antagonist[a]	Target
GW9662	PPARγ

[a] See Table 6 for source and product number of agonist/antagonist.

Table 9 – Summary of Western Blots[#]

	*PPARα**	PPARγ*	PPARβ*
PDGF	Increased (1) No effect (1)	No effect (1) Increased (1)	No Effect (3) Increased (1)
AICAR	No Effect (1)	No Effect (1) Decreased (1)	No Effect (1)
Adiponectin	No Effect (1)	No Effect (1) Increased (1) Decreased (1)	No Effect (3)
Leptin	Increased (1)	No Effect (1) Increased (1) Decreased (1)	No Effect (3)
c9,t11 CLA	No Effect (1) Increased (3)	No Effect (3) Increased (2)	No Effect (4)
t10,c12 CLA	Increased (3)	No Effect (1) Increased (2)	No Effect (3) Increased (1)
CLO	No Effect (1)	No Effect (1) Increased (1)	No Effect (1)
L165041	No Effect (1)	No Effect (2) Increased (1)	No Effect (2) Decreased (1)
PIO	No Effect (1)	No Effect (1) Increased (1)	
WY14643	Decreased (1)	No Effect (1) Increased (1)	No Effect (1)
ROS	Decreased (1)	No Effect (2) Increased (1)	
GW50156	No Effect (1)	No Effect (1) Increased (1)	No Effect (1)

* Ratio of PPAR detected with agonist divided by baseline detection.
No Effect = Western blots which had a ratio (treatment:control) between 0.5-1.99.
Increased= Western blots which had a ratio greater than 2.
Decreased= Western blots which had a ratio less than 0.5.

Number in brackets indicates how many experiments showed this trend. Statistical significant testing could not be completed as each result represents one blot, and there were not enough repeats to detect significant changes. The changes seen were based on subjective visualization as described above.

PART 5 - DISCUSSION

1. Animal Study

1a) Serum Adiponectin

The *fa/*fa Zucker rats had higher serum adiponectin compared their lean counterparts. However, lean Zuckers are more efficient at producing adiponectin as they have higher adiponectin levels per gram of adipose compared to *fa/fa* Zuckers (*Figure 1, Figure 3)*. Past research in our lab also found the *fa/fa* rats to have higher serum adiponectin levels (Amy Noto, 2004). This does not agree with most data as it is common to see lean subjects with higher serum adiponectin levels compared to obese counterparts (Ouchi et al, 2004; Kumada et al, 2003; Hotta et al, 2000).

Additionally, only the animals fed the t10,c12 CLA diet had significantly higher serum adiponectin levels compared to control animals. This suggests that, in regards to serum adiponectin levels, the t10,c12 CLA isomer has the ability to increase serum adiponectin. The c9,t11 CLA isomer does not have an effect in modulating serum adiponectin levels. The t10,c12 CLA isomer could elevate serum adiponectin by augmenting production or by altering the sensitivity of the adiponectin receptors.

Previous work in our lab did show significant changes in serum adiponectin levels when CLA was administered at 1.5% (w/w) CLA (current study used 0.4-2.2% CLA). The dietary treatments in this study with approximately the same % of CLA compared to our last study were the TOG, BIO and NUCHEK diets. The TOG mixture did show an elevation in serum adiponectin levels in *fa/fa* rats, as did the t10,c12 CLA isomer which was present only at 0.4%. Thus, the total % CLA in the diet does not seem to contribute to our inconsistent findings.

However, when comparing serum adiponectin levels in both the lean and the *fa/fa* rats, there was a 1.2-fold difference between genotypes (*Table 10).* Serum adiponectin in both *fa/fa* and lean Zucker rats was two-fold higher in the CLA 2001 study compared to the CLA 2003 study. This inter-animal variability is impossible to control for, and may be responsible for the lack of significance in serum adiponectin levels in this study.

While our findings are not entirely supported in the literature, it is important to point out the incongruencies in this data. Our measurements of serum adiponectin account for all forms of adiponectin; globular and full-length. Additionally, adiponectin also exists as oligomers that apparently determine its function. It is not known what forms of adiponectin were analyzed in the other studies.

Globular trimers of adiponectin are a ball-like structure which consists of the carboxyl-terminus (Tsao et al, 2003). These structures can form trimers (full-length or globular) and hexamers (full-length only), the latter of which are most abundant in serum (Tsao et al, 2002). These different oligomers also possess different functions. For example, hexameric forms do not activate AMPK, but trimers do, suggesting that they work through different signal transduction pathways and possibly different receptors.

Our measurements account for all forms of adiponectin. Further discussion will identify our interest in activation of AMPK, thus only one form of adiponectin may be relevant to us.

However, studies discussing concentrations of adiponectin do not identify the form being measured, and in some cases (as in ours) it may be impossible to determine.

Evidence for an inverse relationship between serum adiponectin levels in the human and risk of atherosclerosis has led to the postulate that exogenous supplementation of adiponectin may be employed for therapeutic intervention in those with angiogenesis and/or vascular complications of diabetes (Ouchi et al, 2004). Before this can be fully determined, future research should look closer at the relationship between the different oligomeric forms of adiponectin and their functions throughout the body. Identifying the size of adiponectin that has

beneficial properties is needed if adiponectin has any hope as a therapeutic agent in the future. Additionally, identifying how t10,c12 CLA elevates levels of adiponectin and which form it affects also must be discovered before it can be boasted that only one CLA isomer can increase levels of adiponectin in the serum.

Additionally, the practicality of using CLA as a nutraceutical is also a concern. The average human intake of CLA per day is 0.02% (w/w), thus, a dramatic increase would be necessary each day. Additionally, the diet does not provide a rich source of the t10,c12 CLA isomer, indicating a need for a CLA supplement.

Table 10 - Comparison of CLA Studies: Serum Adiponectin

Diet	CLA 2001 Study #€	CLA 2003 Study # *
Control Lean	9.61 ± 0.84	4.08 + 0.28
Control *fa/fa*	10.58 ± 0.54	5.29 + 0.36
CLA Lean *	11.78 ± 0.60	4.56 ± 0.39
CLA *fa/fa* *	14.33 ± 0.65	6.48 ± 0.68

* Serum adiponectin for CLA-fed animals is mean of CLA diet BIO. This is the most similar to the CLA diet administered in the CLA 2001 Study (1.2% CLA). Data is expressed as (µg/mL) ± SEM.

Serum adiponectin (µg/mL) is expressed as mean ± SEM.

€ Data from Amy Noto, 2004. Rats fed 1.5% CLA or 0% CLA.

1b) Serum Leptin

Zucker *fa/fa* rats had significantly higher serum leptin concentrations compared to lean rats (*Figure 5)*. These results were expected as previous work clearly illustrates a direct relationship between leptin levels and adipose levels (Funahashi et al, 1999; Poirier et al, 2005). High serum leptin levels have been associated with an increased risk of atherosclerosis in humans (Ciccone et al, 2001).

Taken together, diet did not have an effect on leptin concentrations. However, when looking at the *fa/fa* group, the t10,c12, TOG, BIO, and NCK dietary groups all had significantly lower leptin levels compared to those groups fed either the control diet or the c9,t11 CLA diet (*Figure 7)* . This suggests that beneficial changes in leptin levels in the obese subject may be attributed to the t10,c12 CLA isomer alone and/or in combination with additional CLA isomers. The c9,t11 CLA isomer does not contribute to changes in serum leptin levels. This is in agreement wih past work in OLETF rats by Nagao et al, 2003a.

The findings of the present study are in agreement with other researchers using OLETF rats. Masso-Welch et al (2004) also found reduced serum leptin concentrations in mice after feeding individual CLA isomers (5-10 g/kg diet) for 7 weeks. The t10,c12 CLA isomer decreased serum leptin while the c9,t11 CLA had no effect (Masso-Welch et al, 2004).

This clearly illustrates that the two main CLA isomers have distinctly different physiological functions in the body. In *fa/fa* rats, the t10,c12 CLA isomer potentially exhibits anti-atherosclerotic properties (Hotta et al, 2000; Ciccone et al, 2001).

While levels of serum leptin are important, another major factor is the amount of and function of leptin receptors. Zucker *fa/fa* rats have leptin receptors that do not function properly. Thus, the cell cannot recognize that leptin is present as it cannot be picked up by the receptor, resulting in an increased production of leptin. The t10,c12 CLA isomer appears to play a role in lowering total serum leptin, suggesting that it may signal to inhibit the production of leptin, thus, it may aid in regaining the functioning of the feedback mechanism of the leptin receptor needed to maintain serum leptin levels. Future research must determine this as well as the differences in these isomeric effects.

In conclusion, CLA does not have an overall diet effect, but diet differences are seen in the *fa/fa* group alone. The t10,c12 CLA isomer induces an increase in adiponectin levels, as well as in reducing serum leptin levels.

These are important conclusions, as it is known that both isomers function differently and the physiological effects are concentration dependent.

CLA is promoted for its ability to aid in fat reduction, cancer prevention and diabetes, as well as atherosclerosis. While there is an abundance of data to support this, there is limited data available as to its function and to the particular effects of each isomer. Our findings can help to shed some light on this issue.

CLA isomer mixtures sold on the market today contain primarily the c9,t11 CLA isomer, with t10,c12 CLA being the next most abundant. CLA-containing foods also primarily consist of the c9,t11 CLA isomer. However, our research supports the view that it is the t10,c12 CLA that plays the major role in controlling serum adipokine levels. Thus, promoting the use of CLA-rich foods may be premature as the c9,t11 CLA in it may not offer any health benefits. While this is only one piece of evidence, it is important to look into CLA's isomeric effects more carefully and determine how they each work in the body. Additionally, the safety and efficacy of CLA isomers need to be established before any recommendations for human populations can be suggested.

2. Porcine Smooth Muscle Cell Cultures

2a) Adiponectin and AICAR

There are few studies available to either support or oppose my observations of adiponectin's effects on SMCs. Most work to date has used adipocytes or endothelial cells, and are not human cells (Hedin et al, 2004).

The results of this thesis show that adiponectin increases protein synthesis in quiescent SMCs, and has no effect on PDGF-stimulated cells (*Figure 8)*. This suggests that adiponectin promotes normal metabolic functions, as protein synthesis is needed for the normal metabolism of cells. In a proliferating (stimulated by PDGF) or an injured cell, increased protein synthesis can have both positive and negative effects. For example, when DNA synthesis is unchanged, an increase in protein synthesis dictates cellular hypertrophy, which is a normal adaptive response to a stressed cell, and needed for maintenance and survival of cells. However, my results show that adiponectin also causes an increase in DNA synthesis in healthy SMCs, thus, it is mitogenic.

Additionally, my results do not support that adiponectin has any anti-proliferative effects as it is not able to inhibit SMC proliferation in PDGF-stimulated cells *(Figure 9)*.

Two studies in SMCs have been conducted and both oppose my findings. One study added 5 µg/ml of wild-type adiponectin to human aortic smooth muscle cells (HASMC) and showed that adiponectin reduced SMC proliferation in PDGF-stimulated cells. This study also looked at the expression of ADIPOR1 and ADIPOR2 and found that down-regulating these proteins still resulted in a decrease in SMC proliferation. Thus, they concluded that adiponectin does not work through the ADIPOR1 or R2 receptors in these cells (Wang et al, 2005). This study suggested that adiponectin binds to mitogenic growth factors such as PDGF, reducing their stimulatory effects. The second study by Arita et al (2002) also looked at the effects of adiponectin on HASMC and their results agree with Wang et al (2005). They also suggested that adiponectin binds directly to PDGF and stops thymidine uptake via the extracellular signal-regulated kinase (ERK) 1/2 pathway.

Differences in the study design could be the cause of contradictory results. A five-fold higher concentration of adiponectin was used in comparison to ours. Human serum concentrations range from 3-30 µg/mL (Arita et al, 1999). We used 1 µg/mL, while the others used 5 µg/mL. It appears that optimally we should have used a higher concentration to more closely mimic human serum concentrations.

These two studies utilized the same cell type from a human source, starving-down cells for 24 hours, after which ^{3}H-Thymidine was added for 6 hours.

Previous work in our laboratory indicates that it takes 100-125 hours (4-5 days) until SMCs become completely quiescent (stop proliferation). Similarly, once in the quiescent state, cells must be stimulated for 15-20 hours until they regain proliferative status (Saward & Zahradka, 1997). Based on these findings, a standard protocol has been developed in our lab for measuring ^{3}H-Thymidine uptake. Cells are starved down for 5 days to ensure they have reached a quiescent state. Stimulants are then added and left overnight to allow cells to become proliferative once again. Thus, the contradictory studies discussed did not allow their cells to become completely quiescent, which may have caused the differences in results.

While the data are limited, all publications to date suggest that adiponectin inhibits SMC proliferation in the vasculature. One study which is quoted in numerous papers supporting this anti-proliferative action of adiponectin is Matsuzawa et al (2002). However, this paper does not contain one piece of scientific evidence to support the claim that adiponectin has anti-proliferative effects (Matsuzawa et al, 2002).

SMC proliferation is important for the growth of healthy cells and not all SMC proliferation contributes to atherosclerotic progression. Instead, atherosclerosis occurs when the adaptive, healthy SMC's proliferative response is overcome by uncontrolled migration and proliferation, which subsequently leads to increased vascularization and blocking of the arteries. Thus, my results do not indicate that

adiponectin is atherogenic, per say, but instead that it acts as a mitogen to promote SMC proliferation. Likewise, adiponectin does not act as an anti-proliferative agent in stimulated SMC. This could be beneficial, as adiponectin may promote the regeneration of damaged tissues. Conversely, this could also be detrimental in those who are genetically unable to control SMC proliferation and healing, as it may lead to an abundance of SMC growth, vessel blocking lesions and increases in foam cells. Thus, creating a balance between complete and accelerated healing is important.

Although the limited information available does not agree with this thesis, some studies have shown that adiponectin acts as a mitogen in other cell types such as 3T3-L1 fibroblasts (Fu et al, 2005). This study is the first to suggest that adiponectin may have both endocrine and autocrine/paracrine functions in adipose tissue.

Resistin, another adipocytokine has also been shown to increase proliferation of HASMCs (Calabro et al, 2004). Cells were treated for 48 hours, similar to our study, and cell number was measured. The phosphatidylinositol 3-kinase (PI3K) and ERK pathways were activated, suggesting resistin increases HASMC proliferation through both the ERK1/2 and Akt signaling pathways.

To identify the mechanism behind adiponectin's effects in smooth muscle cell proliferation, certain signal transduction pathways were examined. Adiponectin

increased the phosphorylation of AMPK (*Figure 11)*. Similarly, AICAR, a known AMPK activator, also increased phospho-AMPK protein kinase (pAMPK) levels (*Figure 11)*.

The PI3K/Akt pathway is an important mediator of cell growth and survival in response to growth factors. PI3K activates Akt, which then mediates cell survival and growth signals. Akt has been found to influence cell-cycle progression of SMCs. Akt activity (which is also stimulated by insulin) has been shown to be an important regulator of adiponectin secretion (Ohashi et al, 2004).

Adiponectin increased the phosphorylation of Akt *(Figure 12)*, suggesting that adiponectin-dependent increases in pAMPK may be linked to increased pAkt, resulting in an increase in SMC proliferation in healthy cells. AMPK has previously been shown to be an upstream activator of Akt in endothelial cells (Ouchi et al, 2004). However, AICAR did not affect the activation of Akt, nor did it increase SMC proliferation. Thus, activation of AMPK alone is not responsible for the increase in phosphorylation of Akt. Additionally, AMPK is not down-stream from Akt. Finally, in comparing both adiponectin and AICAR's effects on SMCs, it may be that Akt plays a role in increasing SMC proliferation.

Production of nitric oxide (NO) causes vasodilation and helps to maintain cardiovascular homeostasis. Adiponectin (10 μg/ml) has been shown to increase NO production in bovine aortic endothelial cells. Inhibitors of PI3-kinase

(wortmannin) blocked NO production in response to adiponectin. Adiponectin also increased the phosphorylation of Akt (Ser 473). Further, blocking Akt with a dominant-negative mutant did not affect production of NO, but blocking AMPK did inhibit NO production by adiponectin (Chen et al, 2003). Thus, even though adiponectin activates both AMPK and Akt, AMPK was the important regulator in activating the eNOS in endothelial cells, independent from Akt activation. However, this has only been seen in endothelial cells with full-length adiponectin. Globular adiponectin in rat adipose was not able to stimulate Akt (Wu et al, 2003). Thus, their study, like ours, also found that adiponectin increases activation of AMPK and Akt, and that both protein synthesis and DNA synthesis were increased with adiponectin. AMPK and Akt did not work synergistically in cell growth and proliferation.

Since adiponectin has been shown to have proliferative effects on SMCs, it is important that the mechanism by which it acts be determined. Future research must focus on alternative pathways that increase SMC proliferation. Stimulation of pMAPKp42/44 has shown to increase SMC proliferation (Calabro et al, 2004). This pathway, ERK1/2, is an important pathway in cell growth, thus, discovering if adiponectin plays a role in controlling its functioning may provide useful information.

Additionally, adiponectin may not have a direct effect on SMC proliferation, but may play a different role. For example, mice without adiponectin have increased

neointimal thickening, suggesting that adiponectin may play a role in maintaining the vessel wall (Hug & Lodish, 2005). Adiponectin may thus operate indirectly, since NO is known to inhibit SMC proliferation.

2b) Leptin

Leptin increased protein synthesis in quiescent cells, but had no effect in PDGF-stimulated cells (*Figure 13, Figure 14)*. Leptin also had no effect on SMC proliferation (*Figure 15)*.

One previous study has looked at leptin's effects on vascular SMCs. Results in rat aortic SMCs showed that leptin stimulated both proliferation and migration. Furthermore, leptin activated MAPK and PI3K (Oda et al, 2001). This study used 0.1 µg/ml of leptin, and cells were treated for 3 days with leptin. This study differed from ours as it only starved cells for 48 hours and previous discussion states that cells may not reach full quiescence until 100-125 hours. The quantification method utilized in this study was cell counting, thus, the different techniques could also contribute to divergent responses.

The phosphorylation of AMPK was examined to identify the mechanisms through which leptin increases protein synthesis. Leptin was shown to activate AMPK (*Figure 17)*, in agreement with Steinberg et al (2004) who looked at leptin's effects in skeletal muscle.

Further mechanistic action was determined by testing Akt. Leptin (and PDGF) both activated Akt (*Figure 18)*.

In comparing the leptin, adiponectin and AICAR results, Akt activation was not related to an increase in SMC proliferation based on the observation that leptin increases activation of Akt, but has no effect on SMC proliferation. However, both adiponectin and leptin increased pAkt, and both increased protein synthesis. Thus, it is possible that the activation of AMPK is required for the development of new proteins in quiescent cells. This has also been postulated by Wojtaszewski et al (2003).

One study with 3T3-L1 adipocytes found that phosphorylated Akt increased leptin levels 20-fold, showing that Akt induces leptin production in 3T3-L1 adipocytes directly. So, although the direction is unclear, there is definitely a relationship between Akt and leptin (Song et al, 2005).

Leptin has also been shown to activate the ERK1/2 pathway (Oda et al, 2001) and STAT3 pathways in pre-adipocytes. However, in these cell lines, no effects on PI3K or Akt were seen (Machinal-Quelin et al, 2002).

Thus, additional work should be directed towards determining the effects of these adipokines on the ERK1/2 pathway, and to fully determine what pathways

pAMPK plays a role in. While it is a highly-activated kinase, its exact functions have mostly been postulated up to now.

One may question the validity of cell culture work, as most work is done providing a super-physiological dose to cells and measuring change. It is important to note that the concentrations of leptin used in our cell culture work (0.1 µg/mL) are within the range of those serum concentrations found in obese humans and animals (0.0125 µg/ml to 0.1 µg/ml) (Oda et al, 2001).

2c) Conjugated Linoleic Acid

Until recently, CLA had been studied as a mixture. However, it is now known that each of the CLA isomers act differently in the body. My research supports the view that each of the two main CLA isomers act independently from each other, and have different effects in regards to protein synthesis.

To date, no research has been done on the effects of CLA on smooth muscle. Because CLA is reported to have anti-atherosclerotic properties (Lee et al, 1994) and because an increase in smooth muscle proliferation is associated with atherosclerotic progression (Plutzky, 2003), we looked at these relationships in our study.

The t10,c12 CLA isomer had no effect on protein synthesis while the c9,t11 CLA isomer decreased total protein synthesis (*Figure 19, Figure 20)*. Neither of the CLA isomers had any effect on DNA synthesis (*Figure 21, Figure 22, Figure 23)*.

CLA has been shown to inhibit the proliferation of 3T3-L1 preadipocytes (Satory & Smith, 1999), as well as numerous cancer cell lines (Luongo et al, 2003; Chujo et al, 2003). One study looked at the difference between individual isomers and found that while both isomers inhibited the proliferation of human breast cancer cells, the c9,t11 isomer had the strongest effect (Chujo et al, 2003).

Although these conclusions cannot be assumed to apply to all cell types, it is evident that isomeric differences are being seen in regards to cell proliferation, be it protein synthesis, preadipocyte proliferation, or inhibition of cancer growth.

Our results show that in SMC, CLA does not act as an anti-atherosclerotic agent. The c9,t11 CLA isomer decreased protein synthesis, suggesting a possible detrimental effect of this isomer. Reducing protein synthesis in healthy cells makes it difficult for the cell to participate in normal metabolism and growth. Thus, the main CLA isomer that is found in food may not be the most beneficial.

Differences in the activation of AMPK also existed between isomers, with c9,t11 CLA increasing pAMPK, and t10,c12 having no effect (*Figure 25)*. Akt was not activated by either of the CLA isomers (*Figure 26)*. These results again show the

differing mechanistic actions of the two main CLA isomers. Since there was no change in activation of Akt or no increase in overall protein synthesis, these findings support the possibility that the phosphorylation of Akt may be a major factor in increasing protein synthesis.

This work clearly shows that each of the main CLA isomers has significantly different functions in the body, and that these effects differ even in the same cell types in different growth stages. These two isomers do not appear to act synergistically. Additionally, CLA does not have anti-atherogenic effects in vascular tissue, thus, if CLA does play a role in protection from atherosclerosis, it is not due to direct actions on SMC metabolism. Potentially, CLA may show its effects by activating various adipokines, as the t10,c12 CLA isomer has shown to increase adiponectin levels, and decrease leptin levels in *fa/fa* rats. Additional work must be done to determine if CLA affects globular adiponectin levels in the serum. As further discussion will show, this may be the more physiologically active form of adiponectin in regards to decreasing SMC proliferation.

2d) Globular Adiponectin (gAcrp30)

The globular head of adiponectin (gAcrp30) has shown different effects in the atherosclerotic process, compared to those of the full-length oligomer. Similar to CLA, this adipokine was once thought to function in its entirely, however, new data suggests that different oligomeric forms act quite differently in the body.

Our results support this as full-length and gAcrp30 acted on SMC proliferation in opposite directions. GAcrp30 inhibited SMC proliferation and acted against mitogenic stimulation of the cell, while full-length adiponectin increased SMC proliferation (*Figure 9, Figure 27)*.

GAcrp30 has been shown to have more beneficial effects compared to full-length adiponectin in regards to improved fatty acid oxidation, and lowering serum TGs in muscle (Fruebis et al, 2001; Onay-Besikci et al, 2004). Furthermore, ADIPO1 receptors (receptors that bind to gAcrp30) have also been shown to be lower in those with diabetes mellitus and in obesity (Wang et al, 2004). The functioning of these receptors may also be an issue. The current work illustrates that full-length and gAcrp30 act differently in SMCs, and have different mechanistic actions. The implications for atherosclerosis development is humans requires further investigation.

It has further been suggested that trimers of adiponectin can activate AMPK, while hexamers cannot. GAcrp30 forms trimers, while full-length adiponectin can form both trimers and hexamers (Tomas et al, 2002).

Many similar functions of the two forms of adiponectin have been identified. Both forms have shown to increase fatty-acid oxidation, PPARα ligand binding, phosphorylation of AMPK, ACC and p38 MAPK. MAPKp44/42, a kinase which is stimulated in SMC proliferation is not affected. Surprisingly, neither form of adiponectin affect its phosphorylation (Tomas et al, 2002).

GAcrp30 also has been shown to increase pAMPK. Tomas et al (2002) proposed that in the muscle, gAcrp30 may function by inactivating ACC and thus increasing fatty acid oxidation. In SMCs, gAcrp30 may also decrease SMC proliferation by activating pAMPK. Further work must be done to determine gAcrp30's exact mechanisms of action.

Expression of both forms of adiponectin is dependent on sex, and the adiponectin receptors exhibit different responses to each oligomer (Bouskila et al, 2005). Thus, a more thorough understanding of how gAcrp30 functions, especially in regards to SMC proliferation is vital. While full-length adiponectin may be mitogenic in SMCs, the globular form may offer potential as an anti-atherosclerotic agent.

2e) AMPK

Total AMPK levels represent the total amount of AMPK in SMCs available for activation. Long-term AMPK levels did not significantly change regardless of the agent added (*Figure 10, Figure 16 and Figure 24)*. Thus, AMPK may also serve as a control when trying to account for loading variabilities.

2f) Peroxisome Proliferator Activated Receptors (PPARs)

My results showed that PPARs were not affected by CLA, adiponectin or leptin (as well as any of the other PPAR agonists) (T*able 9).*

Some research suggests that CLA works through PPARs (Moya-Camarena et al; 1999, Granlund et al, 2003) and while there is some evidence to support this in the liver, no work has been done in SMCs.

However, some studies also suggest that CLA does not work through PPARs. For example, CLA (c9,t11 or t10,c12) did not alter cyanide-insensitive palmitoyl CoA oxidase or carnitine acetyl transferase, both markers of peroxisome proliferation in the liver in hamsters fed 6.6 g CLA/kg of diet (De Deckere et al, 1999). Although this study was focusing on other tissues, it demonstrated beneficial effects on lipid levels independent of PPARs. This is somewhat against the current belief as most scientists assume that CLA's beneficial effects are due

to changes mediated via PPARs. This work showed that CLA isomers do not affect PPARs in the SMCs.

Isomers of CLA have been shown to be high-affinity ligands for PPARα (Moya-Camarena & Belury, 1999) and this has been continually supported in studies with liver cells as this is the tissue where PPARα is predominantly located. In contrast, SMCs have low levels of PPARγ, which is found predominantly in the adipose. Thus, change in SMCs may not be seen as they are not concentrated tissues of PPARs.

As well, PPARβ is ubiquitously expressed throughout the body, thus, its function may be modulatory and its concentrations may not be high enough to exert enough of a change in SMCs to be considered significant.

PART 6 - SUMMARY AND CONCLUSIONS

1. Major Research Findings

Animal Study

Adiponectin

- Serum adiponectin levels are higher in *fa/fa* rats compared to lean rats.
- The t10,c12 CLA elevated serum adiponectin in animals.

Leptin

- Serum leptin levels were significantly lower in lean compared to *fa/fa* rats.
- CLA (specifically the t10,c12 isomer) only had an effect on reducing serum leptin levels in *fa/fa* rats. Any diet mixture containing t10,c12 CLA significantly lowered serum leptin levels in *fa/fa* rats.

Porcine Smooth Muscle Cell Cultures

Adiponectin

- Adiponectin stimulates protein synthesis and SMC proliferation in quiescent SMCs, but has no effect in proliferating cells.
- Adiponectin acts as a mitogen in healthy SMCs, and, therefore, did not exert any anti-proliferative effects in SMCs.
- The mechanisms of adiponectin's mitogenic effects does not involve activation of Akt.

- The phosphorylation of Akt by adiponectin does not result in an increase in SMC proliferation.
- The phosphorylation of AMPK and Akt are not directly correlated to protein synthesis and increased SMC proliferation.

Leptin

- Leptin increases protein synthesis in healthy cells, but has no effect on DNA synthesis.
- Leptin increased both the phosphorylation of AMPK and Akt.

CLA

- CLA isomers have different effects on SMC proliferation. While neither of the isomers affected DNA synthesis, c9,t11 CLA decreased protein synthesis, and t10,c12 CLA had no effect.
- CLA does not have anti-atherosclerotic effects in SMCs.
- The phosphorylation of Akt may mediate the increase in protein synthesis.

Globular Adiponectin (gAcrp30)

- Full-length adiponectin and gAcrp30 have opposite effects on SMC proliferation. GAcrp30 decreased SMC proliferation, while full-length adiponectin increased it. Thus, gAcrp30 has anti-mitogenic properties in SMCs.

Peroxisome Proliferated-Activated Receptors (PPARs)

- PPARs are not affected by CLA in SMCs.
- PPARs are not affected by any of the PPAR agonists, leptin or adiponectin in SMCs.

2. Implications for Future Research

- Both gAcrp30 and full-length adiponectin must be individually studied to distinguish their distinct physiological properties in the body.
- More clinical trials must be developed to assess changes in adiponectin and leptin levels in high-risk populations for metabolic syndrome, as well as in obese subjects. Additionally, investigation the effect of CLA and adipokine levels in the human is also important.
- While doses of up to 3.4 g/day have been shown to be safe in humans (Rainer et al, 2004), more conclusive work is needed to determine whether a toxicity threshold exists for CLA in humans. If not, the development of a high-dose t10,c12 CLA-rich supplement may be indicated. Additional work altering farming practices and food processing would be necessary to develop t10,c12 CLA-rich food sources and/or supplements.
- Total dietary intake and composition may influence the effect of CLA isomers in the body. Data supports the view that the source of protein fed in test diets alters the overall effects of CLA. For example, a soy-based diet supplemented with CLA was more effective in regards to reducing

lipid accumulation when compared to the same CLA supplemented diet with casein as the source. However, those fed the casein CLA-supplemented diet had a greater effect on serum cytokine measurements, namely leptin and adiponectin (Akahoshi et al, 2004).

- A further look into adiponectin's possible autocrine/paracrine functions.
- Identification of the mechanism through which adiponectin acts as a mitogen, for example, MAPKp42/44.
- Identification of the mechanisms through which adiponectin and leptin increase protein synthesis. The regulation of eukaryotic elongation factor-2 (eEF2) has been shown to play a role in protein synthesis, and AMPK may play a role in this process. Thus, the influence of adiponectin and CLA on eEF2 may offer insight into its roles in vascular tissue.
- Further testing of Akt activation and its relationship to protein synthesis in SMCs.
- Further work with gAcrp30 and its effects on SMC proliferation, and mechanisms must be determined.
- Distinguishing the actions of each of the adiponectin receptors and how they are altered in metabolic syndrome and obesity.

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PART 8 - APPENDICES

APPENDIX A - GW501516 Study

GW501516 (2-Methyl-4-((4-methyl-2-4(trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)

GW501516 (2-Methyl-4-((4-methyl-2-4(trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl) phenoxy-acetic acid) is a selective PPARβ agonist (Khan et al, 2003).

While the effects of the PPARγ and PPARα agonists have been studied previously in this laboratory, PPARβ activity has not. Thus, GW5's effects in regards to cell proliferation were investigated.

Western blotting of SMC determined that PPARα, γ, and β were all present in porcine SMCs.

Leucine Incorporation

^{3}H-Leucine incorporation was used to assess protein synthesis in SMCs after treatment with GW5. ^{3}H-Leucine was added to SMC culture media and allowed to incorporate into proliferating proteins (as described in Methods).

At lower concentrations, GW5 decreased protein synthesis compared to baseline (*Figure 29*). There was a concentration dependent effect at 4 and 6 uM GW5, but no significant decrease at 10 GW5.

PDGF increased protein synthesis. However, GW5 had no effect on PDGF-stimulated cells on protein synthesis (*Figure 30*).

This indicates that in quiescent cells, at lower concentrations, GW5 decreases protein synthesis. However, in proliferating cells, GW5 has no effect on production of proteins.

Figure 29 – Effect of GW501516's on Protein Synthesis in Porcine Smooth Muscle Cells

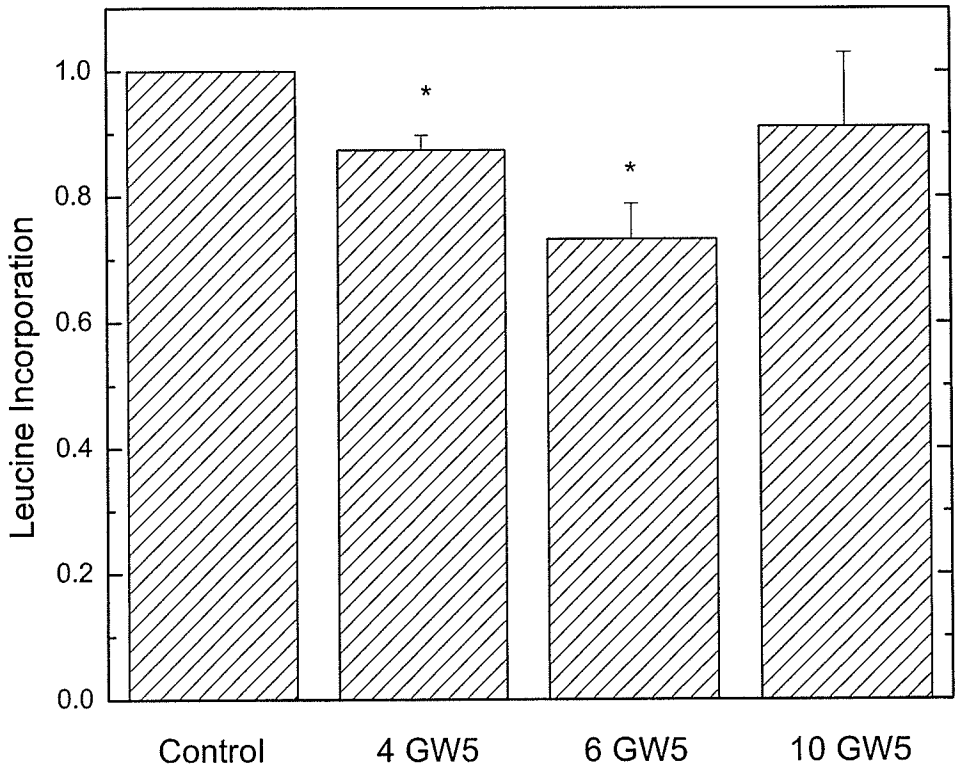

Figure 29 - The effect of 4, 6 and 10 μM GW501516 on ^{3}H-Leucine incorporation was compared to control. Data are standardized to control and expressed as mean ± SEM of samples in triplicate. The 4 and 6 μM GW5 decreased protein synthesis, but 10 μM GW5 had no effect in quiescent cells.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

Figure 30 – Effect of GW501516's on Protein Synthesis in PDGF-stimulated Porcine Smooth Muscle Cells

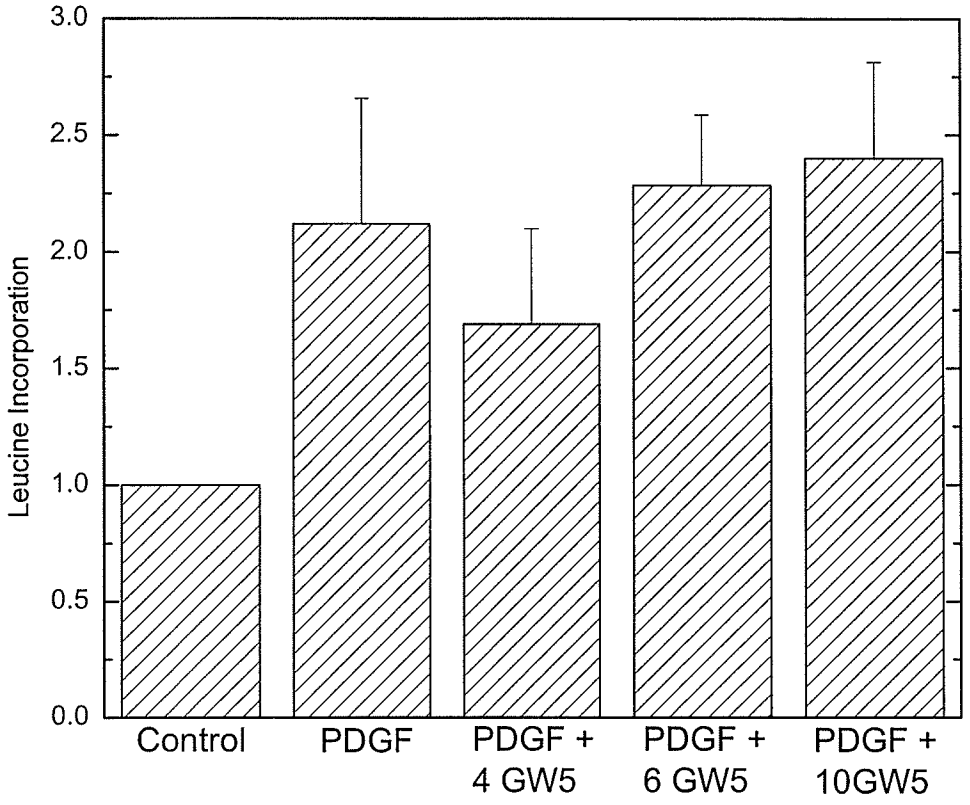

Figure 30 - The effect of 4, 6 and 10 μM GW501516 (GW5) compared to control and to the mitogenic effects on 0.1 μg/mL platelet-derived-growth factor BB (human) (PDGF) on ^{3}H-Leucine incorporation is illustrated. Data are standardized to control and expressed as mean ± SEM of samples in triplicate. PDGF-stimulated cells were not affected by GW501516 at any concentration in relation to protein synthesis.

Thymidine Incorporation

^{3}H-Thymidine incorporation was used to assess DNA synthesis in SMC, since SMC proliferation is a central feature in vascular disease.

PDGF increased DNA synthesis, as expected. GW5 had no effect on DNA synthesis in quiescent or in stimulated cells (*Figure 31*).

No significant differences were seen with varying concentrations (data not shown).

Figure 31 – Effect of GW501516 on Smooth Muscle Cell Proliferation

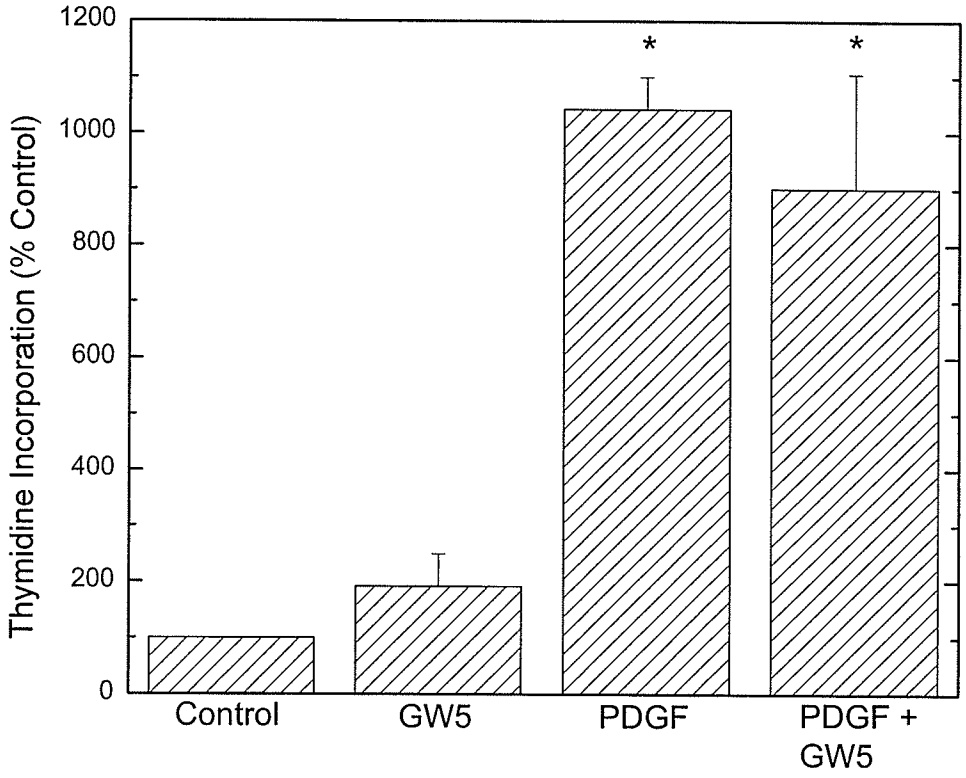

Figure 31 - The effect of 10 μM GW501516 (GW5) compared to control and to the mitogenic effects on 0.1 μg/mL platelet-derived-growth factor BB (human) (PDGF) on ^{3}H-Thymidine incorporation is illustrated. Data is expressed as mean ± SEM of samples in triplicate. GW5 did not affect protein synthesis in quiescent or PDGF-stimulated cells.

* Statistically significant differences from control (unstimulated) at $p<0.05$.

Conclusion and Discussion

In healthy (quiescent) cells, GW5 decreases protein synthesis, although there is no effect on protein synthesis in proliferating cells.

While there was a concentration-dependent effect for GW5 at 4uM and 6uM, 10uM did not seem to have an effect. This could possibly be due to the fact that at higher concentrations, 10uM GW5 becomes too concentrated in the cell, making it unable to alter protein synthesis.

Past research in this laboratory has looked at PPARα and PPARγ agonists as potential SMC proliferators. Both PPARα and PPARγ were present in human arteries, especially in SMCs (Zahradka et al, 2003). The PPARα agonist, WY14643, and a PPARγ agonist 15-deoxy-prostaglanding J_2 (15d-PGJ_2) both reduced SMC proliferation in stimulated cells, suggesting that both of these PPARS affect lesion progression (Zahradka et al, 2003).

This new study discovered that PPARβ agonists do not have a significant effect on SMC proliferation. This is the first line of evidence that suggests that PPARβ does not play a role in SMC proliferation and does not affect the development of atherosclerotic lesions. Thus, if there is a relationship between SMCs and PPARs, PPARα and PPARγ may be those that have greater anti-atherosclerotic effects.

GW5 was an inhibitor of protein synthesis in healthy cells, suggesting that it negatively affects cell growth. It is natural for healthy cells to undergo protein synthesis for metabolism and survival needs. GW5 inhibits this natural process, potentially making it more difficult for the cell to be viable. Additionally, GW5 had no effect on SMC proliferation. Taking these factors into account, it is concluded that PPARβ does not have any proliferative effects in either healthy or proliferating cells.

As to the best of the writer's knowledge, no work has investigated the effects of GW5 in SMC culture. Studies that do look at this PPARβ agonist exist, although they are on different cell lines. For example, Stephen et al (2004) demonstrated an increase in proliferation in breast and prostrate cancer cells as well as in HUVECs suggesting the possibility that PPARβ antagonists may help in the treatment of breast and prostate cancer (Stephen et al, 2004).

GW5 has also been shown to increase cyclooxygenase-2 (COX-2) in hepatocellular carcinoma cells, which is associated with carcinogenesis and an increase in cell proliferation in many cell types (Glinghammer et al, 2003).

Of the limited evidence available, it seems that GW5 may have the ability to increase cell proliferation in specific cell types. However, SMCs do not appear to be affected by this PPARβ agonist.

Figure 29 - GW501516'2 Effects on Protein Synthesis in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1	1	1	1 ± 0.00
4 µM GW501516	0.85	0.85	0.92	0.87 ± 0.06
6 µM GW501516	0.63	0.83	0.74	0.73 ± 0.06
10 µM GW501516	1.08	0.60	1.23	0.91 ± 0.12

* Cells were stimulated for 1 hour prior to Leucine incorporation.

Figure 30 - GW501516'2 Effects on Protein Synthesis in PDGF-stimulated Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1	1	1	1 ± 0.00
PDGF	2.23	1.14	2.99	2.12 ± 0.54
PDGF + 4 µM GW501516	1.29	1.27	2.51	1.69 ± 0.41
PDGF + 6µM GW501516	1.83	2.17	2.85	2.28 ± 0.30
PDGF + 10 µM GW501516	2.46	1.65	3.08	2.40 ± 0.41

* PDGF was added at 0.1 µg/mL. Cells were stimulated for 1 hour prior to leucine incorporation.

Figure 31 - GW501516'2 Effects on Smooth Muscle Cell Proliferation

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100	100	100	100 ± 0.00
GW501516	306.49	123.82	143.97	191.43 ± 57.82
PDGF	928.61	1075.36	1122.76	1024.24 ± 58.44
PDGF + GW501516	-	638.48	759.19	700.77 ± 204.92

* PDGF was added at 0.1 µg/mL. Cells were stimulated for 12 hours prior to thymidine incorporation.

APPENDIX B - RAW DATA FOR FIGURES 1-33

Figure 1 – Serum Adiponectin in lean vs *fa/fa* Zucker Rats

	Mean ± SEM (µg/mL)
Lean [#]	4.47 ± 0.14
fa/fa [#]	6.15 ± 0.19 *

* Significantly different from control ($p<0.05$).
[#] n=59 rats per genotype.

Figure 2[a] – Serum Adiponectin by Diet Group

Diet	Mean ± SEM (µg/mL)
C9,t11 CLA [#]	4.87 ± 0.25
T10,c12 CLA [€]	5.82 ± 0.35 *
TOG [€]	5.57 ± 0.33
BIO [#]	5.52 ± 0.44
NCK [#]	5.42 ± 0.38
CTRL [#]	4.69 ± 0.26

* Significantly different from Control and c9,t11 CLA. ($p<0.05$).
[#] n=20 rats per diet.
[€] n=19 rats per diet.
[a] See Table 2-4 for Diet Composition

Figure 3 - Serum Adiponectin (per gram of adipose tissue) in Lean and *fa/fa* Zucker Rats

	Mean ± SEM[a] {(µg/mL) / g adipose}
Lean [#]	0.47 ±0.02
fa/fa [#]	0.14 ± 0.01 *

* Significantly different from control ($p<0.05$).
[#] n=59 rats per genotype.
[a] Per gram of adipose tissue was based on the sum of epididymal and peri-renal adipose tissue.

Figure 4[a] – Serum Adiponectin (per gram of adipose tissue) by Diet Group

Diet	Mean ± SEM[b] {(µg/mL) / g adipose}
C9,t11 CLA [*]	0.28 ± 0.03
T10,c12 CLA [#]	0.30 ± 0.04
TOG [#]	0.28 ± 0.04
BIO *	0.32 ± 0.05
NCK *	0.35 ± 0.06
CTRL *	0.29 ± 0.04

* n=20 rats per diet.
[#] n=19 rats per diet.
[a] See Table 2-4 for Diet Composition.
[b] Per gram of adipose tissue was based on the sum of epididymal and peri-renal adipose tissue.

Figure 5 – Serum Leptin in Lean and *fa/fa* Zucker Rats

Genotype [#]	Mean ± SEM (ng/mL)
Lean	4.32 ± 0.32
fa/fa	112.57 ± 2.47*

* Significantly different from control ($p<0.05$).
[#] n=60 rats per genotype.

Figure 6[a] – Serum Leptin by Diet Group

Diet *	Mean (ng/mL)
C9,t11 CLA	66.96 ± 14.18
T10,c12 CLA	57.71 ± 12.36
TOG	57.43 ± 12.42
BIO	53.58 ± 11.89
NCK	50.22 ± 11.03
CTRL	64.76 ± 14.18

* n=20 rats per diet.
[a] See Table 2-4 for Diet Composition.

Figure 7[a] – Serum Leptin in lean and *fa/fa* Zucker Rats by Diet

Group €	Mean ± SEM (ng/mL)
Lean	
C9,t11 CLA	5.72 ± 1.19
T10,c12 CLA	5.11 ± 0.74
TOG	4.19 ± 0.77
BIO	3.09 ± 0.42
NCK	3.69 ± 0.53
CTRL	4.10 ± 0.63
fa/fa	
C9,t11 CLA	128.19 ± 3.82
T10,c12 CLA	110.31 ± 5.43 * #
TOG	110.67 ± 4.54 * #
BIO	104.08 ± 5.44 * #
NCK	96.76 ± 5.68 * #
CTRL	125.43 ± 5.57 #

* Significantly different from control ($p<0.05$).
# Significantly different from c9,t11 CLA ($p<0.05$).
€ n=10 rats per group.
[a] See Table 2-4 for Diet Composition.

Figure 8 – Effect of Adiponectin on Protein Synthesis in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100 ± 0.00
Adiponectin	145.40	154.40	161.50	153.77 ± 4.67
PDGF	1253.80	1012.40	611.50	959.23 ± 187.31
PDGF + Adiponectin	1117.90	1125.60	1055.40	1099.63 ± 22.23

* PDGF was added at 1 mM, adiponectin was added at 1 µg/µL. Cells were stimulated for one hour prior to Leucine incorporation.

Figure 9 – Effect of Adiponectin on Smooth Muscle Cell Proliferation

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100 ± 0.00
Adiponectin	1070.6	979.40	729.70	926.57 ± 101.89
PDGF	1099.9	1258.00	909.50	1089.13 ± 100.75
PDGF + Adiponectin	1184.00	1203.50	1452.80	1280.10 ± 86.53

* PDGF was added at 1 mM, adiponectin was added at 1 µg/µL. Cells were stimulated for 24 hours prior to thymidine incorporation.

Figure 10 – Effect of Adiponectin and AICAR on total AMPK levels in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	0.97	2.20	4.20	2.46 ± 0.94
Adiponectin	0.01	4.22	1.55	1.92 ± 1.23
AICAR	1.32	1.63	4.84	2.59 ± 1.12

* PDGF was added at 1 mM, adiponectin was added at 1 µg/µL, and AICAR was added at 1 mM. Cells were stimulated for 12 hours.

Figure 11 – Effect of Adiponectin and AICAR on the Phosphorylation of AMPK in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	5.48	6.80	4.50	3.88 ± 0.80
Adiponectin	9.66	6.80	4.50	6.99 ± 1.49
AICAR	2.62	13.62	6.24	7.50 ± 3.24

* PDGF was added at 1 mM, adiponectin was added at 1 µg/µL, and AICAR was added at 1 mM. Cells were stimulated for 15 minutes.

Figure 12 – Effect of Adiponectin on the Phosphorylation of Akt in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	4.68	142.20	33.55	60.15 ± 3.31
Adiponectin	8.48	45.74	7.68	20.63 ± 12.56
AICAR	1.49	13.36	1.97	5.60 ± 3.88

* PDGF was added at 1 mM, adiponectin was added at 1 µg/µL, and AICAR was added at 1 mM. Cells were stimulated for 15 minutes.

Figure 13 – Effect of Leptin on Protein Synthesis in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100.00 ± 0.00
Leptin	129.27	171.46	1283.90	528.37 ± 378.04

* Leptin was added at 0.1 µg/mL. Cells were stimulated for one hour prior to leucine incorporation.

Figure 14 – Effect of Leptin on Protein Synthesis in PDGF-Stimulated Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100.00 ± 0.00
PDGF	136.16	154.81	111.16	134.04 ± 8.94
Leptin	155.22	157.89	104.90	139.34 ± 12.19

* PDGF was added at 1 mM, leptin was added at 0.1 µg/mL. Cells were stimulated for one hour prior to Leucine incorporation.

Figure 15 – Effect of Leptin on Smooth Muscle Cell Proliferation

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100.00 ± 0.00
Leptin	122.93	36	59.78	72.90 ± 25.94
PDGF	928.61	1075.36	1122.76	1042.24 ± 58.44
PDGF + Leptin	991.96	164.69	-	1156.64 ± 827.27

* PDGF was added at 1 mM, and leptin was added at 0.1 µg/mL. Cells were stimulated for 12 hours prior to thymidine incorporation.

Figure 16 – Effect of Leptin on Total AMPK Levels in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	3.49	4.20	0.97	2.89 ± 0.98
Leptin	0.80	2.76	0.98	1.51 ± 0.63
AICAR	1.63	4.84	1.32	2.56 ± 1.12

* PDGF was added at 1 mM, and leptin was added at 0.1 µg/mL. Cells were stimulated for 12 hours.

Figure 17 – Effect of Leptin on the Phosphorylation of AMPK

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	5.48	3.20	3.50	4.06 ± 0.71
Leptin	6.85	5.1	7.03	6.33 ± 0.62

* PDGF was added at 1 mM, and leptin was added at 0.1 µg/mL. Cells were stimulated for 15 minutes.

Figure 18 – Effect of Leptin on the Phosphorylation of Akt in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	5.48	2.94	3.22	3.88 ± 0.80
Leptin	6.85	7.03	4.10	5.99 ± 0.95
AICAR	2.62	13.62	4.50	6.91 ± 3.40

* PDGF was added at 1 mM, leptin was added at 0.1 µg/mL, and AICAR was added at 1 mM. Cells were stimulated for 15 minutes.

Figure 19 – Effect of Conjugated Linoleic Acid Isomers on Protein Synthesis in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100.00 ± 0.00
t10,c12	99.70	112.40	87.30	99.8 ± 7.25
Control	100.00	100.00	100.00	100.00 ± 0.00
c9,t11	49.00	50.40	32.80	44.07 ± 5.65

* t10,c12 and c9,t11 was added at 60 µM. Cells were stimulated for one hour prior to leucine stimulation.

Figure 20 – Effect of Conjugated Linoleic Acid on Protein Synthesis in PDGF-stimulated Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100.00 ± 0.00
PDGF	1253.38	10112.44	611.46	959.09 ± 187.22
PDGF + c9,t11	1305.71	1390.90	1495.93	1397.41 ± 54.92
PDGF + t10,c12	1166.83	1174.90	1236.04	1192.59 ± 21.85

* PDGF was added at 0.1 µg/mL, while t10,c12 and c9,t11 was added at 60 µM. Cells were stimulated for one hour prior to leucine stimulation.

Figure 21 – Effect of Conjugated Linoleic Acid on Proliferation in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Standardized to Baseline	Mean ± SEM
Control	2498.03	2302.03	2089.03	1.00	1.00 ± 0.05
60 µM c9,t11	2257.03	1436.68	1527.36	0.76	0.76 ± 0.11
120 µM c9,t11	1573.69	2084.37	1401.36	0.73	0.73 ± 0.09
Control	393.01	305.34	-	1.00	1.00 ± 0.13
60 µM t10,c12	421.68	301.01	355.01	0.89	1.03 ± 0.10
120 µM t10,c12	398.01	233.01	304.34	0.74	0.89 ± 0.14

* t10,c12 and c9,t11 was added at 60 and 120 µM. Cells were stimulated for 12 hours prior to thymidine incorporation.

Figure 22 – Effect of c9,t11 Conjugated Linoleic Acid on Proliferation in PDGF-Stimulated Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100 ± 0.00
PDGF	504.80	591.30	205.50	532.87 ± 29.22
PDGF + c9,t11	430.60	484.40	717.00	544.00 ± 87.88
PDGF + t10,c12	558.40	337.50	465.90	453.93 ± 64.05

* PDGF was added at 0.1 µg/mL, while t10,c12 and c9,t11 was added at 60 µM. Cells were stimulated for 12 hours prior to thymidine incorporation.

Figure 23 – Effect of t10,c12 Conjugated Linoleic Acid on Proliferation in PDGF-Stimulated Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100.00 ± 0.00
PDGF	933.91	1147.87	896.05	992.61 ± 78.40
c9,t11 CLA	1250.42	1140.46	1168.83	1186.57 ± 32.96
t10,c12 CLA	1207.92	1536.29	1404.28	1382.82 ± 95.40

* PDGF was added at 0.1 µg/mL, while t10,c12 and c9,t11 was added at 60 µM. Cells were stimulated for 12 hours prior to thymidine incorporation.

Figure 24* - Effect of Conjugated Linoleic Acid on total AMPK Levels in Porcine Smooth Muscle Cells

Treatment	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	3.49	4.20	0.97	2.89 ± 0.98
c9,t11 CLA	0.80	2.76	0.98	1.51 ± 0.63
t10,c12 CLA	1.63	4.84	1.32	2.59 ± 1.12

* PDGF was added at 0.1 µg/mL, while t10,c12 and c9,t11 was added at 60 µM. Cells were stimulated for 12 hours.

Figure 25 – Effect of Conjugated Linoleic Acid on the Phosphorylation of AMPK in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	2.07	0.72	3.22	2.00 ± 0.72
c9,t11 CLA	0.92	0.43	17.04	6.13 ± 5.46
t10,c12 CLA	0.66	0.73	2.44	1.28 ± 0.58

* PDGF was added at 0.1 µg/mL, while t10,c12 and c9,t11 was added at 60 µM. Cells were stimulated for 15 minutes.

Figure 26 – Effect of Conjugated Linoleic Acid on the Phosphorylation of Akt in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
PDGF	100.00	33.55	4.14	45.90 ± 28.35
c9,t11 CLA	3.48	0.01	0.05	1.18 ± 1.15
t10,c12 CLA	0.01	0.43	0.21	0.21 ± 0.12
AICAR	1.49	13.36	1.97	5.60 ± 3.88

* PDGF was added at 0.1 µg/mL, while t10,c12 and c9,t11 was added at 60 µM, and AICAR was added at 1 mM. Cells were stimulated for 15 minutes.

Figure 27 – Effect of Globular Adiponectin on Proliferation in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	100.00	100.00	100.00	100.00 ± 0.00
G Adipo	66.41	55.31	59.44	60.39 ± 3.24
PDGF	242.13	486.37	560.63	429.71 ± 96.21
PDGF + G Adipo	51.84	111.53	166.34	109.90 ± 33.06

* PDGF was added at 0.1 µg/mL, and G Adipo was added at 1.5 µg/mL. Cells were stimulated for 12 hours minutes prior to thymidine incorporation.

Figure 28 – Effect of Globular Adiponectin and AICAR on the Phosphorylation of AMPK in Porcine Smooth Muscle Cells

Treatment*	Count 1	Count 2	Count 3	Mean ± SEM
Control	1.00	1.00	1.00	1.00 ± 0.00
G Adipo	2.56	2.19	1.89	2.21 ± 0.19
AICAR	2.25	1.87	2.96	2.36 ± 0.32

* G Adipo was added at 1.5 µg/mL and AICAR was added at 1 mM. Cells were stimulated for 15 minutes.